Mulberry anthocyanin extract ameliorates insulin resistance by regulating PI3K/AKT pathway in HepG2 cells and db/db mice

Fujie Yan a,b,c, Guanhai Dai d, Xiaodong Zheng a,b,c,*

a Department of Food Science and Nutrition, Zhejiang University, Hangzhou 310058, People’s Republic of China
b Zhejiang Key Laboratory for Agro-food Processing, Zhejiang University, Hangzhou 310058, People’s Republic of China
c Fuli Institute of Food Science, Zhejiang University, Hangzhou 310058, People’s Republic of China
d Institute of Basic Medicine, Zhejiang Academy of Traditional Chinese Medicine, Hangzhou 310007, China

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Abstract

This study evaluated the capacity of mulberry anthocyanin extract (MAE) on insulin resistance amelioration in HepG2 cells induced by high glucose and palmitic acid and diabetes-related metabolic changes in type 2 diabetic mice. In vitro, MAE alleviated insulin resistance in HepG2 cells and increased glucose consumption, glucose uptake and glycogen content. Enzyme activities of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were decreased due to PPARγ coactivator 1α (PGC-1α) and forkhead box protein 01 (FOXO1) inhibition. Furthermore, phosphorylation of protein kinase B (AKT) and glycogen synthase kinase-3β (GSK3β) in model cells was recovered after treated with MAE, leading to an up-regulation of glycogen synthase 2 (GYS2), and this effect was blocked by the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002. In vivo, MAE supplementation (50 and 125 mg/kg body weight per day) markedly decreased fasting blood glucose, serum insulin, leptin, triglyceride and cholesterol levels and increased adiponectin levels in db/db mice. The improvement of related metabolic parameters was in part associated with the impact of MAE on activating AKT and downstream targets in liver, skeletal muscle and adipose tissues. In summary, these findings suggest that MAEs have potential benefits on improving dysfunction in diabetic mice and mitigating insulin resistance in HepG2 cells via activation of PI3K/AKT pathways.

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Keywords: Mulberry anthocyanins; Glucose metabolism; HepG2 cells; db/db mice; AKT

1. Introduction

Diabetes has been emerging as a leading health burden in 21st century since its incidence is rapidly increasing worldwide [1]. Considering adverse effects of drugs, natural products from fruits and vegetables have been drawn extensive attentions on preventive and therapeutic interventions for metabolic syndrome.

Mulberry possesses many pharmacological effects, which has been traditionally used in Chinese medicines. Its deep colored fruit is rich in polyphenolic compounds including water soluble anthocyanins and flavonoids, showing a great antioxidant activity in vitro [2]. Actions of mulberry on liver protection, anti-inflammation and anti-cancer both in cultured cells and animal models have been demonstrated, which is partly associated with the ability of free radical scavenging. Mulberry anthocyanin extract (MAE) administration significantly increased plasma antioxidant capacity in Wistar rats, although concentration of anthocyanins detected in plasma was very low, implying that anthocyanins were responsible for elevating some antioxidant compounds levels in blood after digestion and absorption [3]. Obesity is considered as a major factor to develop type 2 diabetes mellitus (T2DM), and controlling weight gain is very important to prevent and treat diabetic disorders. Wu et al. [4,5] illustrated that dietary supplemented with MAE could protect against diet-induced obesity in C57BL/6 mice due to oxidative stress and inflammation alleviation. Ou et al. [6] and Peng et al. [7] indicated the hypolipidemic effects of mulberry water extracts on oleic acid-induced HepG2 cells and hamsters supplied with high-fat diet. However, there seems to be very little focus on hypoglycemic effect of mulberry fruit, although it is generally accepted that mulberry leaf exhibits an obvious ability on diabetes amelioration [2,8,9]. 1-Deoxynojirimycin isolated from mulberry leaves was discovered to be an inhibitor of α-glucosidase, suppressing activities of carbohydrate-degrading enzymes. Besides, polysaccharide from mulberry plants showed a protective function on damaged pancreatic islets and β-cell [10]. Insufficient investigation has been performed that cyanidin-3-glucoside (C3G) isolated from mulberry fruit showed protective function on erectile in diabetes mellitus rats and restrained cell death in pancreatic β-cells induced by high glucose or H2O2 [11–13]. In addition, MAE was found to lower glucose levels and prevent islet degeneration in Zucker diabetic fatty rats [8]. Our previous research showed that MAE might regulate glucose metabolism through phosphatidylinositol-3-kinase (PI3K)/
protein kinase B (AKT)/glycogen synthase kinase-3β (GSK3β) and PI3K/AKT/forkhead box protein O1 (FOXO1) modulation in HepG2 cells [14]. Nevertheless, there is still a lack of research about precise therapeutic and protective effects of MAE on type 2 diabetes in vivo and insulin resistance (IR) in vitro. Moreover, capacity of MAE at molecular levels on regulating some targets that related to insulin signaling pathway remains largely unclear.

In this study, we firstly aimed to investigate the underlying protective action of MAE against IR in vitro by using HepG2 cells as a model and possible mechanisms involved. Secondly, we assessed if dietary supplementation with MAE could improve glucose metabolic disorders in db/db mice, as well as whether activation of AKT and its downstream target proteins in liver, muscle and adipose were relevant to the effect of MAE.

2. Materials and methods

2.1. Materials

Fresh mulberry (Morus alba L.) was purchased from a local fruit market in Hangzhou. 2-NBDG was obtained from ApexBio. Antibodies for p-AKT, AKT, p-GSK3β, GSK3β, p-FOXO1, FOXO1, β-actin and tubulin, the ECL Western blotting system and SYBR Green I (Roche) on the ABI Step One RT-PCR system. Gene-specific primers (Table S1) were designed with Primer Blast.

2.2. Extraction and determination of anthocyanins from mulberry

Anthocyanins were isolated and purified as previously described [14]. Briefly, fruit was extracted with a threefold volume of 95% ethanol containing 1% HCl for 24 h at 4 °C. Filtered fluid was evaporated at 38 °C and then the concentrates were loaded onto an equilibrated macroporous resin column eluted with 10% formic acid in methanol for further purification. MAE was obtained by lyophilization and stored at −80 °C before use. Composition and content of anthocyanins in MAE were determined by an HPLC instrument (Thermo UltiMate 3000). A linear gradient from 5% A (acetonitrile) to 40% B (water containing 10% formic acid) in 30 min was used for HPLC assay. The column was operated at a temperature of 30 °C and absorption spectrum was recorded at 520 nm.

2.3. Cell culture and treatments

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C, 5% CO2 atmosphere. After reaching 70–80% confluence, the cells were washed with phosphate-buffered saline (PBS) twice and incubated with normal glucose (5.5 mM) or high glucose (30 mM) plus palmitic acid (PA; 0.2 mM) in the absence or presence of MAE with different concentrations for 24 h. Cells treated with normal glucose (5.5 mM) were used as the negative control and cells treated with metformin (2 mM) were used as the positive control.

2.4. MTT assay

Cells were seeded into a 96-well plate, and MTT diluted with PBS at a final concentration of 0.5 mg/ml was added to each well after treatments. After 4 h of incubation at 37 °C, the formazan precipitate was dissolved in 0.5 ml distilled water and boiled for 20 min after adding 0.2% antarhine diluted with 98% H2SO4. OD values were detected at 620 nm and glycogen contents were estimated by the method of Lv et al. [16] and normalized to protein level by the ECL Western blotting system and an independent third-party testing agency (KingMed Diagnostics, China).

2.5. Glucose consumption assay

The glucose consumption was estimated by the method of Liu et al. [16] with modification. Cells were seeded into a 96-well plate at a density of 4×104 cells/well with five wells left as blanks. The medium was removed 24 h later and glucose consumption was calculated by the glucose concentrations of blank wells minus glucose concentrations in plated wells. The MTT assay was used to adjust the glucose consumption. There were five replicates for each treatment and the experiment was repeated twice.

2.6. Glucose uptake assay

Cells were seeded into 24-well plates at a density of 5×104 cells/well, and after the treatments, cells were exposed to 0.1 mM 2-NBDG and 100 nM insulin for 30 min at 37 °C. Images were obtained using identical acquisition settings on a fluorescence microscope (Zeiss) [17].

2.7. Glycogen content assay

The cells were seeded into 6-well plate and washed twice with PBS and homogenized in 30% KOH after treatments. Samples were boiled for 20 min, added 1.5 ml ethyl alcohol and then centrifuged at 12,000 g for 15 min. Precipitates were dissolved in 0.5 ml distilled water and boiled for 20 min after adding 0.2% antarhine diluted with 98% H2SO4. OD values were detected at 620 nm and glycogen contents were employed glucose as a standard level and normalized to protein level by the Bradford reagent.

2.8. Animal experiments

Four-week-old male C57BL6J genetic background (db/db) mice and their nondiabetic lean littermates (m/m; n=10), db/db mice given by gavage with water (n=12), and three groups in which db/db mice were given by gavage with metformin in dose of 200 mg/kg body weight, MAE in doses of 50 and 125 mg/kg body weight every day (n=12), which translates into 300 and 750 mg/day for a 60 kg male. Recent human trails have indicated that such doses are safe and well tolerated [18,19]. All groups were fed with the same standard diet. At week 7 of the intervention, mice were fasted 5 h and orally administered with 0.5 ml glucose dissolved in purified water to deliver 2 g/kg body weight glucose. Glucose was measured at 0, 30 and 120 min with a blood glucose monitor from a tail prick. After 8 week of interventions and 6 h fast, mice were killed and liver, skeletal muscle and epididymal adipose tissues were frozen stored at −80 °C for further assays.

2.9. Biochemical determinations

Enzymatic assays for serum glucose, total triglyceride (TG), total cholesterol (TCH), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and glycated serum protein (GSP) concentration levels were determined by commercially available kits (Nanjing Biotech Co., China). Insulin levels were characterized by a corresponding mouse ELISA kit according to the manufacturer’s instructions. Levels of adiponectin and leptin were assayed by an independent third-party testing agency (KingMed Diagnostics, China). Hepatic concentrations of TG and glycogen contents in liver and muscle were estimated with commercially enzymatic kits (Nanjing Biotech Co., China). Protein concentrations were assayed with a BCA protein assay kit (Beyotime Biotechnology, China).

2.10. Quantitative real-time PCR analysis

Total RNA from HepG2 cells or tissues was extracted using RNeasy Plus kit (code D9108B; TaKaRa, Japan) according to the manufacturer’s instructions. Real-time polymerase chain reaction (RT-PCR) was performed using the SYBR Green Kit (Roche) on the ABI Step One RT-PCR system. Gene-specific primers (Table S1) were designed with Primer Blast.

2.11. Western blot

Equal amounts of protein from tissues and cell homogenates were subjected to SDS-PAGE and transferred to PVDF membranes. Membranes were probed with primary antibodies and then detected with horseradish peroxidase-conjugated secondary antibodies using ECL detection system. β-Actin (cell, liver and adipose) and tubulin (skeletal muscle) were used as a loading control. Densitometry analysis was performed using the Image-pro plus 6.0 software.

2.12. Statistical analyses

Data are means±S.D. Statistical analyses of the data were performed with SPSS for Windows Version 11.5. One-way analyses of variance (ANOVA) and Duncan’s multiple range test were used to detect statistical significance, and differences were considered significant when P<0.05. Two-tailed, unpaired t tests were also used to determine statistical significance.

3. Results

3.1. MAE prevented both glucose consumption and uptake decrease in HepG2 cells induced by high glucose and PA

HepG2 cells were exposed to 30 mM glucose and 0.2 mM PA for 24 h followed by insulin (100 nM) incubation for 30 min. An obvious decrease of glucose consumption and uptake was investigated in model cells, compared to the control. Besides, the dose of inductive
agents had little cytotoxicity (Fig. 1A), which demonstrated that a model of IR in hepatic cells was successfully built.

Metformin, as a positive control, showed the best protective effect on glucose consumption, which even had a higher value than the control. Compared with the IR model, MAE significantly increased the glucose consumption by 18.8%, 17.7% and 31.8% at 50, 100 and 250 μg/ml, respectively (Fig. 1B). Additionally, both metformin and MAE at three concentrations were able to avoid the inhibited glucose uptake caused by high glucose and PA, showing similar results with glucose consumption (Fig. 1C and D). In order to investigate further mechanisms, we selected MAE at 100 and 250 μg/ml for following experiments.

3.2. MAE prevented high glucose- and PA-induced hepatic gluconeogenesis

mRNA expression levels of PPARγ coactivator 1α (PGC-1α) and FOXO1 in IR model cells was up-regulated 38% and 40% compared to the control, which were totally repressed in cells treated with metformin and MAE at 100 or 250 μg/ml for 24 h (Fig. 2A and B). Meantime, HepG2 cells with IR exhibited FOXO1 phosphorylated level down-regulation, while p-FOXO1/FOXO1 ratio was augmented when model cells incubated with metformin and MAE (Fig. 2C). High glucose and PA incubation increased enzyme activities of phosphoenolpyruvate carboxykinase (PEPCK; 79%) and glucose-6-phosphatase.
(G6Pase; 37%), and yet this up-regulation was restrained by MAE treatment. As a positive control, metformin showed a best inhibitory effect on both PEPCK and G6Pase activities (Fig. 2D and E).

3.3. MAE inhibited down-regulation of PI3K/AKT pathway induced by high glucose and PA

Gene expression of AKT2 was inhibited and GSK3β was activated in IR model cells, but these adverse effects were improved when cells were treated with MAE (Fig. 3A and B). Moreover, MAE produced a significant increase in p-AKT/total-AKT ratios in a dose-dependent fashion in comparison with IR model HepG2 cells, resulting in GSK3β phosphorylation enhancement, which directly contributed to the activation of glycogen synthase 2 (GYS2). Results of Fig. 3D and E also proved that MAE at 250 μg/ml protected GYS2 expression decrease at both gene and protein levels in cells with IR (1.81-fold and 1.68-fold, vs. IR group). In this line, glycogen content was apparently recovered when cells were treated with metformin or MAE (Fig. 3F). Then we used a specific PI3K inhibitor, LY294002, to pretreat HepG2 cells for 2 h followed by the addition of MAE in the presence of high glucose and PA for 24 h. The ability of MAE on inhibiting down-regulation of AKT phosphorylation in IR model cells was obviously observed, which confirmed that the capacity of MAE on glucose metabolism regulation was PI3K mediated. Effects of MAE on GSK3β and FOXO1 phosphorylation, two AKT downstream targets, were also blocked when cells were exposed to LY294002, suggesting that GSK3β and FOXO1 phosphorylation detected here might well due to AKT (Fig. 4).

3.4. Effects of MAE on serum parameters in db/db mice

At the beginning of the experiment, no significant difference in fasting glucose levels occurred among intervention groups and db/db groups. At the end of the study, db/db mice given a gavage of MAE showed 38.7% and 41.3% lower glucose levels compared to groups given a gavage of water. No obvious difference was observed in body weight and food intake among four groups (Fig. 5A). However, the db/db group exhibited a dramatic increase in serum TG, TC, HDL, LDL, and HDL values compared with the m/m group, which was markedly alleviated by administration of MAE. Higher serum insulin concentrations and homeostatic model assessment (HOMA)-IR indexes were found in db/db group. The adverse effects on these metabolic parameters were mitigated by MAE supplementation. In addition, the db/db group also showed higher concentrations of leptin and lower concentrations of adiponectin that were significantly altered by MAE treatment (Table 1).

3.5. MAE improved glucose homeostasis in plasma, liver and skeletal muscle

At week 7 of the intervention, glucose tolerance was tested and the areas under the curve (AUCs) were 17.7% and 24.3% lower for the MAE-50 mg/kg and MAE-125 mg/kg groups in comparison with the db/db group (Fig. 5B and C). In livers, TG and glycogen contents were reduced by 21%/24% and 68%/55% separately in db/db mice treated with MAE at two doses (Fig. 5D and E). In particular, MAE markedly increased glycogen content in skeletal muscle (Fig. 5F).

3.6. MAE acted through an AKT activation mechanism

In a further attempt to determine the mechanisms, we assessed changes of glucose metabolism-related genes. The db/db mice orally administrated with MAE showed a great increase in mRNA expression of hepatic GYS2, as well as a decrease of GSK3β (Fig. 6A). Furthermore,
MAE resulted in an elevation of GYS1 gene expression in skeletal muscle (Fig. 6B). Particularly, epididymal adipose mRNA expression of leptin was down-regulated while adiponectin was up-regulated in the MAE group compared to the db/db group, which was consistent with changes in serum (Fig. 6C).

AKT, the insulin receptor downstream kinase, plays a major role in glucose homeostasis. Accordingly, we evaluated the effects of MAE supplementation on the motivation of this pathway in db/db mice. MAE produced a 1.34-fold, 4.88-fold and 1.18-fold increase, respectively, at AKT mRNA levels in liver, skeletal muscle, and adipose

Fig. 2. MAE treatment decreased expressions of genes and proteins associated with gluconeogenesis. PGC-1α (A) and FOXO1 (B) mRNA expressions assessed by quantitative RT-PCR. G6Pase (C) and PEPCK (D) activities measured by ELISA kits. Values are expressed as a fold relative to the control condition. (E) phosphorylated and total FOXO1 protein levels in total cell extracts. Results were expressed as the ratio phosphorylated/total protein levels. Equal loading of Western blots was ensured by β-actin. Vertical lines represent standard deviations of three replicates. Values with different letters above are significantly different; *P < 0.05, one-way ANOVA test.
tissues, compared to the db/db group (Fig. 6). Consistently, in the db/db group, p-AKT/total AKT rations in three tissues above were much lower than the m/m group, while MAE inhibited these decreases. Moreover, an up-regulation of GSK3β phosphorylation was detected in liver while a down-regulation was found in muscle in db/db mice compared to the m/m group. In addition, proportion of p-FOXO1 also declined in diabetic mice. However, these adverse effects were recovered by MAE intervention to some extent (Fig. 7).

4. Discussion

A healthy diet is an essential factor to lower the risk of T2DM development. It is necessary to search for certain foods and their bioactive compounds to recover or prevent the manifestation of metabolic disease. Anthocyanins, kinds of a subclass of flavonoids, have flavylium cation structures that are different from other flavonoids [20]. They are of great interest because people often
consumed dark-colored fruits, vegetables and pigmented grains that are abundant in anthocyanins in a normal diet. There has been an accumulation of data demonstrating beneficial effects of anthocyanins on T2DM no matter in epidemiology and clinical trials or laboratory experiments [21,22]. The putative mechanisms underlying antidiabetic potentials of anthocyanins represent a complicated interaction of multiple signaling pathways, enzymes and transcription factors. Mulberry fruits are rich in anthocyanins and MAE we purified revealed three main anthocyanins recognized as C3G (47%), cyanidin-3-rutinoside (27%) and pelargonidin-3-glucoside (1.4%), which was in accordance with other researches [23]. Some compelling studies have suggested that C3G has an effect on activation of AMPK, which is
crucial for insulin sensitivity and glucose homeostasis. Besides, several researchers have found that glucose uptake enhancement by C3G is associated with GLUT4 translocation acceleration and PPARγ activation [24–28]. Nevertheless, the most promising downstream targets are still not exactly identified.

Cells with IR are a better in vitro model to investigate type 2 diabetes and many parameters related to glucose metabolism must be altered, compared to the normal cells. Our previous research was about the direct hypoglycemic effect of MAE on HepG2 cells [14]. In this study, we wanted to further understand the protective effect of

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**Fig. 5. Impact of MAE on weight, food intake, blood glucose, glycogen synthesis and TG content in db/db mice.** (A) Average body weight and food intake. GTT (B) and AUC (C) of mice gavage with distilled water, metformin or MAE at week 7. (D) Liver TG. (E) Liver glycogen content. (F) Muscle glycogen content. Values are mean±S.D., n=8. Means without a common letter differ; P<0.05, one-way ANOVA test.
MAE on HepG2 cells that formed into IR. Therefore, we firstly chose 30 mM glucose plus PA to develop an IR model in HepG2 cells and our observations showed decreases of glucose consumption, glucose uptake and glycogen content in model cells, which were in agreement with previous findings [29–32]. MAE treatments exhibit an evident increase on glucose consumption, glucose uptake and glycogen content in model cells, which means that MAE can mitigate these adverse effects caused by high glucose and PA.

Both PEPCK and G6Pase play vital roles in glucose homeostasis because they catalyze committed steps of gluconeogenesis [33]. FOXO1 is a member of the forkhead family transcription factors and its transcriptional activity is regulated by phosphorylation and intracellular localization. Besides, FOXO1 can directly bind to PEPCK and G6Pase target DNA sequence to regulate their expression levels in liver [34]. Here, we showed that MAE enhanced the phosphorylation of FOXO1 in response to the IR model cells. These changes lead to PEPCK and G6Pase down-regulation involving a decrease of gluconeogenesis and can result in antidiabetes. Guo et al. [36] reported that C3G promoted phosphorylation and nuclear exclusion of FOXO1 in C57BL/6J mice fed a high-fat diet and db/db mice after refeeding. Given this findings along with our present data, we strongly suggest that these beneficial effects of MAE can be due to restrain up-regulation of FOXO1 and PGC-1α in HepG2 cells with IR so that both PEPCK and G6Pase activities were decreased.

db/db mice have a mutation in the leptin receptor gene that caused abnormal splicing, leading to over high level of leptin and insulin [37]. db/db mice are more beneficial for diabetes research since their blood glucose can keep at a high level for a relatively long time, although a diet-induced obesity would be more relevant to human obesity considering the leptin receptor mutation. For this reason, we chose db/db model in order to better investigate the effectiveness and related mechanisms of MAE on antidiabetes in vivo. Adverse metabolic effects associated with T2DM in db/db mice were attenuated after supplemented with MAE at two doses (50, and 125 mg/kg), including fasting blood glucose, GSP, serum insulin and leptin level decline, adiponectin level elevation, and glucose tolerance improvement, although effects were little less than the antidiabetic drug metformin. Leptin is secreted from adipocytes and its increased level results in negative energy balance. Hypothalamus owns receptor which is an important site of leptin action [38]. Leptin can affect the hypothalamus to secrete many hormones to regulate food intake and body weight. However, our results showed that serum leptin level change did not cause weight and food intake changes. We supposed that MAE improved leptin resistance in adipose tissue, leading to decreased serum leptin, accompanied with serum and hepatic TG contents decrease. However, leptin-receptor deficiency was still existent in the hypothalamus so that food intake was not well controlled, explaining that no significant weight change was observed among groups with different treatments in db/db mice even if leptin levels altered apparently. Adiponectin, another fat-derived hormone, is reported to reverse IR [39]. In the meantime, MAE intervention inhibited leptin up-regulation and adiponectin down-regulation at mRNA levels in adipose tissue, resulting in the changes of serum lipids levels. Therefore, we conclude that MAE is able to improve IR by decreasing leptin and insulin secretion as well as increasing serum adiponectin level.

AKT is considered as a key regulator relevant to glucose metabolism including glucose transport, glycogen synthesis and gluconeogenesis suppression [40]. Activation of AKT will lead to an inhibition of GSK3β by phosphorylation, and then subsequently dephosphorylate and activate glycogen synthase so that glycogen synthesis is accelerated. Besides, AKT stimulation can restrain the gluconeogenesis by inhibiting PEPCK and G6Pase expressions and phosphorylating FOXO1 [41]. Treatment of HepG2 cells with high glucose/PA for 24 h induced an obvious impairment in the
Table 1
Serum parameters of the male db/db mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Groups</th>
<th>m/m (ng/ml)</th>
<th>db/db (ng/ml)</th>
<th>Met (ng/ml)</th>
<th>MAE (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>Glucose mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0 week</td>
<td>6.15±0.38d</td>
<td>13.28±3.10d</td>
<td>14.18±3.09d</td>
<td>13.10±4.79d</td>
<td>12.57±4.18d</td>
</tr>
<tr>
<td>8 weeks</td>
<td>7.67±0.70d</td>
<td>16.74±2.20d</td>
<td>8.93±1.07d</td>
<td>10.26±2.11d</td>
<td>9.82±2.10d</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>3.39±0.43d</td>
<td>5.86±0.57d</td>
<td>4.47±1.36d</td>
<td>3.78±0.58d</td>
<td>4.28±0.52d</td>
</tr>
<tr>
<td>HOMA-IR (mM-mU/l)</td>
<td>1.16±0.01d</td>
<td>4.36±0.06d</td>
<td>1.77±0.06d</td>
<td>1.72±0.05d</td>
<td>1.87±0.08d</td>
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<tr>
<td>TG (mM)</td>
<td>0.98±0.13d</td>
<td>1.38±0.53d</td>
<td>0.71±0.13d</td>
<td>0.84±0.15d</td>
<td>0.73±0.14d</td>
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<tr>
<td>GSP (mM)</td>
<td>4.29±0.26d</td>
<td>5.91±1.06d</td>
<td>5.04±0.17d</td>
<td>4.91±0.09d</td>
<td>4.55±0.09d</td>
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<tr>
<td>TCH (mM)</td>
<td>1.82±0.29d</td>
<td>6.04±0.85d</td>
<td>2.47±0.33d</td>
<td>3.71±0.88d</td>
<td>2.89±0.50d</td>
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<tr>
<td>LDL (mM)</td>
<td>0.39±0.09d</td>
<td>2.81±0.19d</td>
<td>1.73±0.31d</td>
<td>1.51±0.30d</td>
<td>1.13±0.24d</td>
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<tr>
<td>Leptin (ng/ml)</td>
<td>5.98±0.63d</td>
<td>8.51±0.70d</td>
<td>6.02±1.28d</td>
<td>5.75±0.93d</td>
<td>5.47±0.58d</td>
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<tr>
<td>Adiponectin (ng/ml)</td>
<td>1064.36±91.85ab</td>
<td>803.83±47.54c</td>
<td>1088.91±140.34ab</td>
<td>1168.24±185.27a</td>
<td>1018.47±52.16b</td>
</tr>
</tbody>
</table>

Values are mean±S.D., n=10. Means without a common letter differ; P<.05. m/m, nondiabetic lean littermates; db/db, db/db mice gavage with water; Met, db/db mice gavage with 200 mg/kg/day metformin; MAE, db/db mice gavage with 50 or 125 mg/kg/day MAE.

Fig. 6. Effects of MAE on gene expressions in liver, skeletal muscle and adipose tissues from db/db mice. (A) mRNA levels of AKT2, GSK3β and GYS2 in liver. (B) mRNA levels of AKT2, GSK3β and GYS1 in muscle. (C) mRNA levels of AKT2, adiponectin and leptin in epididymal adipose tissue. Results were normalized against β-actin (liver and adipose) or GAPDH (muscle) and expressed relative to m/m mice. Data represent mean±S.D. of three independent experiments. Values having different superscripts are significantly different; P<.05, one-way ANOVA test.
phosphorylated levels of AKT and GSK3β, as well as GYS2 content, whereas this inhibition was recovered by MAE, explaining a rise of glycogen content. However, the change tendency of hepatic glycogen content in mice was different from the result in cells. Chan et al. [42] have studied hepatic carbohydrate metabolism in db/db mice and they demonstrated that hepatic glycogen turned over more rapidly compared to their normal littermates. In the current study, the supplementation of MAE lowered the hepatic glycogen content in db/db mice and results were consistent with changes on phosphohorylation of GSK3β and FOXO1 in the liver, indicating that these alterations seemed to be related to gluconeogenesis suppression. Meanwhile, in skeletal muscle, phosphohorylated AKT expression was partially recovered in MAE-treated db/db mice accompanied with increased levels of GSK3β phosphohorylation, resulting in an increase of GYS1 level which explained the recovery of glycogen concentration.

PI3K consists of a catalytic subunit (p85) that is required for tyrosine-phosphorylated receptor tyrosine kinases and a regulatory subunit (p110) that is coupled with G-protein-mediated activation of itself. It is regarded as a second messenger, playing a crucial role in cellular chemical signals transfer. AKT is one of the most important downstream nodes, and the PI3K/AKT pathway is involved in the regulation of glucose metabolism at several levels [43,44]. MAE produced a distinct reduction in values of p-AKT/total-AKT, p-GSK3β/total GSK3β and p-FOXO1/total FOXO1 in cells pretreated with LY294002, compared with the cells without the inhibitor, demonstrating that the ability of MAE on the protection of IR is PI3K-dependent.

![Fig. 7. Effects of MAE on protein expression in liver, skeletal muscle and adipose tissues from db/db mice. (A) Phosphorylated and total AKT, GSK3β and FOXO1 protein levels in liver. (B) Phosphorylated and total AKT and GSK3β protein levels in muscle. (C) Phosphorylated and total AKT protein levels in epididymal adipose tissue. (D) Bands were quantified and results were expressed as the ratio phosphorylated/total protein levels, and expressed relative to control values (m/m mice). Data represent mean±S.D. of four mice per group. Values having different superscripts are significantly different; P<.05, one-way ANOVA test.](image-url)
In conclusion, using in vitro studies, we suggest that activation of the PI3K/AKT pathway by MAE significantly mitigates high glucose/PA-induced IR in HepG2 cells. In vivo studies, we present evidence that MAE also activates AKT phosphorylation and its downstream targets in insulin-sensitive tissues, which are associated with metabolic changes regulation in db/db mice. The beneficial effects can be in part due to serum adiponectin level increase as well as leptin and insulin level decrease. Therefore, MAE may serve as a strategy to ameliorate IR and metabolic disorders with T2DM.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2016.07.004.

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