# Protective effects of asiatic acid in a spontaneous type 2 diabetic mouse model

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**Abstract.** Asiatic acid (AA) has been demonstrated to exhibit anti-diabetic activity. However, the mechanisms and underlying signaling pathways remain to be elucidated. The present study was performed to confirm the protective effect of AA and demonstrate its ability to regulate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/glycogen synthase kinase-3β (GSK-3β) signaling pathway in db/db mice. Db/db mice fed on a high-fat diet were used to model diabetes mellitus. Modeled mice were divided randomly into the model control, pioglitazone hydrochloride tablet (PH) and AA groups. Age-matched C57 BL/6J mice served as normal controls. Lipid and glucose levels, and glycogen synthesis rates were assessed following treatment. Pathological changes were detected using hematoxylin and eosin staining. Expression of the PI3K/AKT/GSK-3β signaling pathway at the mRNA level was measured using quantitative polymerase chain reaction analysis. The model control group revealed typical characteristics of obesity and diabetes, including high glucose and lipid levels, and decreased glycogen synthesis. Four weeks of treatment with AA or PH ameliorated these abnormalities.

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AA and PH treatments mitigated the upregulation of PI3K, AKT, insulin receptor, and insulin receptor substrate-1 mRNA expression in modeled mice. Furthermore, AA and PH treatments decreased GSK-3β and glucose-6-phosphatase mRNA expression compared with the normal control group. The results of the present study confirmed that AA possesses anti-diabetic activity in db/db mice. The PI3K/AKT/GSK-3β signaling pathway may mediate this protective effect.

## Introduction

Diabetes mellitus is among the most common chronic metabolism diseases, and its incidence continues to increase. Epidemiologically, 250 million patients are diagnosed with diabetes annually, and this number is projected to increase to about 380 million by 2025 (1). Diabetes mellitus is divided into two subgroups: type 1 diabetes mellitus (T1DM) and types 2 diabetes mellitus (T2DM). T1DM is an autoimmune disease characterized by destruction of islet beta cells in the pancreas, which results in the complete cessation of insulin production (2). By contrast, T2DM is a heterogeneous disorder caused by a progressive decline in insulin resistance in the liver and peripheral tissues, accompanied by the inability of beta cells to compensate for insulin resistance (3). Abnormality of glucose metabolism is the central characteristic of diabetes mellitus. Accordingly, the pathogenesis of glucose metabolism is a major topic of research in this field.

The key enzymes, glucose-6-phosphatase (G-6-P) and glycogen synthase kinase-3 (GSK-3) regulate glycogen and gluconeogenesis. G-6-P is the rate-limiting enzyme for the transformation of glucose-6-phosphate to glucose, affecting glycogen output and blood glucose level (4). GSK-3 is the rate-limiting enzyme responsible for gluconeogenesis (5). Two types of GSK-3-GSK-3α and GSK-3β-have been reported to regulate G-6-P expression (6). GSK-3β can phosphorylate glycogen synthase and inhibit the synthesis of glycogen. In turn,

this process leads to the phosphorylation of insulin receptor substrate-1 (IRS-1), interrupting insulin signaling and producing insulin resistance (7). Thus, the regulation of key enzymes involved in the inhibition of glycogen synthesis is a critical step in the search for an effective treatment for diabetes mellitus.

T2DM is a multifactorial disease, with genetic and environmental factors contributing to its development (8). Although western medicine has achieved much improvement in T2DM medications, side effects and high costs continue to pose challenges (9). As a chronic disease, T2DM requires long-term treatment, and side effects such as weight gain, bone loss, and increased cardiovascular risk are unavoidable. Moreover, continuous health care utilization poses huge economic burdens on societies and families. Hence, alternative agents with fewer side effects and lower costs are urgently needed. Herbal medications can be good alternatives, replacing or at least supplementing western medications (10,11). Because most of these medications are extracted from plants, they are characterized by low cost and few side effects. In Chinese medicine, several thousand years of history and experience in the use of herbal medications to prevent and treat T2DM have been documented.

*Centella asiatica* (*C. asiatica*) has been used widely in Chinese medicine to treat varicose veins and chronic venous insufficiency, and in ointments to treat psoriasis and help heal minor wounds (12). Asiatic acid (AA) is an active component of *C. asiatica* with many biological activities, including anti-oxidant (13), liver-protecting (14), lipid-lowering (15), anticancer (16), and anti-diabetic (17) effects. Mechanical studies have suggested that AA lowers glucose levels through anti-inflammatory action, regulation of glucose metabolism enzymes, and anti-fibrotic action in the pancreatic islets (15,18). However, the signaling pathway involved in the effects of AA on glucose level in diabetes mellitus remains unknown.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is important in the regulation of the insulin signaling cascade (19). The activation of PI3K/AKT negatively mediates GSK-3β and affects glycogen synthesis. In this study, a T2DM (db/db) mouse model was used to evaluate the PI3K/AKT/GSK-3β signaling pathway involved in the protective effects of AA on diabetes symptoms.

## Materials and methods

*Chemicals and reagents*. AA (lyophilized powder) was purchased from Chengdu PuRuiFa Technology Development Co. Ltd (Chengdu, China). Pioglitazone hydrochloride (PH) (batch no. Zhunzi H20040631) was purchased from Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China). Assay kits for glucose (glucose oxidase), total cholesterol (COD-PAP), triglycerides (TG; GOP-PAP), high-density lipoprotein (HDL) cholesterol (direct hydrogen peroxide scavenging method), and low-density lipoprotein (LDL) cholesterol (direct surfactant removal method), and hematoxylin and eosin (H&E) and glycogen staining kits were obtained from Beijing LEYBOLD Cable Technology Co., Ltd. (Beijing, China). An immunohistochemical kit and insulin receptor (InsR) antibody were obtained from Boosen Biological Technology Co., Ltd. (Beijing, China).

*Animal modeling and treatments*. Twelve-week-old male db/db mice and age-matched male C57BL/6J mice were provided by the Model Animal Research Center of Nanjing University. This study was approved by the ethics committee of Beijing University of Chinese Medicine. All animals were housed at the Beijing Animal Experimental Center at a temperature of 23±2°C and humidity of 55±10% with a 12/12-h light/dark cycle. The animals were provided with food and water *ad libitum*. Db/db mice were fed a full-formula high-fat diet (composition: basal diet, cholesterol, egg-yolk powder, lard, bile salt). C57BL/6J mice were fed a common basal diet. After 1 week of feeding, tail blood was collected. A non-random blood glucose concentration >11.1 mmol/l was considered to indicate successful diabetic modeling.

Eighteen modeled mice were divided randomly into control, PH, and AA groups. C57BL/6J mice were used as normal controls and were given a normal diet. The other mice were fed a high-fat diet. PH was used as the positive control. PH (12 mg/kg) and AA (50 mg/kg) were administered orally once per day for 4 consecutive weeks. Saline served as the negative control. The animals' general condition (mental state, activity, hair color) was observed and body weight was recorded each week. After 4 weeks of treatment, the mice were fasted for 12 h. Tail blood was collected for the measurement of blood glucose and performance of oral glucose tolerance test (OGTTs) and insulin tolerance test (ITTs) at 0, 30, 60, and 120 min. Arterial blood was obtained for the measurement of fasting plasma glucose (FPG), carbohydrate (CHO), TG, HDL, LDL, insulin, and free fatty acids (FFAs). Insulin resistance was calculated using the following formula: homeostasis model assessment-estimated insulin resistance (HOMA-IR) index=glucose level x serum insulin level/22.5 (20).

*H&E and periodic acid-Schiff staining*. Liver tissue fixed in paraformaldehyde was used for H&E and periodic acid-Schiff (PAS) staining. After fixation in 10% neutral formalin, the tissues were embedded in paraffin, sectioned (5-µm slice thickness) and stained with H&E or PAS.

*Immunohistochemistry*. Fixed liver tissue was cryoprotected in 30% sucrose for 1 h at 4°C and sectioned at 20-µm intervals with a freezing microtome (Leica, Mannheim, Germany). The sections were then incubated with the primary antibody (1:1,000) at 4°C overnight. After washout with phosphate-buffered saline, the second antibody was added and incubated for 2 h at room temperature. After 3,3-diaminobenzidine (DAB) colorization, the slices were covered and observed under a microscope (Olympus Corp., Tokyo, Japan). The optical density was analyzed using Image-Pro Plus software.

*Quantitative PCR*. Total RNA was extracted from liver tissues using TRIzol reagent. RNA concentrations were determined spectrophotometrically, and 1 µg total RNA was reverse transcribed using an avian myeloblastosis virus reverse-transcriptase kit (Promega Corp., Madison, WI, USA). PCR primers were as follows: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5'-TGAAGCAGGCATCTGAGGG-3' (sense), 5'-CGAAGGTGGAAGAGTGGGAG-3' (antisense); InsR: 5'-TTTGTCATGGATGGAGGCTA-3' (sense), 5'-CCT CATCTTGGGGGTTGAACT-3' (antisense); IRS-1: 5'-TCCTAT CCCGAAGAGGGTCT-3' (sense), 5'-TGGGCATATAGCCAT CATCA-3' (antisense); PI3K: 5'-GCTCCTGGAAGCCAT
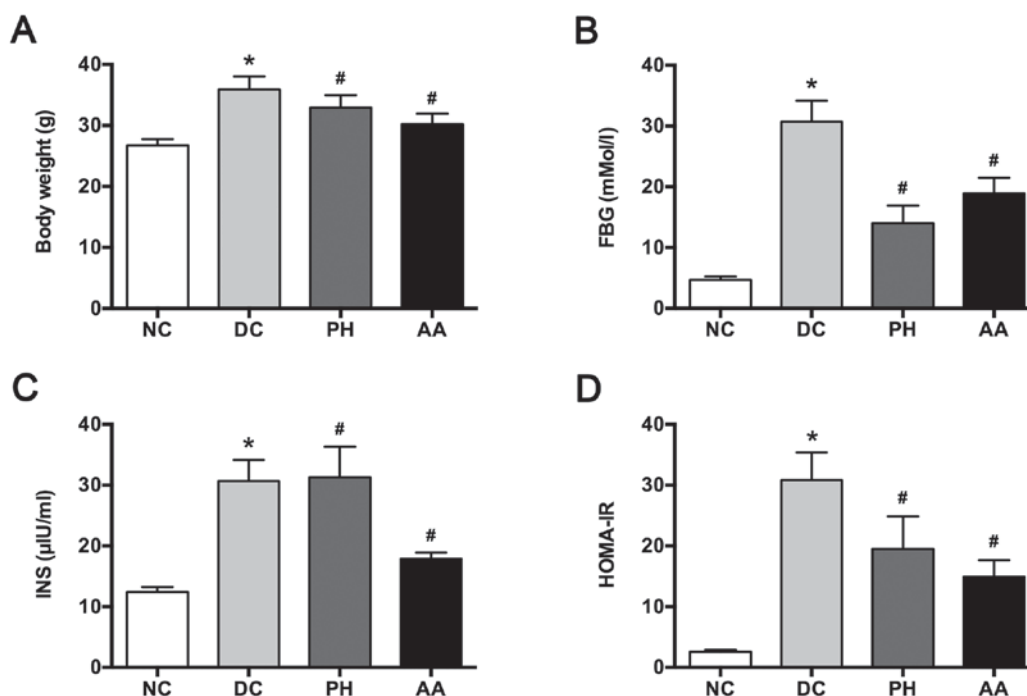
Figure 1. Effects of AA on (A) body weight, (B) FPG, (C) INS, and (D) HOMA-IR. Values are mean ± SD for six mice. *P<0.05 vs. NC, #P<0.05 vs. DC. NC, normal control; DC, diabetic control; PH, pioglitazone hydrochloride tablets; AA, asiatic acid; FPG, fasting plasma glucose; INS, insulin; HOMA-IR, homeostasis model assessment-estimated insulin resistance.

TGAGAA-3' (sense), 5'-CGTCGATCATCTCCAAGTCCAC-3' (antisense); Akt-1: 5'-CCCTTCTACAACCAGGACCA-3' (sense), 5'-ATACACATCCTGCCACACGA-3' (antisense); GSK-3β: 5'-TATTTCCTGGGGACAGTGGT-3' (sense), 5'-ATTTGCTCCCTTGTTGGTGT-3' (antisense); G-6-P: 5'-AGCTCCGTGCCTATAATAAAGCAG-3' (sense), 5'-CATACGTTGGCTTTTTCTTTCCTC-3' (antisense). The amplification reactions were carried out with a 7500 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA), with initial hold steps (50°C for 2 min, followed by 95°C for 10 min) and 40 cycles of a two-step PCR (95°C for 15 sec and 60°C for 1 min). The comparative computed tomography method was used to determine the amount of target, normalized to an endogenous reference (GAPDH) and relative to a calibrator ($2^{-\Delta\Delta Cq}$).

*Statistical analyses*. Data are presented as means ± standard deviations. One-way analysis of variance with post hoc Bonferroni tests for multiple comparisons was performed. P<0.05 was considered to indicate a statistically significant difference.

## Results

After 4 weeks of treatment, mice in the normal control group displayed normal activity and appearance. In contrast, the activity and appearance of mice in the model control group were abnormal. AA and PH treatments ameliorated those abnormalities. Body weight increased significantly after modeling compared with the normal control (P<0.05), AA and PH treatments decreased body weight significantly (P<0.05; Fig. 1A). The FPG level was elevated in the model group, but this condition was reversed significantly by the AA and PH treatments

(Fig. 1B). The increased INS level after modeling was attenuated by AA, but aggravated by PH (Fig. 1C). HOMA-IR values were elevated significantly in the model control group compared with the normal control group (P<0.05; Fig. 1D). PH and AA treatments significantly attenuated this elevation (P<0.05).

OGTT and ITT values were also higher in the model control group than in the normal control group at each time point (P<0.05). AA and PH treatments reversed those abnormalities (P<0.05 *vs*. model group; Fig. 2).

After 4 weeks of treatment, serum CHO, TG, HDL, LDL, and FFA levels were markedly elevated in the model group. AA treatment attenuated CHO, HDL, and FFA levels, and PH treatment attenuated CHO, TG, and FFA levels (P<0.05; Table I).

H&E staining showed that hepatic cells were arranged radially around the central vein in the normal control group (Fig. 3). Cellular structure was normal, with centralized nuclei. In the model control group, H&E staining displayed steatosis in hepatic cells. Moreover, the cells were arranged irregularly. Portal-area inflammation infiltration was observed in the model control group. AA and PH treatments ameliorated these pathological changes, and steatosis disappeared. Hepatic cells were arranged regularly in these groups, and minimal inflammation infiltration was observed in the portal area.

PAS staining revealed a large number of purple glycogen granules in hepatic cells in the normal control group (Fig. 4). In contrast, few such granules were found in the model control group. AA and PH treatments achieved remarkable recovery of glycogen, although the numbers of granules remained smaller than in the normal control (P<0.05).

Liver InsR, detected by immunohistochemical staining, was expressed mainly on cell membranes (Fig. 5). After 4 weeks of treatment, InsR expression was reduced significantly in the model group compared with the normal control.

Table I. Effects of asiatic acid on lipid levels in db/db mice.

| Group | n | CHO (mM) | TG (mM) | HDL (mM) | LDL (mM) | FFA (mM) |
|---|---|---|---|---|---|---|
| NC | 6 | 1.80±0.24 | 0.62±0.13 | 0.76±0.11 | 1.03±0.14 | 0.40±0.01 |
| DC | 6 | 2.40±0.61[a] | 1.24±0.21[a] | 0.56±0.15[a] | 1.55±0.55[a] | 0.49±0.04[a] |
| PH | 6 | 1.86±0.20[b] | 0.88±0.26[b] | 0.63±0.20 | 1.32±0.12 | 0.56±0.06[b] |
| AA | 6 | 1.67±0.46[b] | 1.21±0.27 | 0.75±0.14[b] | 1.53±0.26 | 0.45±0.01[b] |

Data are presented as mean ± standard deviation. [a]$P<0.05$ vs. NC, [b]$P<0.05$ vs. DC. NC, normal control; DC, diabetic control; PH, pioglitazone hydrochloride tablet; AA, asiatic acid; CHO, cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FFA, free fatty acids.
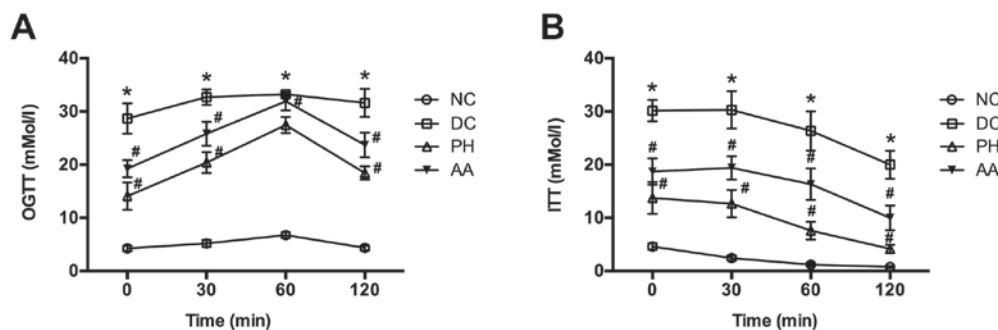


Figure 2. Effects of AA on (A) OGTT and (B) ITT values. Values are mean ± SD for six mice. *$P<0.05$ vs. NC, #$P<0.05$ vs. DC. NC, normal control; DC, diabetic control; PH, pioglitazone hydrochloride tablets; AA, asiatic acid; OGTT, oral glucose tolerance test; ITT, insulin tolerance test.
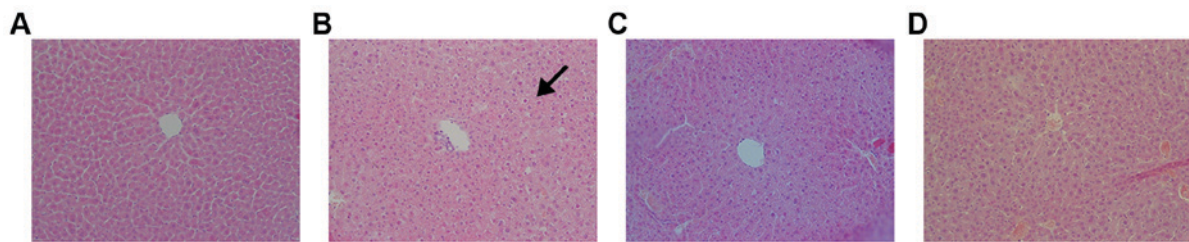


Figure 3. Liver hematoxylin and eosin staining in db/db mice in different groups. (A) Normal control, (B) diabetic control, (C) pioglitazone hydrochloride tablets, and (D) asiatic acid. Magnification, x200.
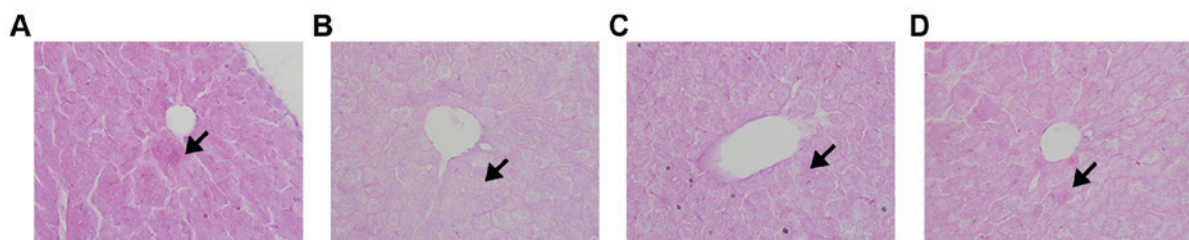


Figure 4. Liver periodic acid-Schiff staining in db/db mice in different groups. (A) Normal control, (B) diabetic control, (C) pioglitazone hydrochloride tablets, and (D) asiatic acid. Magnification, x400.

By contrast, AA and PH treatments significantly elevated InsR expression ($P<0.05$).

InsR, IRS-1, PI3K, and Akt-1 expressions were increased significantly in the model group compared with the normal control group ($P<0.05$; Fig. 6). After 4 weeks, AA and PH treatments significantly mitigated the expressions of InsR, IRS-1, PI3K, and Akt-1 ($P<0.05$). In addition, diabetic modeling elevated G-6-P and GSK3β expression, which was reversed by AA and PH treatments ($P<0.05$).

## Discussion

The db/db mouse model is used widely to study spontaneous T2DM (21). Genetically, leptin gene mutation leads to
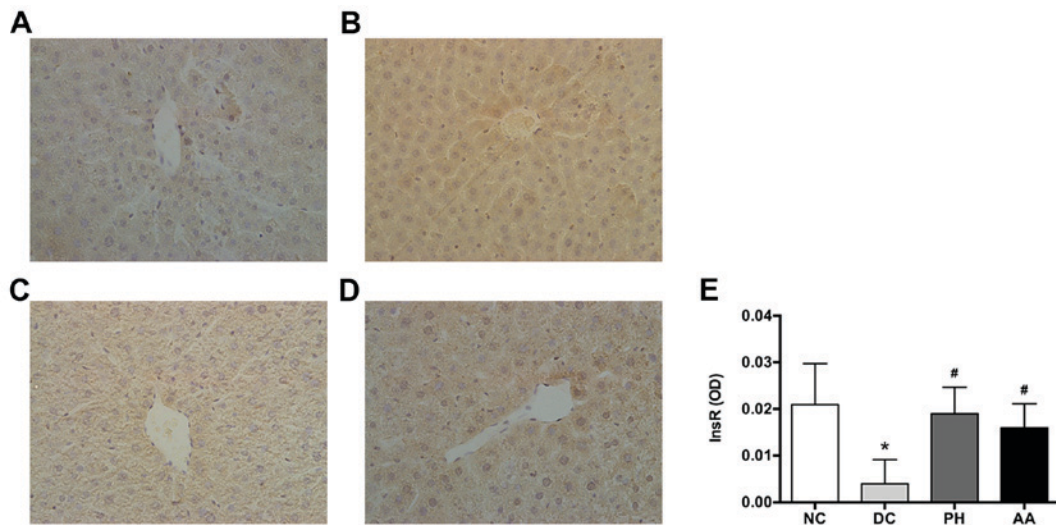
Figure 5. Effect of asiatic acid (AA) on InsR expression. (A) Normal control (NC), (B) diabetic control (DC), (C) pioglitazone hydrochloride tablets (PH), (D) asiatic acid (AA), and (E) quantification of OD. Values are means ± SDs for six mice. $^{*}P<0.05$ vs. NC, $^{#}P<0.05$ vs. DC.



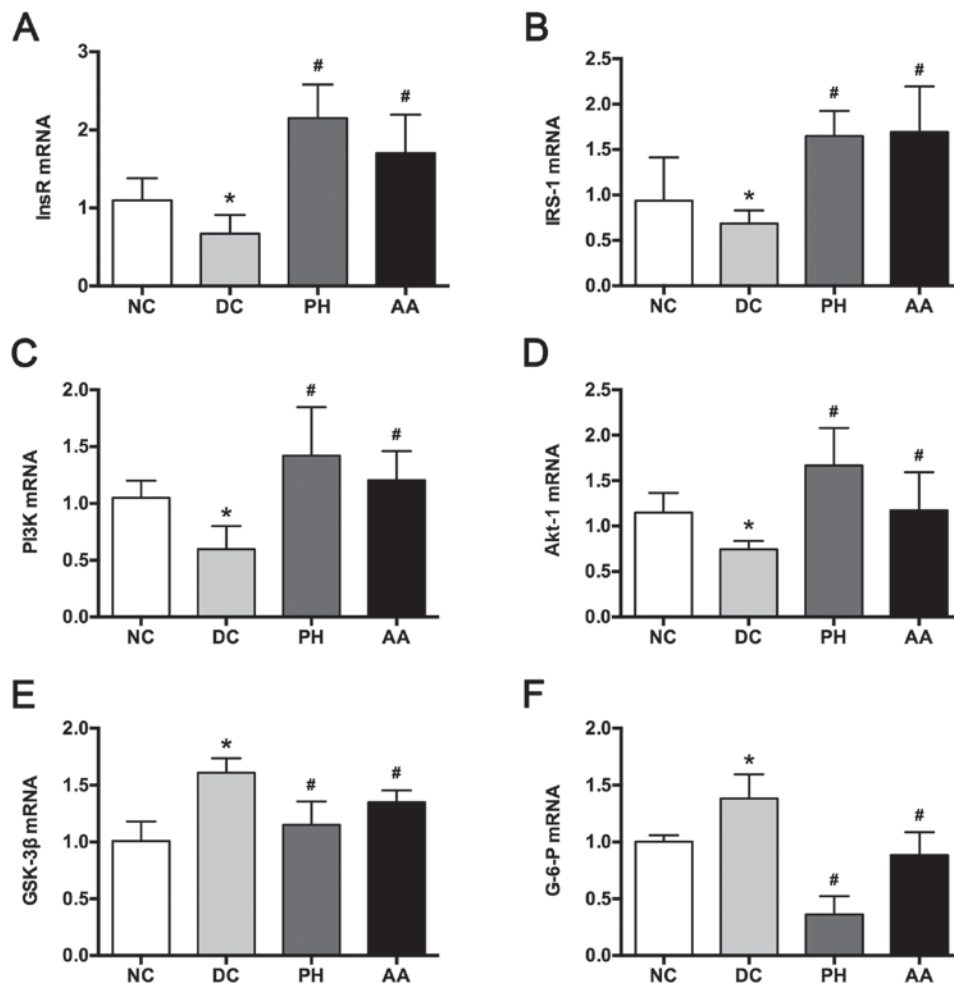Figure 6. Effects of AA on (A) InsR, (B) IRS-1, (C) PI3K, (D) Akt-1, (E) GSK-3β and (F) G-6-P mRNA expression. Values are mean ± SD for six mice. $^{*}P<0.05$ vs. NC, $^{#}P<0.05$ vs. DC. NC, normal control; DC, diabetic control; PH, pioglitazone hydrochloride tablets; AA, asiatic acid; InsR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3K, phosphatidylinositol 3-kinase; Akt-1, protein kinase B; GSK-3β, glycogen synthase kinase-3β; G-6-P, glucose-6-phosphatase.

abnormality of the leptin signaling pathway. Major symptoms that appear in this model include obesity, insulin resistance, high glucose levels, and fatty liver, which are always found in 6-week-old and typically displayed in 8-12-week-old mice (22). T2DM is characterized by absolute or relative deficiency of insulin secretion. Previously, AA was reported

to mitigate the symptoms of streptozotocin (STZ)-induced diabetes, possibly through anti-oxidant and anti-inflammatory actions, amelioration of glycogen metabolism, protection of islets, and insulin resistance (23). In the present study, we further demonstrated the protective effects of AA against diabetic symptoms in a genetic model. Moreover, we showed that the PI3K/AKT/GSK-3β signaling pathway plays a role in these protective effects.

Typical symptoms of diabetes were observed in 6-week-old db/db mice fed a high-fat diet, including elevated FPG, INS, HOMA-IR, and glucose levels and ITT values. The protective effects of AA observed in this model provide support for the potential effects of this acid in glucose metabolism. In addition to its support of glucose control, AA also decreased FFA and CHO levels and increased HDL levels. In combination with morphological findings, these results suggest that AA is capable of lowering lipid levels. Ramachandran also reported that AA prevents lipid peroxidation in STZ-induced diabetes (24).

Glucose metabolism is regulated by the insulin signaling pathway. When combined with InsR, insulin initiates the phosphorylation of its intracellular substrates. PI3K serves as a docking protein for InsR. Activation of PI3K and its downstream protein kinase B (Akt) is essential for almost all insulin-induced glucose or lipid metabolism, such as glucose uptake, glycogen synthesis, and suppression of triglyceride synthesis (25,26). GSK-3 differs from other kinases in that it remains active in its dephosphorylated form. It is inactivated by phosphorylation by other protein kinases, such as AKT. Insulin activates Akt to inhibit the activity of GSK-3α or GSK-3β isoforms by phosphorylating their $NH_2$-terminal serine residues, terminating their inhibition of glycogen synthase (27). GSK-3β is the predominant regulator of glycogen synthase in skeletal muscle (28). In diabetes, GSK-3β level and activity are increased (29). In the present study, we demonstrated for the first time that AA decreased PI3K and AKT expression and regulated GSK-3β expression in diabetes.

G-6-P is a rate-limiting enzyme for gluconeogenesis and glycogen decomposition, which directly affects the hydrolysis of G-6-P to glucose. Consistent with previous reports (30), we showed that G-6-P expression was up-regulated in our diabetic model. Considering the inhibition of G-6-P by PI3K, GSK-3β might be important in this process. The effects of AA on G-6-P expression may be exerted *via* PI-3K, AKT, and GSK-3β.

Although our data support the effect of AA on PI3 K/AKT/GSK-3β in ameliorating diabetic symptoms, direct evidence of how AA functions through PI3K/AKT/GSK-3β remains lacking. AA and its derivatives as potential inhibitors of glycogen phosphorylases (31). Future studies should examine the mechanism by which AA regulates the expression of PI3K/AKT/GSK-3β.

In conclusion, we demonstrated that AA had clear lipid- and glucose-lowering effects in a db/db mouse model. AA functions through PI3K/AKT/GSK-3β to facilitate glycogen synthesis. This experimental evidence provides new clue for the development of anti-diabetic drugs.

## Acknowledgements

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