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Original Paper

Antidiabetic Effect of Salvianolic Acid A on Diabetic Animal Models via AMPK Activation and Mitochondrial Regulation

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Key Words
Salvianolic acid A • Diabetes mellitus • Antidiabetic • Mitochondria • AMPK • ATP • Mitochondrial membrane potential

Abstract
Background/Aims: Diabetes mellitus (DM) characterized by hyperglycemia contributes to macrovascular and microvascular complications. Salvianolic acid A (SalA) is a polyphenolic compound isolated from the root of *Salvia miltiorrhiza* Bunge, which is a traditional Chinese medicine widely used to treat cardiovascular diseases. However, little is known about its antidiabetic effect. Our study aimed to investigate the *in vivo* and *in vitro* antidiabetic effect of SalA and the underlying mechanisms. ***Methods:*** Alloxan-induced type 1 diabetic mice and high-fat diet (HFD) and low-dose streptozotocin (STZ)-induced type 2 diabetic rats received SalA treatment. Blood glucose, oral glucose tolerance test (OGTT), 24-h food and water intake were monitored. *In vitro,* glucose consumption and uptake were measured in HepG2 cells and L6 myotubes. Mitochondrial function was detected in hepatic and skeletal muscle mitochondria. AMP-activated protein kinase (AMPK) and Akt were analyzed by western blot. ***Results:*** In both type 1 and type 2 diabetic animals, SalA lowered fasting blood glucose (FBG) and fed blood glucose in dose-dependent manner, as well as reduced 24-h food and water intake. *In vitro,* SalA caused dose-dependent increase in glucose consumption and enhanced glucose uptake. SalA significantly increased ATP production from 10 min to 12 h in HepG2 cells and L6 myotubes. Interestingly, SalA decreased mitochondrial membrane potential (MMP) in HepG2 cells. Furthermore, SalA improved hepatic and skeletal muscle mitochondrial function, increased ATP production, and concurrently decreased MMP. In particularly, SalA activated AMPK phosphorylation through Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMPK signaling pathway, independent of liver kinase 1 (LKB1)/AMPK pathway.

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However, SalA didn't show any effect on insulin secretagogue and activation of PI3K/Akt signaling pathway. ***Conclusion:*** SalA exhibits the antidiabetic effects in diabetic animal models through improving mitochondrial function, increasing ATP production, and decreasing MMP via CaMKKβ/AMPK signaling pathway.

Introduction

Diabetes mellitus (DM) and its complications are running rampantly in the world, which is associated with increased morbidity and mortality. As hyperglycemia is a fundamental abnormality contributing to macrovascular and microvascular complications, glucose-lowering strategy is very important in the treatment of type 2 diabetes to reduce the risk for diabetes complications [1, 2]. Since oral antidiabetic agents were available in clinic 60 years ago, more attentions have been focused on hypoglycemic efficacy. However, more and more emerging drug safety issues have gained extensive concerns [3]. The use of metformin is contraindicated in patients with renal dysfunction [4]. In addition, food and drug administration (FDA) issued a new warning about the side effects of thiazolidinediones (TZDs), such as weight gain, edema, heart and liver failure. Therefore, development of new antidiabetic agents with greater safety and efficacy is of great necessity. Recently, mitochondrial function and AMP-activated protein kinase (AMPK) are known to be closely involved in glucose metabolism, which have been regarded as the potential therapeutic targets for diabetes and diabetes complications.

Medicinal plants have been identified and widely used for the treatment of various diseases in developing countries [5, 6]. Plants have the ability to synthesize a wide variety of chemical compounds, which give a great opportunity for the discovery of antidiabetic agents. Salvianolic acid is a polyphenolic compound isolated from the root of *Salvia Miltiorrhiza* Bunge, which is a traditional Chinese medicine and widely used in the Asian countries for the treatment of cardiovascular and cerebrovascular diseases with well-known safety [7]. Salvianolic acid A (SalA)(2R)-3-(3,4-Dihydroxyphenyl)-2-[(E)-3-[2-[(E)-2-(3,4-dihydroxyphenyl) ethenyl]-3,4-dihydroxyphenyl]prop-2-enoyl]oxypropanoic acid) has been reported to have extensive pharmacological effects including antioxidant, anti-platelet aggregation, anti-cerebral and myocardial ischemia [8-12]. Salvianolic acid B, as one of salvianolic acid, was found to protect against high-fat diet-induced obesity recently [13]. In our previous studies, we have demonstrated that SalA ameliorated diabetes complications, such as vascular disease and peripheral neuropathy [14-16].

Here, our study reported that SalA lowers blood glucose, improves glucose tolerance, as well as reduces food and water intakes. Further studies demonstrated that SalA regulates glucose metabolism by increasing ATP production, decreasing mitochondrial membrane potential (MMP) and improving mitochondrial function via Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ)/AMPK signaling pathway, not phosphatidyl inositol 3' kinase (PI3K)/Akt signaling pathway. All these results indicated that SalA might be a novel and promising drug candidate for diabetes treatment.

Materials and Methods

Chemicals

Compounds were purchased from the following sources: Metformin was from Bristol-Myers Squibb Co., China. SalA is a water-soluble component from *Salvia Miltiorrhiza* Bunge, which was isolated by Beijing Collab Co., China as a lyophilized powder, with a purity of over 94% by HPLC analysis.

Experimental Animals

Male ICR mice (18-20 g, 8 weeks old) and male Wistar rats (180-200 g, 8 weeks old) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The animals were housed under standard environmental conditions (22-25 ℃, humidity 60-70%, 12 h light/dark cycles). This study was

carried out in accordance with the Guide for the Animal Care & Welfare Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences.

Type 1 diabetic mice and treatments

After fasted for 16 h, male ICR mice were injected intravenously with 63 mg/kg alloxan (Sigma, USA). After 72 h, mice with fasting blood glucose (FBG) higher than 10 mM were considered as diabetic mice. The diabetic mice were orally administered with SalA (0.4, 1.3 mg/kg), metformin (200 mg/kg), or vehicle (DM group) once a day for 15 days. Age-matched mice were treated with vehicle as normal control (NC) group.

Type 2 diabetic Rats and treatments

Male Wistar rats were randomly divided into NC group and diabetic group. NC rats were fed with standard diet (41.47% carbohydrate, 14.42% fat, 21.06% protein), while rats in diabetic group were fed with high-fat and high-sucrose diet (standard rat diet supplemented with 10% sucrose, 10% lard, 2% cholesterol, and 0.2% cholic acid) for 4 weeks, and then injected intraperitoneally with streptozotocin (STZ, 30 mg/kg, Sigma, USA) [17-19]. Seven days after STZ injection, the animals with FBG higher than 10 mM were considered as diabetic rats. The diabetic rats were randomly divided into 4 groups: untreated diabetic group (DM group), metformin-treated diabetic group (Metformin 150 mg/kg) and SalA-treated diabetic group (SalA 1 and 3 mg/kg). All diabetic rats were fed with a high-fat diet (standard rat diet supplemented with 10% lard, 2% cholesterol, 0.2% cholic acid), while NC rats were fed with a standard diet for 4 weeks.

Measurements of blood glucose, glycosylated hemoglobin and oral glucose tolerance test

Blood glucose and glycosylated hemoglobin (GHB) were tested according to manufacturer's instruction (Beijing Biosino Co, China). To determine FBG, animals were fasted for 4 h. Fed blood glucose samples were collected without fasting and free to feed. Oral glucose tolerance test (OGTT) was performed after the animals were fasted for 4 h followed by glucose solution (2.0 g/kg) by oral gavage. The blood samples were collected at 0, 30, 60, 120 min after glucose loading.

Cell Culture

L6 cells (rat myoblast cell line) were maintained in Minimal Essential Medium (MEM) supplemented with 10% FBS at 37 °C, 5% CO_2. To induce cells differentiation, medium was switched to MEM with 2% FBS for 7 days after complete confluence. The medium was changed every two days. HepG2 cells (human hepatoma cell line), MIN6 cells (mouse insulinoma cell line) and HeLa cells (human cervical cancer cell line) were from National Platform of Experimental Cell Resources for Sci-Tech, Chinese Academy of Medical Sciences. They were cultured in DMEM (5.5 or 25.5 mmol/L) supplemented with 10% FBS at 37 °C, 5% CO_2.

Glucose consumption and glucose uptake

Glucose consumption was conducted as described previously [20]. In brief, HepG2 cells or differentiated L6 myotubes in 96-well plate were treated with different concentrations of SalA (10^{-8}-10^{-6} M) and insulin (3×10^{-8} M) in DMEM or MEM medium (5.5 mM glucose) at 37 °C, 5% CO_2. After treatment for 24h, the supernatant was taken for glucose assay.

The glucose uptake was performed with the radioactive substances [^{3}H]-2-deoxy-D-glucose (Perkin Elmer, USA) as described previously [21]. In brief, differentiated L6 myotubes in 24-well plate were starved with serum-free MEM for 2 h, and pretreated with cytochalasin B (20 μM, Serva, German) for 5 min as non-specific uptake. The cells were then washed and incubated with 400 μl of Krebs-Ringer phosphate–HEPES buffer (131.2 mmol/L NaCl, 4.71 mmol/L potassium chloride, 2.47 mmol/L $CaCl_2$, 1.24 mmol/L magnesium sulfate, 2.48 mmol/L monosodium phosphate, 10 mmol/L HEPES, and 0.5% BSA [pH 7.45]) containing different concentrations of SalA (10^{-8}-10^{-6} M) or insulin (10^{-7} M, Sigma, USA) for 30 min at 37 °C. After adding 100 μl of 2-deoxy-D-glucose (50 μM, Sigma, USA) with [^{3}H]-2DG (0.2 μCi/well) for 10 min, uptake was terminated by washing three times with ice-cold Krebs-Ringer phosphate–HEPES buffer and solubilized in 0.2 mol/L sodium hydroxide (NaOH). The radioactivity was measured by scintillation counter (Perkin Elmer, USA).

Measurement of ATP production

Intracellular ATP level was determined by CellTiter-Glo® kit (Promega, USA) according to manufacturer's instruction. Briefly, HepG2 cells or L6 myotubes were treated with different concentrations

of SalA (10^{-9}-10^{-6} M) for 10 min to 12 h. Then, 100 µl CellTiter-Glo™ reagent was added and mixed thoroughly. The luminescence was recorded by Spectra Max M5 microplate reader (Molecular Devices, USA).

Measurement of mitochondrial function

Isolation of mitochondria. The isolation of mitochondria was performed as previously described [22]. The liver and skeletal muscle tissues were quickly removed after the animals were sacrificed and finely minced in ice-cold isolation buffer (0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, and 1 g/L BSA, pH7.4). The tissue homogenates (10%, w/v) were generated in glass homogenizer. After removing cell debris by centrifugation at 1000 g for 10 min, the supernatant was subjected to further centrifugation at 10,000 g for 10 min to harvest mitochondria with final concentration of 20-25 mg/ml.

Measurement of mitochondrial respiratory activity

Oxygen consumption was measured by Clark type oxygen electrodes (Strathkelvin Instruments, British) at 25 °C. Mitochondrial respiration was detected by adding 1 mg/ml hepatic or skeletal muscle mitochondria in 1 ml of respiration medium (225 mM sucrose, 5 mM potassium phosphate buffer, pH 7.4, 10 mM Tris-HCl, 10 mM KCl, 0.2 mM EDTA and 100 µg/L BSA). The hepatic and skeletal muscle mitochondria were incubated with different concentrations of SalA (10^{-9}-10^{-6} M) at 25 °C for 10 min. Mitochondrial respiration was initiated by adding complex I substrate (10 mM L-glutamate plus 5 mM L-malate), and then adding ADP to a final concentration of 250 µM. State 3 respiration is oxygen consumption in the presence of ADP, while State 4 respiration is consumption of oxygen after ADP depletion. Respiration control ratio (RCR) is the ratio of State 3 to State 4 respiration, and is used as an indicator of mitochondrial respiration activity [23]. The ADP/O ratio is calculated to be the ratio of ADP concentration to consumption of oxygen during State 3 respiration. Oxidative phosphorylation rate (OPR) is obtained from the calculation of State 3 respiration rate and ADP/O ratio.

Measurement of mitochondrial ATP production

The mitochondria were incubated with different concentrations of SalA (10^{-9}-10^{-6} M) at 25 °C for 10 min, followed by adding 10 µl of 2 M L-glutamate plus 0.8 M L-malate, and 15 µl of 0.4 M ADP for another 5 min. The luminescence were detected by adding 100 µl CellTiter-Glo® reagent (Promega, USA) and recorded by Spectra Max M5 microplate reader (Molecular Devices, USA).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (Δψm) was measured with fluorescent dye Rhodamine 123 described previously [24, 25]. In brief, 50 µl of mitochondria (0.5 mg/ml) were added into 150 µl buffer (150 mM sucrose, 5 mM $MgCl_2 \cdot 6H_2O$, 5 mM succinate, 5 mM KH_2PO_4, 20 mM HEPES, pH 7.4), followed by adding 10 µM of Rhodanmine 123 (Sigma, USA) after treatment with SalA (10^{-9}-10^{-6} M) for 30 min. The fluorescence intensity (Ex 488 nm/ Em 535 nm) was recorded. To detect the MMP level in cultured cells, HepG2 cells were incubated with 10 µM of Rhodanmine 123 at 37 °C for 10 min after treatment with SalA (10^{-9}-10^{-6} M) for 30 min. After washing with PBS three times, the confocal images were taken and intensities (Ex 488 nm/ Em 535 nm) were measured. MMP was calculated with the Nernst equation ($\Delta\Psi_m$ (mV) = -59 log [Rh123]in/[Rh123]out).

Insulin release

MIN6 cells were plated into 24-well plate and treated with different concentrations of SalA (10^{-9}-10^{-6} M) for 24 h. Insulin content in the supernatant was determined by radioimmunoassay (Beijing North Institute of Biological Technology, Beijing, China).

The cell viability was measured by MTT assay as previously described [26]. Briefly, after treatment with different concentrations of SalA (10^{-9}-10^{-6} M) for 24 h, MTT (0.5 mg/ml) was added into cells and incubated at 37 °C for 4 h. Then the supernatant was discarded and DMSO was added. The OD value at 570 nm was recorded by Spectra Max M5 microplate reader (Molecular Devices, USA).

Western blotting

The drug-stimulated cells were lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Biochem, PA, USA). Equal amounts of protein were subjected to SDS-PAGE and immunoblotted

with antibodies specific for total Akt, phospho-Akt (Ser473), total AMPK, phospho-AMPK-a (Thr172) and GAPDH (Cell Signaling Technology, MA, USA).

Statistical analysis

All data are expressed as mean ± standard error (S.E.M.) and analyzed using statistical package SPSS 13.0. ANOVA followed by Dunnett's multiple comparison test was used to determine significant differences, with $P<0.05$ as statistically significant.

Results

Metabolic consequences of SalA in diabetic animal models

Polydipsia, polyphagia, polyuria are characteristic of diabetes, which can be improved by hypoglycemic agent treatment. Improvement of blood glucose is usually concomitant with the reduction of food and water intake. In type 1 diabetic mice, 24-h food and water intake were reduced after 7-day SalA treatment , and markedly reduced after 14-day treatment, stronger than metformin (Fig. 1A, 1B). As shown in Fig. 1C, 14-days treatment of SalA (0.4, 1.3 mg/kg) exhibited mild hypoglycemic effect on FBG with about 16% decrease compared with DM group, but without significant difference. Meanwhile, SalA significantly lowered fed blood glucose in a dose-dependent manner, with a little stronger effect than metformin (Fig. 1D). In OGTT, compared with DM group, SalA lowered the blood glucose after glucose

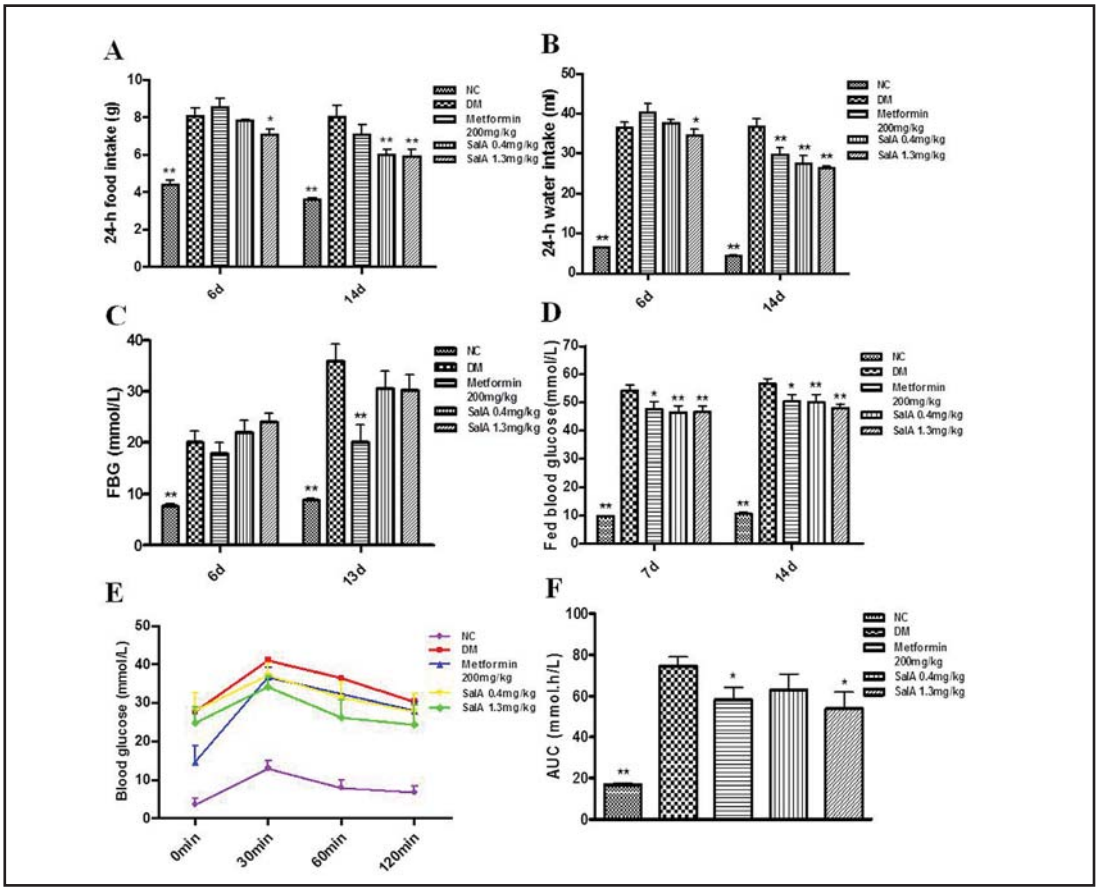

Fig. 1. Antidiabetic effect of SalA in type 1 *diabetic mice.* (A, B) 24-h food and water intake in untreated type 1 diabetic mice or mice treated with metformin or SalA for the indicated time points. (C, D) Fasting and fed blood glucose in untreated type 1 diabetic mice or mice treated with metformin or SalA for the indicated time points. (E, F) Effects of treatment on OGTT and AUC in type 1 diabetic mice for 15 days. Results are represented as mean±S.E.M. (n=10-12). *P<0.05, **P<0.01 vs. DM group.

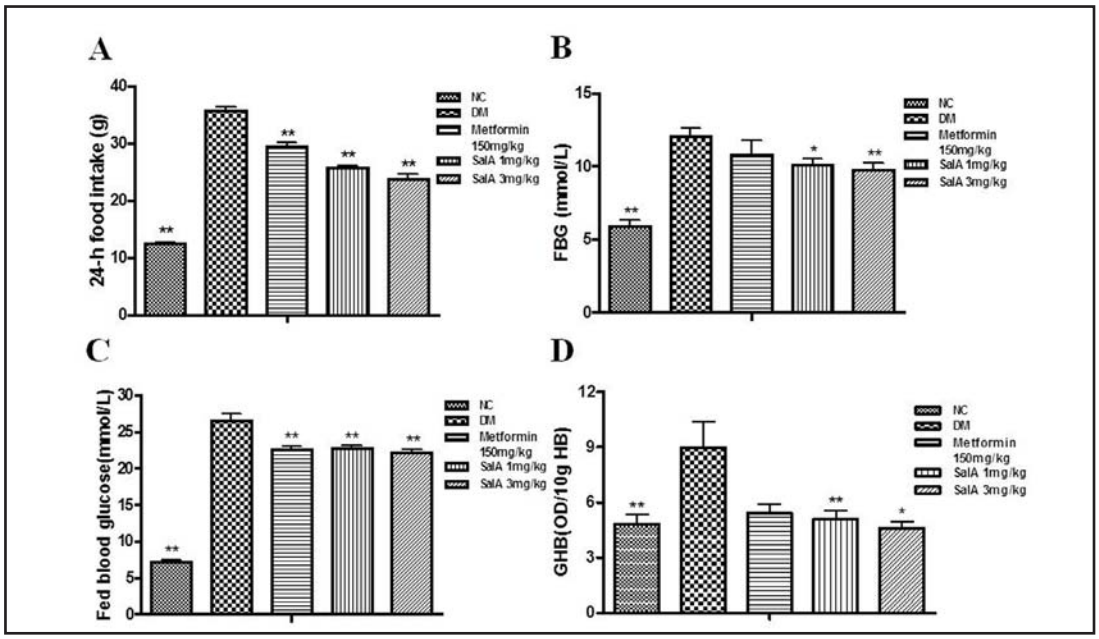

Fig. 2. Antidiabetic effect of SalA in type 2 *diabetic rats.* (A) Effect of 4-week treatment of metformin or SalA on 24-h food intake. (B, C, D) Fasting blood glucose, fed blood glucose, and glycosylated hemoglobin in untreated type 2 diabetic rats or rats treated with metformin or SalA for 4 weeks. Results are represented as mean±S.E.M. (n=10-12). *P<0.05, **P<0.01 vs. DM group.

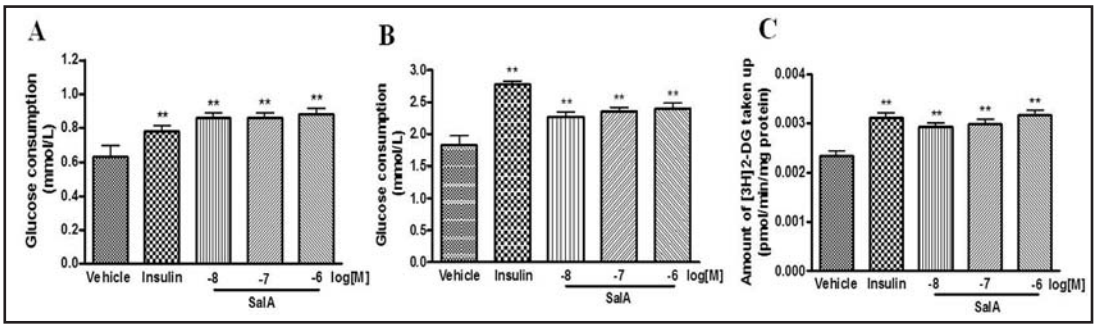

Fig. 3. SalA increased glucose consumption and enhanced cellular glucose uptake. (A, B) SalA increased glucose consumption. After (A) HepG2 cells or (B) L6 myotubes in 96-well plate were treated with different concentrations of SalA (10^{-8}-10^{-6} M) and insulin (3×10^{-8} M) for 24 h, the supernatant was taken for glucose assay. (C) SalA stimulated glucose uptake. Differentiated L6 myotubes in 24-well plate were incubated in serum-free MEM for 2 h, and then pretreated with 20 μM cytochalasin B for 5 min as non-specific uptake. The cells were incubated with SalA (10^{-8}-10^{-6} M) or insulin (10^{-7} M) at the indicated concentrations for 30 min. Glucose uptake was performed after adding [^{3}H]-2DG (0.2 μCi/well) and 2-deoxy-D-glucose (50 μM) for 10 min. Results are represented as mean±S.E.M. from three separate experiments. *P<0.05, **P<0.01 vs. vehicle group.

loading and reduced the area under curve (AUC), indicating that SalA improved the glucose tolerance (Fig. 1E, 1F).

We also confirmed the antidiabetic effect of SalA in type 2 diabetic rat model. Compared with DM group, 24-h food intake was markedly reduced by 4-week administration of SalA (1, 3mg/kg) with significant difference (Fig. 2A). SalA treatment significantly lowered FBG and fed blood glucose in dose-dependent manner, similar to metformin (Fig. 2B, 2C). Glycosylated hemoglobin in the blood reflects the average blood glucose levels over the preceding 2-3 months. Compared with DM group, SalA markedly reduced the glycosylated hemoglobin, which further confirmed the hypoglycemic effects of SalA (Fig. 2D).

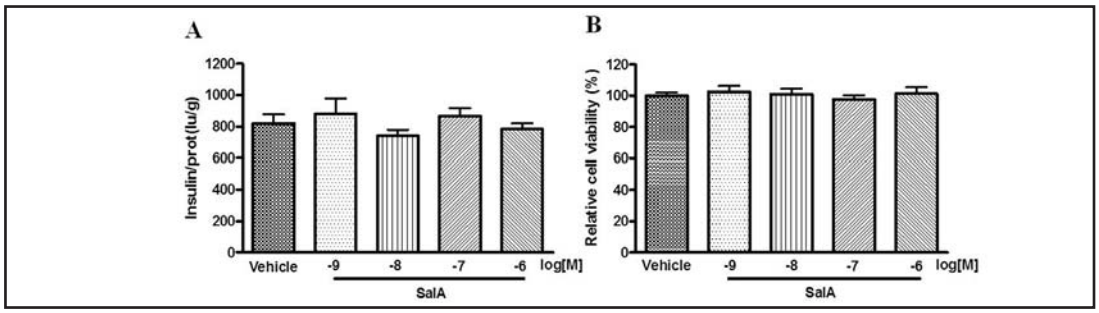

Fig. 4. SalA did not stimulate insulin release. (A) SalA did not stimulate insulin release in MIN6 cells. After MIN6 cells were treated with different concentrations of SalA (10^{-9}-10^{-6} M) for 24 h, the supernatant was then taken to determine insulin concentration. (B) SalA did not affect the cell viability of MIN6 cells. Cell viability was measured by MTT assay. Results are represented as mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs. vehicle group.

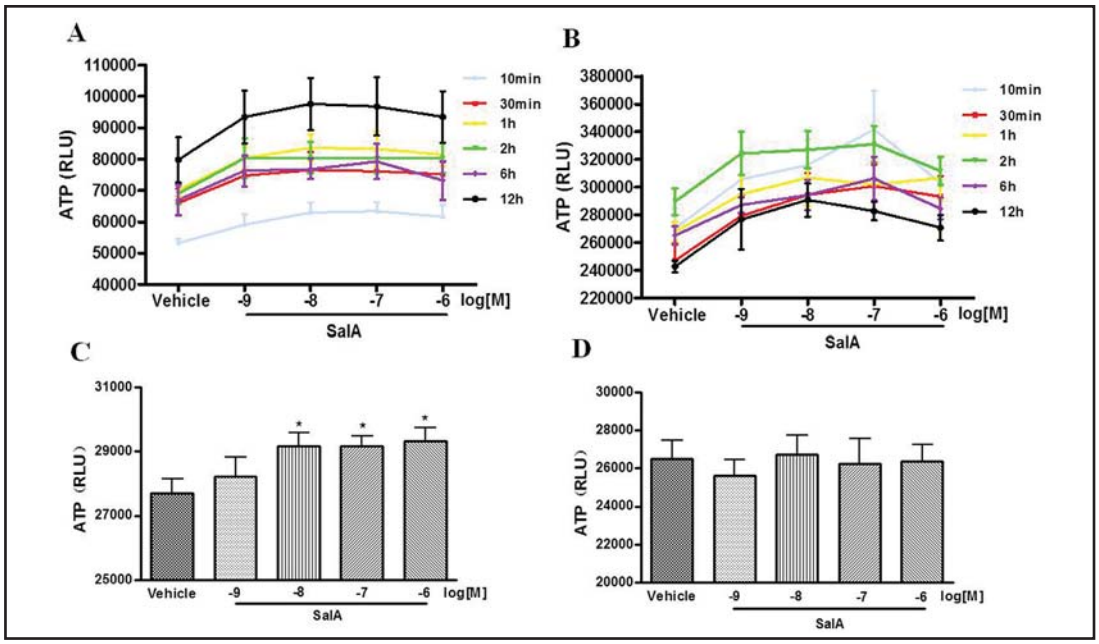

Fig. 5. SalA increased ATP production. After (A) HepG2 cells and (B) differentiated L6 myotubes were treated with SalA (10^{-9}-10^{-6} M), ATP was detected from 10 min to 12 h. After (C) hepatic and (D) skeletal muscle mitochondria were treated with SalA (10^{-9}-10^{-6} M) for 10 min followed by respiratory substrate I (L-glutamate, L-malate) and ADP for 5 min, CellTiter-Glo reagent 100 µl was added and luminescence was detected 10 min later. Results are represented as mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs. vehicle group.

Collectively, SalA lowered fasting and fed blood glucose with the reduction of 24-h food and water intake, exhibiting antidiabetic effect in both type 1 and type 2 diabetic animal models.

SalA increased glucose consumption and enhanced glucose uptake

Since SalA displayed the antidiabetic effect *in vivo*, we then investigated the glucose metabolic activity of SalA *in vitro* to unveil the mechanisms. To this end, we examined the effect of SalA on glucose consumption and uptake. As shown in Fig. 3A and 3B, both HepG2 cells and L6 myotubes exhibited the elevated glucose consumption in a dose-dependent manner under SalA treatment. In addition, glucose uptake was enhanced in L6 myotubes treated by SalA. 10^{-6} M SalA had the strongest effect, similar to 10^{-7} M insulin (36% increase, Fig. 3C). It suggested that SalA can increase glucose consumption and enhance glucose uptake *in vitro*.

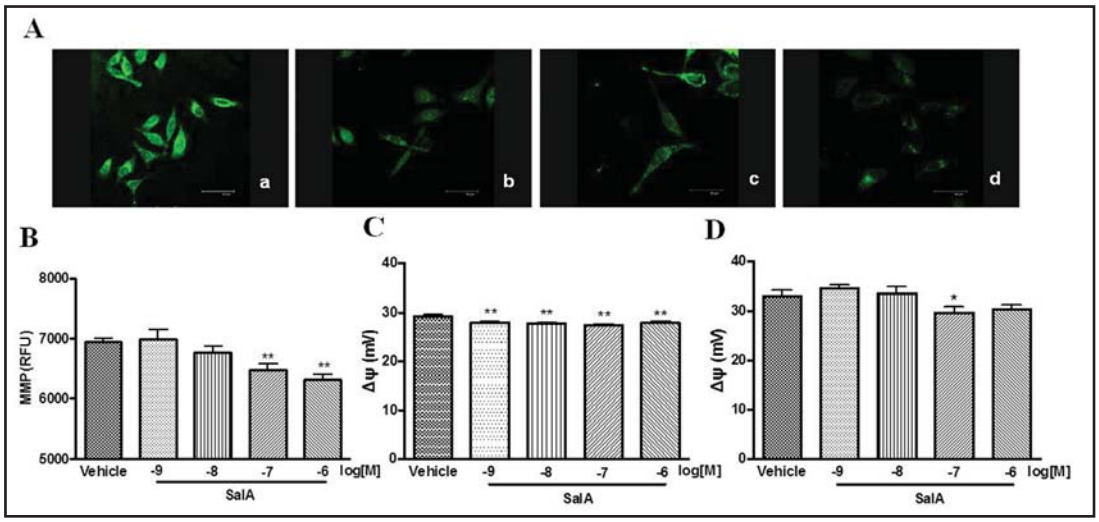

Fig. 6. SalA decreased mitochondrial membrane potential. After HepG2 cells were incubated with SalA (10^{-9}-10^{-6} M) for 30 min, 10 μM of Rhodanmine 123 was added and incubated for 10 min. (A) Confocal images (a: Vehicle, b: SalA 10^{-8} M, c: SalA 10^{-7} M, d: SalA 10^{-6} M) were taken and (B) fluorescence intensity were measured. After mitochondria were incubated with SalA (10^{-9}-10^{-6} M) for 30 min, 10 μM of Rhodanmine 123 was added and incubated for 10 min. The fluorescence intensity in (C) hepatic mitochondria and (D) skeletal muscle mitochondria was recorded. Results are represented as mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs. vehicle group.

SalA did not stimulate insulin release in MIN6 cells

To clarify whether SalA lower the blood glucose through stimulating insulin release like insulin, MIN6 cells were treated with SalA at different concentrations for 24 h. Both of insulin release and cellular toxicity revealed no significant alteration after SalA treatment (Fig. 4A, 4B), which suggested that SalA had no insulin secretagogue effect.

Effects of SalA on mitochondrial function

SalA increased ATP production in mitochondria. ATP production is the main activity of mitochondria. To explore whether SalA targets mitochondrial function, we investigated the effects of SalA on ATP production in both cultured cells and isolated mitochondria. After treatment with SalA in HepG2 cells and L6 myotubes, ATP production displayed significant dose-dependent increase from 10 min to 12 h with the strongest effect at 10^{-7} M and 10^{-6} M (Fig. 5A, 5B). In isolated mitochondria, SalA increased ATP production dose-dependently in hepatic mitochondria, while showed no significant effect in skeletal muscle mitochondria (Fig. 5C, 5D). Altogether, SalA increased ATP production in mitochondria.

SalA decreased mitochondrial membrane potential. Monitoring mitochondrial membrane potential is used to assess mitochondrial function. A lower membrane potential can prevent free radical production. So, we investigated whether SalA works on mitochondrial membrane potential. Interestingly, our results showed that 30-min treatment of SalA dose-dependently reduced Rhodamine 123 in HepG2 cells, suggesting that SalA decreased mitochondrial membrane potential in hepatocytes (Fig. 6A, 6B). In isolated hepatic and skeletal muscle mitochondria, we also found that mitochondrial membrane potential was decreased after 10-min treatment of SalA (Fig. 6C, 6D). Collectively, SalA decreased mitochondrial membrane potential in hepatic and skeletal muscle mitochondria.

SalA improved mitochondrial respiratory functions. Respiratory control ratio is the single most useful general test of function in isolated mitochondria. High RCR indicates good function and lower RCR usually indicates dysfunction [27]. Our previous findings have demonstrated that SalA improved the mitochondrial function in high-fat diet-fed

Table 1. Effects of SalA on mitochondrial respiratory properties in isolated *hepatic* mitochondria. RCR: State 3/State4; ADP/O: nmol ADP/nmol O; State 3: nmol O/min/mg prot; State 4: nmol O/min/mg prot; OPR: nmol ATP/min/mg prot; Complex I substrates: L-glutamate plus L-malate; Complex II substrate: succinate. Results are mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs. vehicle group

Respiratory activities	Complex I substrate				
	RCR	ADP/O	State 3	State 4	OPR
Vehicle	3.54±0.05	2.04±0.04	56.17±1.91	16.17±0.64	115.57±5.78
SalA 10^{-9} M	3.65±0.16	2.17±0.10	61.33±2.67	16.93±0.93	133.66±10.82
SalA 10^{-8} M	3.87±0.10*	2.13±0.06	62.40±2.56	15.91±0.72	117.78±6.70
SalA 10^{-7} M	3.55±0.11	2.17±0.04	57.64±2.05	15.87±0.80	123.06±4.97
SalA 10^{-6} M	3.55±0.10	2.22±0.06*	56.00±2.67	16.16±0.85	123.60±5.06

Table 2. Effects of SalA on mitochondrial respiratory properties in isolated skeletal muscle mitochondria. RCR: State 3/State4; ADP/O: nmol ADP/nmol O; State 3: nmol O/min/mg prot; State 4: nmol O/min/mg prot; OPR: nmol ATP/min/mg prot; Complex I substrates: L-glutamate plus L-malate. Results are mean±S.E.M. (n=6). *P<0.05, **P<0.01 vs. vehicle group

Respiratory activities	Complex I substrate				
	RCR	ADP/O	State 3	State 4	OPR
Vehicle	4.60±0.37	2.23±0.10	219.44±14.23	42.55±1.28	458.22±55.41
SalA 10^{-9} M	6.07±0.29**	2.59±0.09*	219.69±14.88	40.22±2.29	537.24±48.67
SalA 10^{-8} M	5.77±0.23*	2.55±0.08*	220.63±16.53	40.84±2.20	553.34±63.70
SalA 10^{-7} M	5.75±0.31*	2.36±0.10	215.00±11.95	39.19±2.81	492.86±48.77
SalA 10^{-6} M	5.69±0.46*	2.34±0.09	207.50±12.99	40.00±2.34	470.01±39.83

and STZ-induced type 2 diabetic rats [28]. Here, to further verify the effect of SalA on the mitochondrial respiratory function, hepatic and skeletal muscle mitochondria in normal rats were isolated and treated with SalA. In both hepatic and skeletal muscle mitochondria, SalA increased RCR, ADP/O, and OPR under complex I substrates stimulation (Table 1 and Table 2). These results suggested that SalA may improve mitochondrial function.

SalA activated phosphorylation of AMPK via CaMKKβ/AMPK signaling pathway. AMPK is a metabolic master switch regulating mitochondria, glucose uptake and fatty acid oxidation [29]. To explore if AMPK is involved in the antidiabetic mechanisms of SalA, we detected AMPK expression in HepG2 and differentiated L6 myotubes under SalA treatment. Our results showed that AMPK was phosphorylated in a dose- and time-dependent manner under SalA stimulation (Fig. 7A-F).

LKB1 and CaMKKβ are two important upstream kinases for AMPK, while upstream kinase of the major AMPK in L6 cells is LKB1. To identify the upstream kinase of AMPK, we examined the effect of SalA in HeLa cells with only expression of CaMKKβ as AMPK upstream kinase and deficiency in LKB1. We found that SalA also activated phosphorylation of AMPK in HeLa cells, suggesting that LKB1 might not be the upstream kinase of AMPK (Fig. 7G). Pretreatment with specific CaMKKβ inhibitor STO-609 or specific AMPK inhibitor compound C significantly inhibited phosphorylation of AMPK, which wasn't activated by cotreatment with SalA (Fig. 7H). Collectively, these results demonstrated that SalA activates AMPK phosphorylation probably through CaMKKβ/AMPK signaling pathway, independent of LKB1.

SalA did not activate phosphorylation of Akt. To test whether SalA modulates glucose metabolism via PI3K/Akt pathway known to play a major regulatory role in insulin pathway, we detected the Akt expression under SalA treatment. Our results showed that SalA did not phosphorylate Akt in both HepG2 cells and L6 myotubes (Fig. 8A-D). It suggested that SalA exhibited antidiabetic effect not through PI3K/Akt signaling pathway, consistent with the absent insulin-secretagogue effect of SalA.

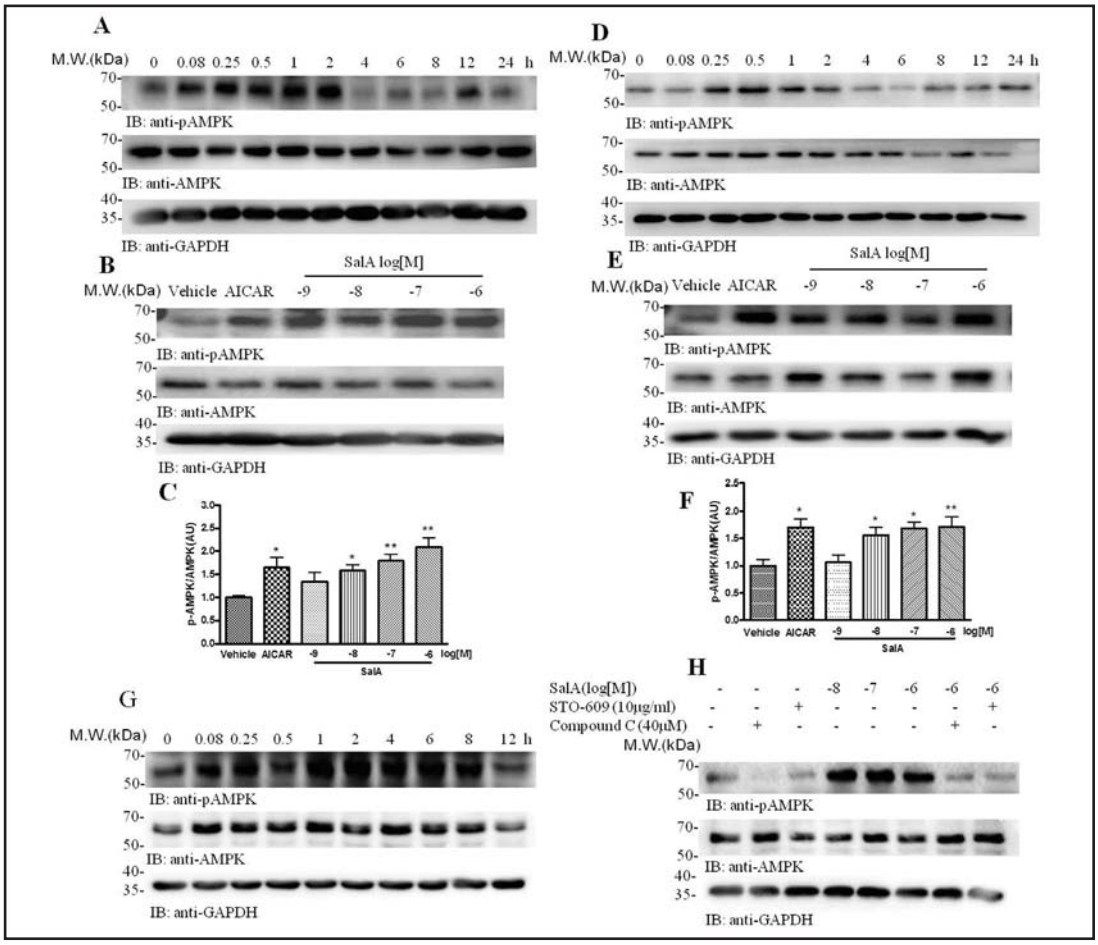

Fig. 7. SalA activated AMPK phosphorylation via CaMKKβ/AMPK signaling pathway. After treatment with SalA (10^{-6} M) from 5 min to 24 h, phosphorylations of AMPK in (A) HepG2 cells and (D) L6 myotubes at time-response effect were determined by western blot. Representative blots for dose-response effect of SalA (10^{-9}-10^{-6} M) in (B) HepG2 cells at 1 h and (E) L6 myotubes at 30 min. Quantification of the ratio of phospho-AMPK to total AMPK in (C) HepG2 cells and (F) L6 myotubes. (G) After HeLa cells were treated with SalA (10^{-6} M) from 5 min to 24 h, phosphorylation of AMPK at time-response effect were detected by western blot. (H) After pretreatment with AMPK inhibitor Compound C (40 μM) or CaMKKβ inhibitor STO-609 (10 μg/ml) for 30 min at 37 °C, HeLa cells were treated with SalA (10^{-8}-10^{-6} M) for 2 h with or without Compound C or STO-609 treatment, phosphorylation of AMPK were detected by western blot. Results are represented as mean±S.E.M. from three separate experiments. **P*<0.05, ***P*<0.01 vs. vehicle group.

Discussion

In this report, we have demonstrated that SalA, a small-molecular-weight natural product from *Salvia Miltiorrhiza* Bunge, exhibits antidiabetic effect in both *in vivo* and *in vitro*. SalA lowered fasting and fed blood glucose in both type 1 diabetic mouse and type 2 diabetic rat models, as well as increased glucose consumption in hepatocytes and enhanced glucose uptake in myotubes. Further findings supported that the antidiabetic effect of SalA may be attributed to significant improvement of mitochondrial function via AMPK signaling pathway.

The majority of ATP is generated by proton gradient in the mitochondria through oxidative phosphorylation during glucose metabolism. ATP is requisite to maintain the cellular processes and adequate ATP supplements by energy metabolisms are crucial for the treatment and prevention of many diseases [30]. It is well known that type 2 diabetes is not

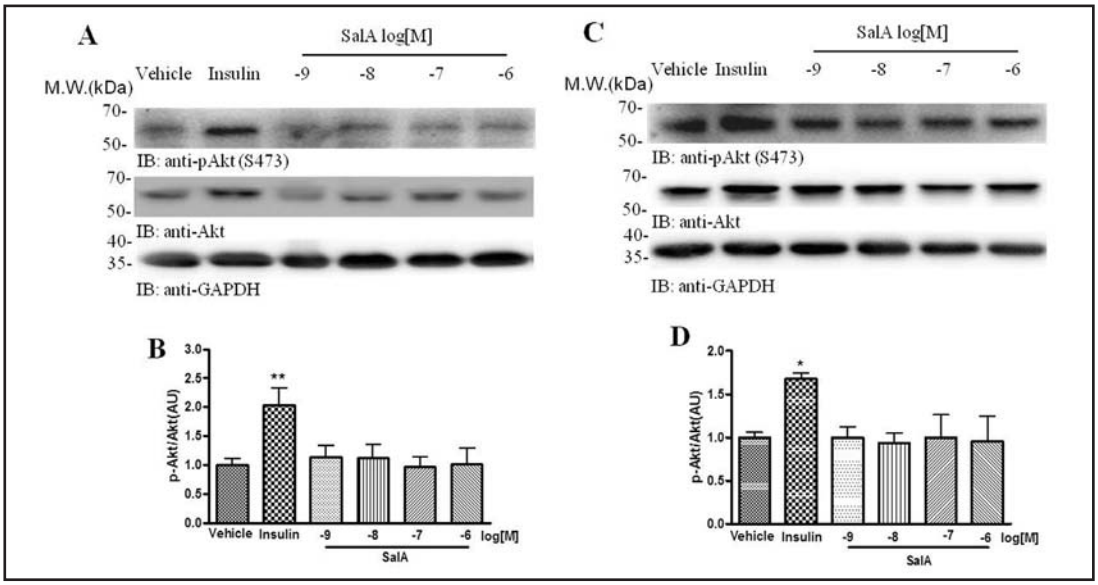

Fig. 8. SalA did not phosphorylate Akt. After treatment with different concentrations of SalA (10^{-9}-10^{-6} M) for 30 min, phosphorylation of Akt was detected by western blot analysis. Representative blots in (A) HepG2 cells and (C) L6 myotubes. Quantification of the ratio of phospho-Akt to total Akt in (B) HepG2 cells and (D) L6 myotubes. Results are represented as mean±S.E.M. from three separate experiments. *P<0.05, **P<0.01 vs. vehicle group.

merely a disease of insulin insensitivity or lack of insulin release but a global dysfunction of mitochondrial energy system [31]. Therefore, treatment of diabetes should focus on the improvement of mitochondrial function [31]. Our previous studies demonstrated that SalA promoted ATP production in myocardial, endothelial and smooth muscle cell (data not shown). In the current study, we found that SalA increased ATP production in both hepatic HepG2 and skeletal muscle L6 myotubes, consistent with improving the mitochondrial respiratory function of liver and skeletal muscle. Taken together, these data indicated that SalA exhibited the antidiabetic effect through targeting mitochondria.

A lower membrane potential can prevent free radical production. ΔΨm is directly related to reactive oxygen species (ROS) production: at higher potentials starting at ΔΨm>140 mV, ROS production increases exponentially [32, 33]. In diabetic patients, hyperglycemia can induce mitochondrial superoxide production, not only contributing to the development of complications, but also being involved during post-hyperglycemia periods of normal glycaemia [34]. Therefore, maintenance of lower ΔΨm, avoiding ROS generation, and providing full capability to produce ATP would be beneficial for diabetes treatment. In our study, SalA promoted the intracellular ATP production, while decreased mitochondrial membrane potential without any cytotoxicity effect on skeletal muscle L6 and hepatic HepG2 cells (data not shown). In addition, SalA increased ADP/O in both skeletal muscle and hepatic mitochondria in normal rats with robust improvement of RCR in skeletal muscle mitochondria. Those results are consistent with our previous findings in high-fat diet-fed and STZ-induced type 2 diabetic rats [3, 16, 28]. More interestingly, the effect of SalA is different from the common antidiabetic agents, as both metformin and TZDs inhibit complex I activity and cell respiration [35-37]. Recent studies have shown that decreasing mitochondrial membrane potential might be an important target for development of antidiabetic agents [38]. In our study, SalA decreased mitochondrial membrane potential level and increased ATP production. These unique functions of SalA make it appealing for diabetes treatment.

AMPK, a serine/threonine protein kinase, acts as a metabolic master switch regulating cellular metabolism and energy balance [39]. Abnormal AMPK activity is closely associated with diabetes. Therefore, AMPK has been recognized as a promising therapeutic target for type 2 diabetes [38, 40]. Some antidiabetic agents, such as metformin, rosiglitazone, and

natural products like berberine and resveratrol, have been demonstrated to activate AMPK signaling pathway [37, 41-43]. We found that AMPK in HepG2 cells and L6 myotubes was phosphorylated by SalA in dose- and time-dependent manner. It has been known that AMPK activity is switched on in several catabolic pathways for ATP generation such as glucose uptake, consumption, and glycolysis [30, 44]. So AMPK is involved in ATP production and maintenance of glucose homeostasis. Thus, SalA activates phosphorylation of AMPK, which triggers intracellular metabolic changes, including increasing glucose uptake in skeletal muscle and decreasing glucose production in liver, and eventually leads to glucose-lowering.

It is well known that LKB1 kinase mediates the therapeutic effect of metformin [45]. Apart from LKB1, two other kinases, CaMKKβ and TGF-β-activated kinase 1 (Tak1), have also been identified as AMPK phosphorylating kinase. Tak1 regulates AMPK activity by phosphorylation on Thr172 [46]. CaMKKβ phosphorylates AMPK responding to the elevated intracellular Ca^{2+} concentrations, independent of any changes in cellular AMP/ATP ratio [47]. We found that SalA was able to phosphorylate AMPK in HeLa cell with deficiency in LKB1, which suggested that SalA activated cellular AMPK activity independent of LKB1. Furthermore, CaMKKβ inhibitor STO-609 blocked AMPK phosphorylation in HeLa cells, suggesting that phosphorylation of AMPK by SalA might be through CaMKKβ pathway, not LKB1, as was different from metformin [45].

Increasing glucose consumption and glucose uptake in periphery tissues (liver and skeletal muscle) contributes to lowering postprandial glucose. It is well known that in addition to fasting hyperglycemia, postprandial hyperglycemia is also one of the earliest abnormalities of glucose homeostasis associated with type 2 diabetes and is markedly exaggerated with fasting hyperglycemia [48]. SalA lowered fasting blood glucose mildly in type 1 diabetic mice and significantly in type 2 diabetic rats. Furthermore, SalA markedly lowered fed blood glucose in both diabetic animal models. Particularly, we did not find the significant toxicity in long-term toxicity test, suggesting that SalA can be used with greater safety (data not shown). Hence, lowering fasting and fed blood glucose with remarkable safety makes SalA attractive in the development of antidiabetic drug.

Taken together, SalA exhibits the antidiabetic effect through improving mitochondrial function and switching on AMPK via CaMKKβ/AMPK signaling pathway. Interestingly, its activities of decreasing mitochondrial membrane potential and increasing ATP production make SalA unique and appealing in antidiabetic effect. These findings indicate that SalA may serve as a novel and potential therapeutic option for diabetes treatment.

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Disclosure Statement

The authors declared no competing interests.

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