

Natural product cycloastragenol: a promising anti-diabetes candidate with therapeutic effects on its related complications in rats

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Abstract

BACKGROUND AND PURPOSE Type II Diabetes mellitus (T2DM) is a worrying chronic metabolic disorder accompanied by multiple serious complications, makes threaten public health. In the present study, we evaluated the therapeutic effects against T2DM of the cycloastragenol (CAG), a key metabolite of astragaloside IV (AST) isolated from traditional Chinese plant *Astragalus membranaceus* and revealed its molecular mechanism. **EXPERIMENTAL APPROACH** The plasma glucose-decreasing effects of AST and CAG were monitored in KunMing mice by performing an OGTT test. Furthermore, the effect of CAG on the metabolism of Zucker diabetic fat (ZDF) rats was demonstrated after treatment for 5 weeks, as well as on diabetes-relevant clinical symptoms and glucose absorbance. The underlying molecular mechanism of the therapeutic effects of CAG was explored in both wild-type and sodium/glucose co-transporter 2 (SGLT2)-overexpressed HEK293 cells. **KEY RESULTS** CAG showed stronger effects in lowering plasma glucose and enhancing glucose tolerance than AST in KM mice and ZDF rats. In the ZDF rats, the potential antidiabetic properties of CAG were associated with amelioration in hyperglycemia, dyslipidemia, myocardial and kidney fibrosis. Interestingly, glucose reuptake was inhibited in the kidneys of mice treated with CAG, while urine glucose and sodium levels were elevated. The underlying mechanism might be that CAG reduced the expression of SGLT2 in the kidney and inhibited glucose transport as well. **CONCLUSIONS AND IMPLICATIONS** CAG can ameliorate the T2DM-related metabolic syndromes of ZDF rats by acting on the SGLT2, which provides a certain references for the application of CAG in T2DM therapy.

Natural product cycloastragenol: a promising anti-diabetes candidate with therapeutic effects on its related complications in rats

Running title: A potential SGLT2 inhibitor from natural products

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Bullet point summary

What is already known

- Astragaloside IV (**AST**) has been reported with the anti-hyperglycemia activity in mice.
- SGLT2 protein is a promising target for treating of T2DM

What this study adds

- The metabolite of **AST**, **CAG**, is more efficiently in combating T2DM.
- **CAG** inhibits glucose absorbance and promotes glucose excretion.
- **CAG** works mainly through the SGLT2.

What is the clinical significance

CAG should be considered as a new-type SGLT2 inhibitor for treating of T2DM.

ABSTRACT

BACKGROUND AND PURPOSE

Type II Diabetes mellitus (T2DM) is a worrying chronic metabolic disorder accompanied by multiple serious complications, makes threaten public health. In the present study, we evaluated the therapeutic effects against T2DM of the cycloastragenol (**CAG**), a key metabolite of astragaloside IV (**AST**) isolated from traditional Chinese plant *Astragalus membranaceus* and revealed its molecular mechanism.

EXPERIMENTAL APPROACH

The plasma glucose-decreasing effects of **AST** and **CAG** were monitored in KunMing mice by performing an OGTT test. Furthermore, the effect of **CAG** on the metabolism of Zucker diabetic fat (ZDF) rats was demonstrated after treatment for 5 weeks, as well as on diabetes-relevant clinical symptoms and glucose absorbance. The underlying molecular mechanism of the therapeutic effects of **CAG** was explored in both wild-type and sodium/glucose co-transporter 2 (SGLT2)-overexpressed HEK293 cells.

KEY RESULTS

CAG showed stronger effects in lowering plasma glucose and enhancing glucose tolerance than **AST** in KM mice and ZDF rats. In the ZDF rats, the potential antidiabetic properties of **CAG** were associated with amelioration in hyperglycemia, dyslipidemia, myocardial and kidney fibrosis. Interestingly, glucose reuptake was inhibited in the kidneys of mice treated with **CAG**, while urine glucose and sodium levels were elevated. The underlying mechanism might be that **CAG** reduced the expression of SGLT2 in the kidney and inhibited glucose transport as well.

CONCLUSIONS AND IMPLICATIONS

CAG can ameliorate the T2DM-related metabolic syndromes of ZDF rats by acting on the SGLT2, which provides a certain references for the application of **CAG** in T2DM therapy.

Keywords: type 2 diabetes mellitus, Cycloastragenol, Astragaloside IV, hyperglycemia, cardiovascular disease, SGLT2.

Abbreviations

T2DM, Type II Diabetes mellitus; CAG, cycloastragenol; AST, astragaloside IV; SGLTs, sodium-glucose cotransporters; ZDF rats, Zucker diabetic fatty rat; FDA, Food and Drug Administration; SLCs, solute carriers; 2NBDG, 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose; OGTT, oral glucose tolerance test; H&E, hematoxylin and eosin; PAS, periodicacid-schiff stain; MetS, metabolic syndromes; HbA1c, glycated hemoglobin; LDL-c, low-density lipoprotein cholesterol; 14C-AMG, [14C]methyl-alpha-D-glucopyranoside, TGF- β 1, transforming growth factor beta-1.

Introduction

Type II diabetes mellitus (T2DM) has become a global public health crisis, which poses a great threat to the health and quality of life of people in all countries, especially in developing countries (Moucheraud et al., 2019). T2DM is a chronic metabolic disease characterized by hyperglycemia and insulin resistance and accompanied by a series of clinical syndromes, including diabetic nephropathy (Tao et al., 2019), diabetic retinopathy (Dow et al., 2018; Ren et al., 2019), and cardiovascular complications (Vaidya et al., 2015). The FDA has approved several new drugs for T2DM, such as metformin (Hostalek et al., 2015; Pernicova & Korbonits, 2014), rosiglitazone (Han et al., 2016) and Dapagliflozin (Scheen, 2017; Vivian, 2015). Unfortunately, although the use of these drugs can lower blood glucose to nearly normal levels, mortality from cardiovascular disease in diabetes either increased or was not affected (Gerstein et al., 2008; Navarro-Perez et al., 2018). Given this, the discovery of new drugs with new targets or specific molecular mechanisms is still of great concern.

Sodium-dependent glucose co-transporters (SGLTs) are solute carriers (SLCs) responsible for glucose (re)absorption (Rives et al., 2017; Wright et al., 2011). SGLTs are encoded by the SLC5A genes, contains multiple isoforms, mostly, SGLT1 and SGLT2. SGLT1 and SGLT2 are expressed in the kidney, and SGLT1 expressed in the small intestine as well (Rieg & Vallon, 2018; Wood & Trayhurn, 2003). Among them, SGLT2 is a high capacity transporter responsible for 90% of glucose reuptake and tissue-specific expressed in the renal tubules of the kidney (Abdul-Ghani et al., 2012; Wang et al., 2017). SGLT2 is a promising target for the treatment of diabetes due to the inhibition on it can reduce the risk of hyperglycemia by decreasing glucose reuptake (Chao & Henry, 2010). Nowadays, eight SGLT2 inhibitors have been approved by the FDA for treating diabetes; they can increase urinary glucose excretion and display anti-hyperglycaemic effects that are primarily independent of insulin (Filippas-Ntekouan et al., 2018; Nauck, 2014; Shyangdan et al., 2016). Moreover, the efficacy and safety of SGLT2 inhibitors, especially in the prevention of cardiovascular diseases, have attracted full attention (Vaduganathan & Butler, 2019). Nevertheless, the SGLT2 inhibitors on the market now are all glycoside compounds, and it is of considerable significance to further study whether we can expand new structure types in this field. For example, finding a novel structure from natural compounds.

Astragalus Membranaceus is used as a traditional Chinese medicine with multiple bioactivities, such as anti-hyperglycemia (Liu et al., 2019), anti-inflammation (Zhou et al., 2017), anti-fibrosis (Shan et al., 2016), and remission of diabetic nephropathy (Liu et al., 2017). Several key ingredients were identified from *Astragalus Membranaceus*, such as astragaloside IV (**AST**), a soluble lanolin alcohol-shaped tetracyclic triterpenoid saponin. Although **AST** shows a potential for treating T2DM (Zhang et al., 2020), it possesses low bioavailability (Cheng & Wei, 2014; Ran et al., 2016). Instead, its aglycone cycloastragalol (**CAG**) exhibits good bioavailability since **CAG** is the primary metabolite of **AST** *in vivo* via intestinal bacterial biotransformation (Szabo, 2014; Zhou et al., 2012). However, the effects of **CAG** on treating diabetes mellitus has not been reported so far. Therefore, the first purpose in the present study is to evaluate the

therapeutic effect of **CAG** on T2DM from the perspective of the pathological feature by using the ZDF diabetic rat model. The second goal was to investigate the molecular patterns of **CAG** in the performance of these effects. These studies will provide some new ideas for the discovery of new anti-T2DM drugs with SGLT2 inhibitory activity.

Materials and Methods

Materials

All the compounds used in the present study were commercially available, including 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Invitrogen, USA, CID: 6711157), 2-deoxy-D-glucose (2DG, Invitrogen, USA, CID: 5287471), cytochalasin B (Shanghai Yuanye Bio-Technology Co., Ltd, China, CID: 5311281), metformin (Shanghai Yuanye Bio-Technology Co., Ltd, China, CID: 4091), Dapagliflozin (Shanghai Yuanye Bio-Technology Co., Ltd, China, CID: 9887712), astragaloside IV (AST, Chengdu Mansit Bio-technology Co, Ltd, China, CID: 13943297), and cycloastragalol (CAG, Chengdu Mansit Bio-technology Co, Ltd, China, CID: 13943286). All detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), and ELISA kits were obtained from Mlbio (USA). All other chemicals were of analytical grade and were obtained from Sigma (USA) and Merck (DE).

Animal experiments

Male KM mice (7-8 weeks, 18-22 g, RRID:MGI:5651867) were purchased from the Laboratory Animal Centre of Sun Yat-sen University (Guangzhou, China) and male Zucker Diabetic Fatty (ZDF) rats including ZDF-Lepr^{fa/fa} rats and ZDF-Lepr^{fa/ctrl} rats (7-8 weeks, 220~240 g, RRID:RGD_2314027, RRID:RGD_2311071) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). All animal procedures were approved by the Sun Yat-sen University Committee on Ethics for the Use of Laboratory Animals in accordance with the Animal Welfare Legislation of China. Animal studies are reported in compliance with the ARRIVE guidelines.

KM mice were kept at 22 ± 1 °C on a 12-hour light/dark cycle with free access to food and water. These mice were randomly divided into 6 groups, including lean group (without glucose administrated), vehicle group, metformin group, Dapagliflozin group, **AST** group and **CAG** group, each group included 10 mice. Metformin (100 mg/kg), Dapagliflozin (1 mg/kg), **AST** (5 mg/kg), **CAG** (5 mg/kg) were dissolved in normal saline containing 1% CMC-Na and taken gavage administration in mice. The lean and the vehicle groups were administered the same volume. After acclimatization, oral glucose tolerance test (OGTT) was performed. Briefly, mice were fasted for a period of 14 h and then administrated with glucose (2 g/kg) orally. The plasma glucose of 0, 15, 30, 60, and 120 min were detected by using a blood glucose meter (Roche), respectively.

ZDF-Lepr^{fa/ctrl} rats were fed a chow diet provided by the Animal Center of Sun Yat-sen University and ZDF-Lepr^{fa/fa} rats were fed high fat diet 5008 (Labdiet, USA) for one month. Randomly selected the rats with blood glucose over 11.1 mmol/L from ZDF-Lepr^{fa/fa} rats before experiments. These ZDF rats were divided into 5 subgroups: lean group (ZDF-Lepr^{fa/ctrl}, saline), vehicle group (ZDF-Lepr^{fa/fa}, model), Dapagliflozin (1 mg/kg/d, *p.o.*), **CAG** (1 and 5 mg/kg/d, *p.o.*). The OGTT experiment was preceded after the administration of **CAG** as described above. The urinary glucose, urinary sodium, and urine efflux were determined after 1- or 5-week treatment, respectively.

Plasma analysis

At the end of the study, fasted mice for eight hours and injected of ketamine/xylazine (*i.p.*). After the mice were fully anesthetized, collected the whole blood from abdominal aorta in a tube containing 1 mM EDTA for the measurement of relevant plasma parameters. Killed the mice by cervical decapitation. Weighed the tissues of interest and freeze-clamped or fixed them in a 4% formaldehyde solution. The whole blood was centrifuged at a speed of 3,000 rpm for 30 minutes, and the supernatant was separated and applied to the determination of cholesterol, low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and triglyceride by using correspondent kits (Nanjing Jiancheng Bioengineering Institute, China), or detection of glycosylated hemoglobin (HbA1c) and insulin by the ELISA detection kit (Abcam, USA).

H&E examination and immunohistochemistry assay

The tissues fixed in 4% formaldehyde were embedded in paraffin after dehydration in a gradient ethanol series (70–100%). Cut embedded sections (4 μm thick) with a rotary microtome and subjected to hematoxylin and eosin (H&E) staining, Masson staining, periodic acid-Schiff (PAS) staining, and Sirius red staining, or labeled with TGF- β 1 (BD Biosciences Cat# 562423, RRID: AB_2737615, Guangzhou, China) for microscopic examination. Sections were viewed with a light microscope (Olympus, Japan) and photographed at $\times 100$ magnification. The sections of histological examination and immunohistochemistry image were firstly labeled by an identification number code without the information of the grouping. The numbers of TGF- β 1 labelled cells in each slide were counted by using the Image J software (Macbiophotonics, Hamilton, Canada). Quantification analysis was performed in six randomly selected fields per sample in a blinded manner.

Construction of HEK293-SGLT2 cell lines

HEK293 cells (CLS Cat# 300192/p777_HEK293, RRID: CVCL_0045) were seeded into a 6-well plate at the density of 0.5×10^6 cells/well and incubated overnight. To construct a cell line with over-expression of SGLT2, the plasmid plvx-puro-SGLT2 (containing SGLT2 gene sequence with lentivirus) were transfected into HEK293 cells using lipofectamine 3000 (Invitrogen, Cat: L3000-015, USA). After six-hour of infection, the culture medium was replaced with fresh Dulbecco's modified Eagle's medium (DMEM). After 48 hours, the cells were treated with puromycin (Thermo Fisher Scientific, USA, CID: 439530) at the final concentration of 2 $\mu\text{g}/\text{mL}$, and the complete culture medium (with 2 $\mu\text{g}/\text{mL}$ of puromycin) was replaced every 48 hours. RT-PCR and Western Blot were performed to determine the expression level of SGLT2.

RNA extraction and RT-PCR

Primer sequences were synthesized by Generay Biotech and the total RNA was extracted from cells using RNAiso Plus kit (Takara, Japan). The mRNA levels of *SLC5A1*, *SLC5A2*, and *Actin* were detected by the quantitative real time PCR. The primer sequences of SGLT1 and SGLT2 are as follows: SLC5A1-F: GTCGGACTGTGGGCTATGTT, SLC5A1-R: GTCTGCAAGGTGTCCGTGTA; SLC5A2-F: ACGCC-CATGTTTCTCATGGT, SLC5A2-R: CCTGGGGCTCATTTCATCTCC.

Western Blot

The cells were washed 3 times with a $1 \times$ PBS buffer, then were lysed in an ice-colded RIPA extract buffer (FUDE, Cat: FD008, Jiangsu, China) supplemented with a mixture of protease inhibitors (SIGMA, Cat: #021M8200, Guangzhou, China). The extracted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Cat: #1620177, USA). After blocking with a TBS/T buffer (0.1%, containing 5% bovine serum albumin) for 30 min at room temperature, the membrane was incubated with different primary antibodies at 4 $^{\circ}\text{C}$ for overnight, including SGLT2 antibody (Abcam, Cat#ab37296), and Actin (Cell Signaling Technology Cat# 4970). The membrane was washed with the TBS/T buffer four times to remove the unbound antibody and then incubated with the HRP-conjugated secondary antibody (Cell Signaling Technology). Protein bands were visualized with an ECL kit (Millipore, Cat: WBKLSO500). The densitometry analysis was performed using the Quantity One Software (Bio-Rad, CA, USA) relative to the loading control.

2-NBDG uptake assay

The cells were seeded into a 24-well glass bottom plate, and incubated at 37 $^{\circ}\text{C}$ in a humidified incubator with 5% CO_2 until reached a density of 60%-70%. Changed with a serum-free medium for two hours before taking experiment. After that, added 150 μL sodium-HEPES buffer containing 240 μM 2-NBDG and 150 μL compound solutions to the plate to a final concentration of 10 μM . Incubated cells at 37 $^{\circ}\text{C}$ for 30 min. Then, washed cells with sodium-HEPES buffer three times and photographed with a Zeiss LSM laser confocal imaging system.

Statistics analysis

The data and statistical analyses were complied with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data were expressed as the mean \pm SEM. Data between two groups were analyzed by t-test using Graphpad Prism (Graphpad Software Inc, California, USA). Statistical analyses for multiple groups was performed by one-way ANOVA followed by Tukey's HSD post hoc tests. A *p*value of [?] 0.05 was considered statistically significant.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org> the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a; Alexander et al., 2017b).

Results

CAG reduces plasma glucose in KM mice by inhibiting glucose uptake

To determine the glucose-decreasing effect of **AST** and **CAG** (Chemical structure were listed in Figure 1A), KM mice were administrated with **AST** and **CAG** for ten days. Metformin and Dapagliflozin were used as positive drugs, respectively. Compared with the vehicle group, metformin and Dapagliflozin efficiently decreased plasma glucose at each time point, and Dapagliflozin showed a higher efficacy. **AST** could not show a significant effect on reducing plasma glucose, while **CAG** showed a more substantial hypoglycemic effect and accompanied by a backward shift of the blood glucose absorption peak (Figure 1B), indicating that **CAG** could decrease glucose absorption in mice. The area under the curve of plasma glucose (AUC) further confirmed the glucose-decreasing effect of **CAG** in mice, which was similar to metformin (Figure 1C).

There are two approaches for glucose-diminishing in mice, including promoting the glucose uptake and mobilization or inhibiting glucose absorption (Pandey et al., 2015). To reveal the underlying possible pattern of the glucose-decreasing in **CAG**-treated mice, we performed glucose uptake assay in cells by using the 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose (2-NBDG) labelling method. Dapagliflozin markedly decreased glucose uptake by a decrease in the signal of the 2-NBDG within cells, and an opposite effect was observed in metformin-treated cells, which was in accordance with the previous studies (Lu et al., 2018). As shown in Figure 2, cells treated with **CAG** showed a similar pattern with that of Dapagliflozin, which was a significant reduction of the 2-NBDG signal. To be noted, metformin, Dapagliflozin, and **CAG** could all enhance insulin sensitivity as judged by an increase in the signal of the 2-NBDG in the combination group compared with groups in the absence of insulin. These results suggested that **CAG**, similar to Dapagliflozin, could inhibit glucose uptake.

CAG ameliorates diabetes-related metabolic syndromes in ZDF diabetes rat

ZDF diabetes rat, a well-known model mimicking the characteristics of T2DM populations, was applied to determine the anti-diabetes effects of **CAG** *in vivo*. The related indexes, including body weight, levels of glucose, HbA1c, triglyceride, cholesterol, LDL-c, and HDL-c in the ZDF rats treated with **CAG** for five weeks, were determined. As shown in Figure 3A, Dapagliflozin decreased plasma glucose after one week of treatment, and this strong glucose-decreasing effect did not maintain to 5 weeks as observed by a slight increase in plasma glucose level in week 2~5. Compared with Dapagliflozin, **CAG** could reduce blood glucose levels with a concentration-dependent mode. Either the hypoglycemic effect or the later increase of glucose level is not as apparent as Dapagliflozin. The OGTT assay further confirmed that **CAG** had a mild hypoglycemic effect on plasma glucose in mice (Figure 3B and 3C).

In clinical, diabetes populations are accompanied by several metabolic syndromes (MetS), bodyweight decrease, and tissue breakdown, especially at the late stage. Thus, we evaluated the effect of **CAG** on bodyweight and observed a progressive decrease in body weight in the vehicle group, while Dapagliflozin and **CAG** efficiently reversed this decrease (Figure 3D). Consistent with the plasma glucose-decreasing effect in KM mice, **CAG** dose-dependently decreased serum glucose and HbA1c levels as well as the insulin excretion

level, whereas Dapagliflozin showed no major effect on insulin level in rats (Figure 3E). Especially for another important clinical diagnosis index for diabetes, glycated hemoglobin (HbA1c)(Rodbard et al., 2007), **CAG** showed significant decreasing effect similar to Dapagliflozin (Figure 3E). Meanwhile, **CAG** could also efficiently decrease plasma triglyceride, low-density lipoprotein cholesterol (LDL-c) and reverse the progressive degeneration of ratio HDL-c/LDL-c in ZDF rats (Figure 3F-3I).

CAG inhibits

coronary artery sclerosis, myocardial and kidney fibrosis in ZDF rats

As mentioned above, complications are one of the main causes of death in patients with diabetes, and whether anti-diabetes drugs can alleviate these complications has become an important consideration in the development of such drugs. The main complication of diabetes is coronary artery sclerosis (Ikonomidis et al., 2016; Naito & Miyauchi, 2017). Using H&E and periodic acid-Schiff staining, we found that the **CAG**-treated mice had thinner coronary arteries than the vehicle group (Figure 4A and 4B). The thinning effect on the coronary arteries of **CAG** was even stronger than that of Dapagliflozin. This result corresponded to an increase in the plasma HDL-c/LDL-c ratio in rats treated with **CAG** (Figure 3I), suggesting that **CAG** can effectively reverse coronary atherosclerosis.

Also, epidemiological studies have shown that with the development of diabetes, multiple tissues collapse, such as myocardial fibrosis and renal degeneration, which can also lead to cardiovascular complications and diabetic nephropathy (Pop-Busui et al., 2017). We then determined the protective effects of **CAG** on cardiovascular and nephropathy complications in ZDF rats. By using the Sirius staining (for collagen) and Masson staining (for fiber), a significant accumulation of collagen in the heart, which is a pathologic indicator of myocardium fibrosis, was observed in the vehicle group (as indicated by the black arrow in Figure 5A), compared with the lean group. Dapagliflozin and **CAG** could efficiently decrease collagen and fiber to a similar level of that in the lean mice (Figure 5A-5C). These ameliorating effects were well coincided with their effects in reducing myocardial fibrosis in ZDF diabetes rats. These two compounds have also been shown to relieve fibrosis in the kidney similar to that in the heart. (Figure 5D-5F). Immunohistochemistry analysis of TGF- β 1 protein, a representative biomarker of fibrosis, further confirmed the anti-fibrosis effects of **CAG** in the heart and the kidney as indicated by a significant decrease in the expression of TGF- β 1 (Figure 5G-5H).

CAG inhibits glucose reabsorption in ZDF rats

Inhibiting glucose reabsorption in kidney and promoting glucose efflux out are using therapeutic approaches for diabetes in clinic. After the treatment of Dapagliflozin or **CAG** for one week, we tested the level of urinary glucose and sodium, and urine efflux volume in ZDF mice at different time points in 24 hours. Dapagliflozin significantly increased the urinary glucose and sodium in each period within a day, while showed nearly no effect on urine efflux volume (Figure 6A-6C). Similar to Dapagliflozin, **CAG** increased urinary glucose, urinary sodium but a marginally decrease in the urinary efflux volume (Figure 6A-6C). Similar results appeared in ZDF rats whose administration was extended to 5 weeks (Figure 6D-6F). Of note, after 5-week administration, both **CAG** and Dapagliflozin decreased the water intake volume (Figure 6G), suggesting the inhibitory effects on glucose reabsorption and the promotion of glucose efflux out.

The anti-diabetic effect of CAG is related to the inhibition of SGLT2

Considering that the antidiabetic effect and manner of **CAG** were similar to that of Dapagliflozin, we further studied the effect of **CAG** on SGLT protein. By using an immunohistochemistry detection, a higher expression of SGLT2 was observed in kidney of ZDF diabetic rats in the vehicle group compared with the lean mice, and a reversal effect of Dapagliflozin was also overserved, which was consistent with the previous reports. A similar decreasing was found in the **CAG**-treated diabetic rats (Figure 7A). Similarly, **CAG** and Dapagliflozin also inhibited the expression of SGLT2 in the HEK293 cell lines with over-expression of SGLT2 (Figure 7B and 7C).

Discussion

The present study investigated the therapeutic effects of **CAG**, the major metabolite of **AST**, for treating type 2 diabetes, and its underlying mechanism for the first time. **AST** is a glucoside isolated from traditional Chinese herb *Astragalus Membranaceus (fisch) bunge*, which is one of the most widely used traditional Chinese medicine for diabetes therapy (Wang et al., 2015). Till today, **AST** shows broadly pharmacological effects, such as reducing the symptoms of metabolic syndrome (Yue et al., 2017; Zhang et al., 2011), preventing the cardiovascular pathological changes and protecting against cardiovascular injury (Xu et al., 2006) and improving renal function (Lu et al., 2015), or reducing the progression of diabetic peripheral neuropathy (Yu et al., 2006). Despite these findings, the molecular mechanism by which **AST** fights diabetes remains elusive. In the present study, we tried to study the anti-diabetes mechanism of **AST** from another perspective, that is, to study the anti-diabetes effect and possible pathways of **CAG**, which is the aglycone of **AST**, as well as the major metabolic form of **AST in vivo**. The study of **CAG** not only helps to explain the antidiabetic action of **AST** in vivo but also helps to discover new active molecules of non-glycosides structure, which is undoubtedly meant for the research in this field. In fact, **CAG** showed an improvement of pharmacokinetic profile over **AST** (Zhou et al., 2012), but whether **CAG** had a promising anti-diabetes effect is not reported.

We examined the effect of **CAG** in the diabetic ZDF rat, which is a well-recognized model with characteristics towards diabetes as a manifestation of accompanied metabolic syndromes (MetS) in humans. Our results showed that **CAG** efficiently decreased plasma glucose, improved glucose tolerance, heart and kidney fibrosis, and coronary artery sclerosis. To be noted, the anti-diabetic effects of **CAG** shared a similar manner with Dapagliflozin rather than metformin, especially the manner in glucose uptake. Together with our further finding that **CAG** could down-regulated SGLT2's expression, **CAG** displays its anti-diabetes effects, at least in part, through the SGLT2 protein. These therapeutic effects suggest that **CAG** may be a considerable potential anti-diabetes candidate for further development.

SGLT2 is a novel plasma glucose regulator that responds for glucose (re)absorption by regulating glucose reabsorption in the kidney. SGLT2 is tissue-specific expressed in the kidney, and its expression level is inducible in response to the change of plasma glucose concentration (Chonlaket et al., 2018). Therefore, SGLT2 inhibitors are a new class of antidiabetic drugs with an insulin-independent mechanism. In particular, SGLT2 inhibitors reduce hospitalization for heart failure or cardiovascular mortality in patients with type 2 diabetes (Reid et al., 2020). We observed a reduction in the thickness of the artery wall in the mice-treated with **CAG**, together with a decrease of the expression of SGLT2 in cells and tissues. Moreover, **CAG** increased the urinary glucose, urinary sodium but decreased the urinary efflux volume, which are precisely the characteristics of SGLT2 inhibitors.

As talking above, there are lots of severe complications related to T2DM arising from chronic hyperglycemia. The majority of individuals with diabetes mainly die from cardiovascular disease. Recently, numerous trials have been carried out to ascertain the benefits of intensive glucose lowering on cardiovascular outcomes diabetes, such as using the SGLT2 inhibitors or combination therapy (Birkeland et al., 2017). The mechanism for SGLT2 inhibitor to reduce cardiovascular failure in T2DM patients includes reducing the oxygen consumption, improving the energy metabolism of the heart, and inhibiting the fibrosis of cardiomyocytes (Lam et al., 2016). Extensive evidence shows that there exists fibrosis in T2DM patients, including myocardial fibrosis (Guido et al., 2017) and kidney fibrosis (Tang et al., 2017). Diabetes-associated fibrosis is mainly mediated by activated fibroblasts, but may also involve fibrogenic actions of macrophages, fibroblasts, and vascular cells (Monami et al., 2017). To our delight, **CAG** showed an excellent effect in reversing myocardial fibrosis and renal fibrosis, although it was not as significant as Dapagliflozin in lowering blood glucose. To be specific, we found **CAG** significantly decreased the fiber contents in the tissues of the heart and kidney, accompanied by a significant reduction in the expression of TGF- β , which is the primary transcription activator involved in the regulation of fibrosis.

In conclusion, the present study identified the treating effects of **CAG** on both metabolic factors and diabetes-related comprehensions. These data showed that **CAG** treatment significantly reduced plasma glucose and improved hyperinsulinemia in ZDF diabetic rats. **CAG** prevents cardio and renal dysfunction by inhibiting

the accumulation of fibrosis in the heart and kidney of ZDF rats. Be noted, these treating effects of CAG were in line with an inhibition of plasma reabsorption in kidneys with SGLT2 as a key effector (Figure 8). Also, this study further provided new clues regarding the role of the SGLT2 inhibitor Dapagliflozin in treating diabetes and its related complications, such as cardiovascular disease and kidney dysfunction. Overall, these findings may provide a new insight into the novel application of **CAG** in the prevention of diabetes as well as the related metabolic comprehensions.

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Conflict of interest

This study does not incur financial or any other conflicts of interest.

Author contribution

This study was designed by T.-M. Ou, Y. Rao, and Q. Zhang. Q. Zhang, S. Q. Chen, J. W. Sun., Z. Y. Sun, W. Peng, G. T. Kuang, X. X. Su, X. N. Wang, performed biologic evaluation, animal study and mechanism study of **AST** and **CAG**. Y. Rao, T.-M. Ou provided reagents, materials and analysis tools. Data was analyzed and interpreted by Y. Rao, T.-M. Ou. The manuscript was written by Q. Zhang, Y. Rao. T.-M. Ou, with input from all authors.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for *Immunoblotting and Immunohistochemistry*, *Animal Experimentation*, and *Design & Analysis* and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Figures Legends

Figure 1 . Effects of **AST** and **CAG** on plasma glucose and glucose tolerance in KM mice. 18-22 g male KM mice were gavage administrated with **AST** (5 mg/kg) and **CAG** (5 mg/kg) by for ten days. Fasted mice overnight and performed the OGTT experiment by orally administrated glucose at a dosage of 2 g/kg, determined plasma glucose at the time point of 0, 15, 30, 60, and 120 min. The curve and area under curve (AUC) were calculated by Origin 8 software. Dapagliflozin (1 mg/kg) and Metformin (100 mg/kg) were used as positive controls. (A) Chemical structures of **AST** and **CAG** . (B) The content of plasma glucose in the OGTT experiment. (C) The calculated AUC in the OGTT experiment. # $p < 0.05$, compared with the lean group; * $p < 0.05$, compared with the vehicle group. N = 8 mice/group.

Figure 2 . Effect of **CAG** on glucose uptake in cells. HEK293 cells with over-expression SGLT2 were treated with **CAG** (10 μ M), Dapagliflozin (10 μ M), and Metformin (1 mM) alone or combined with insulin (10 μ g/mL) for 1 h, then cells were stained with 2-NBDG (120 μ M) and Hoechst 33342 (1 μ g/mL) at 37 $^{\circ}$ C for 30 min. Representative images were captured and fluorescence were quantified. Image magnification,

$\times 100$. # $p < 0.05$, compared with the vehicle cells; * $p < 0.05$, compared with the insulin-treated cells. N = 5 independent experiments.

Figure 3. Determination of the effects of **CAG** on ameliorating diabetes related MetS in ZDF diabetes rats. ZDF diabetes rats were treated with Dapagliflozin (1 mg/kg/d) and **CAG** (1 or 5 mg/kg/d) for five weeks, respectively. Relative parameters were determined. (A) Change of plasma glucose level in ZDF diabetes rats; (B-C) OGTT test after one-week treatment of drugs; (D) Change of body weight of the ZDF rats; (E) Levels of glucose, HbA1c, and insulin in serum, respectively; (F-H) Levels of triglyceride, cholesterol, and cholesterol in serum, respectively; (I) Ratio of HDL-c/LDL-c in serum. # $p < 0.05$ compared with lean group mice; * $p < 0.05$ compared with vehicle group mice. N = 8 mice / group.

Figure 4. Determination of the protective effect of **CAG** on coronary artery in ZDF diabetic rats. Rats were treated with Dapagliflozin (1 mg/kg/d) and **CAG** (1 or 5 mg/kg/d) for five weeks, the tissues coronary artery were collected and subjected to HE and PAS staining. (A) Representative HE and PAS staining image of coronary artery. Image magnification, $\times 200$. (B) Determination of the thickness of coronary artery. # $p < 0.05$, compared with lean group mice; * $p < 0.05$, compared with vehicle group mice. N = 8 mice / group.

Figure 5. Determination of the anti-fibrosis effects of **CAG** in the heart and the kidney of ZDF diabetic rats. Rats were treated with Dapagliflozin (1 mg/kg/d) and **CAG** (1 or 5 mg/kg/d) for five weeks, the tissues of heart and kidney were collected and subjected to pathological determination and immunohistochemistry assay for TGF- $\beta 1$. (A-C) Sirius red and Masson staining of the heart and quantification. Image magnification, $\times 200$. (D-F) Sirius red and Masson staining of the kidney and quantification. Image magnification, $\times 200$. (G-I) Determination of the protein TGF- $\beta 1$ in the heart and the kidney by using an immunohistochemistry assay and quantification. # $p < 0.05$, compared with lean group mice; * $p < 0.05$, compared with vehicle group mice. N = 8 mice / group.

Figure 6. Effects of **CAG** on glucose reabsorption in the kidney of ZDF rats. Rats were treated with Dapagliflozin (1 mg/kg/d) and **CAG** (1 or 5 mg/kg/d). After 1 week of treatment, the urinary glucose (A), urinary sodium (B) and urine efflux (C) within 24 hours were determined. After 5 week of treatment, the total urinary glucose (D), urinary sodium (E) and urine efflux (F) in 24 hours were determined. (G) Effect of **CAG** on the water intake after 1- or 5-week treatment. # $p < 0.05$, compared with lean group mice; * $p < 0.05$, compared with vehicle group mice. N = 8 mice / group.

Figure 7. Inhibition of **CAG** on SGLT2's expression. (A) Effects of **CAG** and Dapaglifloin on the expression of SGLT2 in the kidney of the ZDF rats by using an immunohistochemistry assay. (B) Determination of the SGLT2 expression in HEK293 cell by using Western blot. NC indicates cells without transfection by any plasmid, SGLT2 indicates cells transfected with the plasmid plvx-puro-SGLT2, and Ctrl indicates cells transfected with the control plasmid. (C) The HEK293 cells with over-expression of SGLT2 were treated with Dapagliflozin and **CAG** for 24 hours, and cells were subjected to SGLT2 determination by using Western blot.

Figure 8. Proposed mechanism underlying the anti-diabetes effects of **CAG** in ZDF diabetic rat.







