Danggui Buxue decoction ameliorates lipid metabolic defects involved in the initiation of diabetic atherosclerosis; identification of active compounds

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Abstract

OBJECTIVE: To determine the constituent compounds of Danggui buxue decoction (DBD) involved and the potential mechanisms mediating its effects, with specific reference to lipids playing a role in the initiation of diabetic atherosclerosis.

METHODS: Liquid chromatography-tandem mass spectrometry was used to identify and quantify the absorbed bioactive compounds (ABCs) present in DBD. Goto-Kakizaki (GK) rats were randomly allocated to a diabetes atherosclerosis (DA) group, a DBD group, and an ABC group (10 per group), which were all high-fat diet-fed. The treated rats were administered DBD (4 g/kg) or ABCs (in amounts equal to those present in DBD) once daily for 28 d, and a control group of Wistar rats were administered vehicle. Body mass gain, fasting blood glucose, and homestasis assessment of insulin resistance (HOMA-IR) were measured. Serum triglyceride (TG), cholesterol (CHOL), high density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) and tumor necrosis factor-α (TNF-α) concentrations were determined. Hematoxylin and eosin staining and microscopy were used to characterize the abdominal aorta and the expression of lipogenic genes was quantified in this vessel.

RESULTS: Seven ABCs were identified in rat serum: ferulic acid, formononetin, calycosin, astragaloside, caffeic acid, ligustilide, and butyphthalide. DBD significantly reduced HOMA-IR, the serum concentrations of TG, CHOL, and LDL-C, and the expression of the lipogenic genes monocyte chemotactic protein 1, Fas, intercellular adhesion molecule 1, and Cd36 in aorta; and significantly increased the mRNA expression of Scd1 in aorta.

CONCLUSION: DBD affects lipid metabolism in the early stage of atherosclerosis in diabetic GK rats, with the mechanism likely involving the regulation of lipid metabolic genes in vessels. The contribu-
tion of ABCs to the effect of DBD on lipid metabolism was 24%-101%.

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Keywords: Atherosclerosis; Phytochemicals; Lipid metabolism; Danggui buxue decoction

INTRODUCTION

Macrovascular complications are the leading cause of morbidity and mortality in patients with type 2 diabetes mellitus (T2DM).1,2 The atherosclerosis that develops in diabetic patients often involves the coronary, lower limb, and extracranial carotid arteries, and increases the risk of coronary heart disease 2-4 fold. However, early and mid-stage atherosclerosis remains asymptomatic and acute coronary syndromes do not occur without the preceding development of a coronary plaque. Decades of research have revealed that abnormalities in certain lipid particles in the blood, such as triglyceride (TG), low-density lipoprotein-cholesterol (LDL), and high-density lipoprotein-cholesterol (HDL), are risk factors for the development of atherosclerosis and coronary events. Cholesterol (CHOL) is a well-known coronary risk factor, and studies have shown that the consumption of a high-fat/high-CHOL meal aggravates arterial inflammation and oxidative stress, and impairs endothelial function.1,3 Goto-Kakizaki (GK) rats fed a high-fat diet (HFD) have been demonstrated to develop lesions that closely resemble early atherosclerosis in diabetes patients, characterized by excessive lipid accumulation in vascular walls; thus, they are considered to represent an ideal model of diabetic atherosclerosis.4

Emerging evidence suggests that Traditional Chinese Medicines (TCMs) have potential for the treatment of diabetes and its complications, because of their range of effects.5 Danggui-buxue decoction (DBD), a TCM formula, is composed of Huangqi (Radix Astragali Mongolic) and Danggui (Radix Angelicae Sinensis) in a 5:1 ratio. This formula is thought to replenish vital energy and blood components, to protect against diabetes, and regulate neural plasticity, without generating obvious adverse reactions. In our previous study, we found that DBD reduces the serum concentrations of c-reactive protein (CRP) and tumor necrosis factor-α (TNF-α), improves survival, ameliorates body mass loss, and reduces water intake in GK rats with diabetic atherosclerosisists.5,6

However, according to the philosophy of bioethnopharmacological analytic pharmacology (BAP), absorbed bioactive compounds (ABCs) are more likely to have a therapeutic effect in vivo after oral administration. Therefore, it is important to identify the ABCs in DBD and determine which of the absorbed compounds are bioactive, to verify the efficacy of the TCM and lay the foundation for the wider clinical application of DBD.7,12 Thus, in the present study, we aimed to identify the constituent ABCs and compare their efficacy with that of the parent formula, specifically with regard to lipid metabolism and the initiation of atherosclerosis in diabetic GK rats.

MATERIALS AND METHODS

Drugs and chemicals

Reference grade ferulic acid, astragaloside, formononetin, calycosin, caffeic acid, ligustilide, and butyrophthalide (purity 99.8%) were purchased from the National Institute of Control of Pharmaceutical and Biologic Products (Beijing, China). The herbs were purchased from the pharmacy of Jiangsu Provincial Hospital (Nanjing, China). Experimental animals were purchased from Shanghai Slack Experimental Animals Co., Ltd. (license No. SCXK 2012-0002). TRIzol Plus RNA Purification Kit was purchased from Invitrogen (12183-555) and an RNase-Free DNase Kit was from Qiagen (79254) (Carlsbad, CA, USA). All other reagents were of high-performance liquid chromatography grade or were of the highest purity commercially available.

Preparation of DBD

DBD was prepared by water decoction, as previously described.9 According to the prescription, Huangqi (Radix Astragali Mongolic) 100 g and Danggui (Radix Angelicae Sinensis) 20 g were weighed out and placed in a round-bottom flask, then eight volumes (g/mL) of pure water were added, the ingredients were soaked for 60 min at room temperature, and then the suspension was boiled for 30 min. Subsequently, the suspension was gauze-filtered, and six volumes (g/mL) of pure water were added to the residue, which was stirred, and the decoction continued for 30 min. After the product was filtered, the two filtrates were combined, placed in a rotary evaporator, and rotated to concentrate the filtrate at 60 °C. The concentrated filtrate (100 mL; 1.2 g/mL) was then freeze-dried.

Analysis of the ABCs of DBD using liquid chromatography-tandem mass spectrometry

Sample preparation: the 10 Wistar rats were fasted for 12 h, while continuing to have free access to water. DBD (20 g/kg) was then administered by intragastric gavage and blood samples were obtained 30 min later. These blood samples were centrifuged at 1500 × g for 10 min at 4 °C, and the plasma was collected and stored at −20 °C. Fifty microliters of quercetin (200 μg/mL) was added as an internal standard to 0.2 mL of plasma. The samples were mixed, extracted with 1.8 mL acetonitrile by vortexing for 3 min, centrifuged at 1500 × g for 10 min, and 1.6 mL of the upper organic phase were carefully transferred to a 5 mL tube and evaporat-
ed under nitrogen. After drying, the residue was reconstituted with 200 μL of water: acetonitrile (50: 50, v/v), vortexed for 3 min, and centrifuged at 15 000 rpm for 10 min at 4 °C. The supernatant (50 μL) was then collected and injected into the liquid chromatography-tandem mass spectrometry (LC-MS-MS) system. Chromatography system and instrumentation for the measurement of the plasma concentration of ABCs: The ABCs were analyzed by LC-MS-MS using a Q-TRAP 5500. Chromatographic separations were performed with a Waters Acquity BEH-C18 (2.1 mm × 100 mm, 1.7 μm) and a column temperature of 40 °C. The mobile phase was 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient program used was: 0 min, A: B 75/25 (v/v); 2 min, A: B 75/25; 3 min, A: B 60/40; 5 min, A: B 10/90; 8 min, A: B 0/100; 8 min, A: B 75/25; 12 min, A: B 75/25. The flow rate was 0.3 mL/min. Electrospray Ionization (ESI) was used for mass analysis and detection. The mass spectrometer was operated in the positive and negative-ion mixing detection mode. The ion source vapor temperature was 350 °C, and the drying gas flow 10 L/min. The internal standard was quercetin. The conditions used for mass spectrometry are shown in Table 1. It was determined that the ABCs comprised the following seven components: ferulic acid > caffeic acid > ligustilide > calycosin > formononetin > butyphthalide > astragaloside IV. The chromatograms are shown in Figure 1.

**Experimental animals**

Animal experimentation was performed in compliance with national ethical guidelines and was approved by Nanjing University of Chinese Medicine Animal Care and Use Committee (201809A028). Thirty 4-month-old male GK rats and 10 age-matched non-diabetic Wistar rats were purchased for the study. After 1 week of acclimatization, random blood glucose (RBG) measurements were made in all the rats. Thirty GK rats with an RBG > 11.1 mmol/L were randomly assigned to three groups (n = 10 per group): an early phase of diabetic atherosclerosis (DA) group, a DBD-treated group (DA + 4 g • kg⁻¹ • d⁻¹ intragastric administration of DBD), and an ABC-treated group (DA + amounts of each ABC equal to the composition of the decoction: caffeic acid 5.36 mg/kg, formononetin 2.68 mg/kg, calycosin 2.56 mg/kg, ferulic acid 1.36 mg/kg, astragaloside 0.45 mg/kg, butyphthalide 0.16 mg/kg, and ligustilide 0.16 mg/kg). The three groups were all fed an HFD to induce the early phase of atherosclerosis. The 10 Wistar rats, used as non-diabetic controls (control group), were administered sterile water and consumed standard rat chow, and the other groups were administered DBD or ABCs, respectively, by intragastric gavage for 4 weeks. Blood glucose concentrations were monitored once weekly for the entire study period using a blood glucose meter (LifeScan, Milpitas, CA, USA). The body mass of the rats was measured twice weekly.

**Measurement of fasting blood glucose, serum insulin, and HOMA-IR**

At the end of experiment, after 12 h of food deprivation, fasting blood glucose was measured using a blood glucose meter. An enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Sweden) was used to determine serum insulin concentrations, following the manufacturer’s instructions. Homeostasis assessment of insulin resistance (HOMA-IR) was calculated using the formula: HOMA-IR = fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5.

**Measurement of serum CHOL, TG, HDL-C, LDL-C, and TNF-α**

At the end of the study, and after 12 h of food deprivation, the animals were anesthetized and blood was collected from their caudal venae cavae. The samples were then centrifuged at 1500 × g and 4 °C for 10 min within 1 h of collection, and the sera frozen at −80 °C until analysis. Serum CHOL, TG, HDL-C, and LDL-C were measured using an autoanalyzer (Shimadzu CL-7200, Shimadzu Co., Kyoto, Japan). TNF-α was determined using a Sunny ELISA kit (Multi Sciences, Shanghai, China).

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**Table 1: Mass spectrometric conditions for each compound**

<table>
<thead>
<tr>
<th>Item</th>
<th>Detection mode</th>
<th>DP (Volts)</th>
<th>EP (Volts)</th>
<th>CE (Volts)</th>
<th>CXP (Volts)</th>
<th>Time (msec)</th>
<th>m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferulic acid</td>
<td>ESI-</td>
<td>−85</td>
<td>−10</td>
<td>−20</td>
<td>−13</td>
<td>50</td>
<td>193.0-134.0</td>
</tr>
<tr>
<td>Astragaloside</td>
<td>ESI-</td>
<td>−50</td>
<td>−10</td>
<td>−30</td>
<td>−33</td>
<td>50</td>
<td>783.2-283.0</td>
</tr>
<tr>
<td>Formononetin</td>
<td>ESI-</td>
<td>−215</td>
<td>−10</td>
<td>−28</td>
<td>−21</td>
<td>50</td>
<td>267.0-252.0</td>
</tr>
<tr>
<td>Calycosin</td>
<td>ESI-</td>
<td>−180</td>
<td>−10</td>
<td>−26</td>
<td>−11</td>
<td>50</td>
<td>282.9-268.0</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>ESI-</td>
<td>−80</td>
<td>−10</td>
<td>−20</td>
<td>−11</td>
<td>50</td>
<td>179.0-135.0</td>
</tr>
<tr>
<td>Ligustilide</td>
<td>ESI+</td>
<td>81</td>
<td>10</td>
<td>33</td>
<td>12</td>
<td>50</td>
<td>191.1-191.0</td>
</tr>
<tr>
<td>Butyphthalide</td>
<td>ESI+</td>
<td>76</td>
<td>10</td>
<td>19</td>
<td>16</td>
<td>50</td>
<td>190.7-145.1</td>
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<tr>
<td>Quercetin</td>
<td>ESI-</td>
<td>−54</td>
<td>−10</td>
<td>−30</td>
<td>−16</td>
<td>50</td>
<td>301.1-151.0</td>
</tr>
</tbody>
</table>

Notes: DP: declustering potential; EP: entry potential; CE: collision energy; CXP: cell exit potential; ESI: electrospray ionization.
Histologic staining
At the end of the experiment, abdominal aortae were harvested, weighed, and fixed in 10% buffered formalin. The abdominal aortae were then processed for histologic examination using conventional methods and stained with hematoxylin and eosin (HE). Histopathologic assessments were performed by a qualified pathologist, including determination of the area of the atherosclerotic plaque.

RNA extraction and RT-PCR
Total RNA was isolated from aortae using TRIzol® Plus RNA Purification Kit then mRNA was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Quantitative real-time PCR was performed using CFX384 multiplex real-time fluorescence quantitative PCR (Bio-Rad, USA). Primer 6 and Beacon designer 7.8 software were used to design the PCR primers, and the primer sequences and PCR conditions are listed in Table 2. The relative mRNA expression of Mcp1, Scd1, Fas, Icam1, and Cd36 were calculated using the $2^{-\Delta\Delta C_{p}}$ (Cp = crossing point) method.

Calculation of the contribution of ABCs
We calculated the contribution of the ABCs to the effects of DBD treatment, on the basis that the concentration of each ABC was equal to that in the DBD, using the formula: component effect/parent formula effect × 100%.

Statistical analysis
All data were analyzed using SPSS19.0 Software Statistical Package (SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± standard error of mean. Statistical significance was determined using one-way analysis of variance. $P < 0.05$ was considered to represent statistical significance.

RESULTS
Qualitative and quantitative assessment of the ABCs in DBD
An accurate and rapid LC-MS/MS method was established for the identification of the ABCs in rat plasma following the oral administration of DBD extract at a dose of 20 g/kg. The ABCs of DBD identified were fe-
rulinic acid, astragaloside, formononetin, calycosin, caffeic acid, ligustilide, and butyrophilalde. The rat treatment groups were administered DBD (4 g/kg) or ABCs in quantities equal to their concentrations in the decoction: caffeic acid 5.36 mg/kg, formononetin 2.68 mg/kg, calycosin 2.56 mg/kg, ferulic acid 1.36 mg/kg, astragaloside 0.45 mg/kg, butyrophilalde 0.16 mg/kg, and ligustilide 0.16 mg/kg.

**Effect of DBD and ABCs on body mass and blood glucose**

Compared with the control group, the DA, DBD, and ABC groups were less weight gain. There were no significant differences in body mass gain among the DA, DBD, and ABC groups (Figure 1A), but the blood glucose of the DBD and ABC groups was significantly lower than that of the DA group after 1 week of treatment. Furthermore, the blood glucose concentration of the two treatment groups had decreased to normal after 4 weeks of treatment (Figure 1B).

**Effect of DBD and ABCs on fasting blood glucose, serum insulin, and HOMA-IR**

Compared with the DA group, the FBG of the DBD and ABC group were significantly lower (Figure 2A), while the fasting serum insulin was higher in the ABC-treated rats (Figure 2B). A lower HOMA-IR score was calculated for the DBD group than for the other groups (Figure 2C).

**Effect of DBD and ABCs on serum CHOL, TG, HDL-C, LDL-C, and TNF-α**

As shown in Figure 3, the DBD and ABC groups had lower serum TG than the DA group (P < 0.05), and the cholesterol and HDL-C concentrations were significantly lower in the DBD-treated group than in the DA group (P < 0.05). There were no significant differences in LDL-C among the groups. The serum TNF-α concentration in DA rats [(2.53 ± 0.64) pg/mL] was higher than that in control rats [(1.61 ± 0.35) pg/mL, P < 0.01]. However, treatment with DBD [(1.79 ± 0.16) pg/mL] or ABCs [(1.87 ± 0.35) pg/mL] significantly reduced serum TNF-α (P < 0.05 versus the DA group) to a similar concentration to that of the control group.

**Effects of DBD and ABCs on aortic histology**

The results of the histologic examination of the aortae are shown in Figure 4. The control rats demonstrated no thrombotic plaques, and the intima of each aortic arch was smooth and intact. In contrast, DA group rats displayed external membrane hemorrhage, a large number of aggregating foam cells, resulting in thickening of the intima, and proliferation of smooth muscle cells, resulting in atherosclerotic plaques. However, after 28 d of treatment, the appearance of aortae from the DBD and ABC groups was normal.

**Effect of DBD and ABCs on the mRNA expression of monocyte chemotactic protein 1 (Mcp1), stearyl-CoA desaturase 1 (Scd1), Fas, intercellular adhesion molecule 1 (Icam1), and Cd36**

As shown in Figure 5, Scd1 mRNA expression was significantly higher in the DBD group (P < 0.01), while that of Fas and Cd36 was significantly lower, than in the DA group (P < 0.05). Furthermore, the expression of Mcp1 and Icam1 was significantly lower in the aortae of DBD and ABC-treated rats (P < 0.01).

**Contribution of ABCs**

An example of how the contribution of the ABCs to the effect of the parent preparation was calculated is given below. Compared with the DA group, the fasting blood glucose of the DBD group was 31.4% lower, and that of the ABC group was 26.1% lower. Thus, the contribution of the ABCs to the reduction in fasting blood glucose induced by DBD treatment was 0.3143/0.2610 × 100% = 83%. Likewise, the contributions of the ABCs to CHOL, TG, HDL-C, LDL-C, and TNF-α were 48%, 24%, 101%, 78%, and 89%, respectively.
Figure 2 Preventive effects of DBD and ABCs on the development of insulin resistance in db/db mice
A: fasting blood glucose; B: insulin concentration; C: homeostasis assessment of insulin resistance. Control group (non-diabetic controls) was administered sterile water and consumed standard rat chow. DA group, a DBD-treated group (DA + 4 g·kg⁻¹·d⁻¹ intragastric administration of DBD), and an ABC-treated group (DA + amounts of each ABC equal to the composition of the decoction: caffeic acid 5.36 mg/kg, formononetin 2.68 mg/kg, calycosin 2.56 mg/kg, ferulic acid 1.36 mg/kg, astragaloside 0.45 mg/kg, butyphthalide 0.16 mg/kg, and ligustilide 0.16 mg/kg) were administered DBD or ABCs, respectively, by intragastric gavage for 4 weeks. DA: diabetes atherosclerosis; DBD: Danggui-buxue decoction; ABCs: absorbed bioactive compounds. *P < 0.01, †P < 0.05, versus DA group.

Figure 3 Effects of on serum TG, CHOL, HDL-C and LDL-C content
Control group (non-diabetic controls) was administered sterile water and consumed standard rat chow. DA group, a DBD-treated group (DA + 4 g·kg⁻¹·d⁻¹ intragastric administration of DBD), and an ABC-treated group (DA + amounts of each ABC equal to the composition of the decoction: caffeic acid 5.36 mg/kg, formononetin 2.68 mg/kg, calycosin 2.56 mg/kg, ferulic acid 1.36 mg/kg, astragaloside 0.45 mg/kg, butyphthalide 0.16 mg/kg, and ligustilide 0.16 mg/kg) were administered DBD or ABCs, respectively, by intragastric gavage for 4 weeks. DA: diabetes atherosclerosis; DBD: Danggui-buxue decoction; ABCs: absorbed bioactive compounds. *P < 0.05 vs the DA group.

Figure 4 Histological haematoxylin-eosin staining of the aortic
A: control group; B: DA group; C: DBD group; D: ABCs group. Control group (non-diabetic controls) was administered sterile water and consumed standard rat chow. DA group, a DBD-treated group (DA + 4 g·kg⁻¹·d⁻¹ intragastric administration of DBD), and an ABC-treated group (DA + amounts of each ABC equal to the composition of the decoction: caffeic acid 5.36 mg/kg, formononetin 2.68 mg/kg, calycosin 2.56 mg/kg, ferulic acid 1.36 mg/kg, astragaloside 0.45 mg/kg, butyphthalide 0.16 mg/kg, and ligustilide 0.16 mg/kg) were administered DBD or ABCs, respectively, by intragastric gavage for 4 weeks. DA: diabetes atherosclerosis; DBD: Danggui-buxue decoction; ABCs: absorbed bioactive compounds.
DISCUSSION

The present study was designed to determine the effects of ABCs on lipid metabolism in the early stage of atherosclerosis induced by HFD-feeding in diabetic GK rats. We found that HOMA-IR was lower in the DBD group than in the DA group, but not in the ABC group. It is well known that serum lipid profile is an important factor in the development of diabetes mellitus and atherosclerosis, and cholesteryl ester accumulation in macrophages is a crucial step in the pathogenesis of atherosclerosis. We also found that both the DBD and ABC groups had lower serum TG and HDL-C concentrations than the DA group. Because of Angelicae sinensis radix and ferulic acid could inhibit the activity of rat liver methylvalerate-5-pyrophosphate dehydroxylase, antioxidant and free radical-scavenging effects, they protect the wall of blood vessels, and maintain appropriate lipid balance, together constituting a significant anti-atherogenic effect. Mounting evidence suggests that systemic low-grade inflammation plays a central role in atherosclerosis, and in the origins and complications of T2DM. We have shown that DBD and ABCs reduce the circulating levels of TNF-α in rats with diabetic atherosclerosis and prevent aortic pathology developing.

The pathogenesis of atherosclerosis involves lipid accumulation in arterial walls, and this lipid is delivered by circulating lipoproteins. Mcp1 induces monocyte aggregation in the endothelial gap, which is one of the earliest pathologic events in atherosclerosis. Fas and Scd1 are key lipogenic enzymes, and Icam1 plays a key role in the process of monocyte transmigration into the subendothelial space. Finally, the study of murine models of atherosclerosis has demonstrated that LDL uptake is primarily mediated by Cd36. Thus, the expression of the genes encoding Mcp1, Scd1, Fas, Icam1, and Cd36 is important for the initiation of a lesion in the arterial wall. Our results shown that the expression of Mcp1, Fas, Icam1, and Cd36 in the aorta of rats in the early phase of diabetic atherosclerosis is significantly higher than in control rats. However, administration of DBD or ABCs downregulates the expression of these genes, providing a likely mechanism for their effects on lipid metabolism.

In summary, our results demonstrate that DBD affects lipid metabolism in the early stage of atherosclerosis in diabetic GK rats and the mechanism likely involves the regulation of lipid metabolic gene expression. Our data also show that the seven ABCs identified account for 24%-101% of the effects of DBD on lipid metabolism. The specific effects of these seven compounds from DBD require further investigation in follow-up studies. There were, however, some limitations to the present study. Other substances, such as amino acids, affect macrophage atherogenicity, mainly through modulation of cellular triglyceride metabolism. In addition, previous research has shown that much of the efficacy of Traditional Chinese Medicines may be achieved through effects on the intestinal flora, and some may be the result of pharmacologic effects of metabolites. This may explain why ABCs were less effective than the parent preparation, and these possibilities should be assessed in further studies.

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