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RESEARCH

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Glucose fluctuations aggravate myocardial fibrosis via activating the CaMKII/Stat3 signaling in type 2 diabtetes

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Abstract

Background Glucose fluctuations (GF) are a risk factor for cardiovascular complications associated with type 2 diabetes. However, there is a lack of adequate research on the effect of GF on myocardial fibrosis and the underlying mechanisms in type 2 diabetes. This study aimed to investigate the impact of glucose fluctuations on myocardial fibrosis and explore the potential mechanisms in type 2 diabetes.

Methods Sprague Dawley (SD) rats were randomly divided into three groups: the control (Con) group, the type 2 diabetic (DM) group and the glucose fluctuations (GF) group. The type 2 diabetic rat model was established using a high-fat diet combined with low-dose streptozotocin injection and the GF model was induced by using staggered glucose and insulin injections daily. After eight weeks, echocardiography was used to assess the cardiac function of the three groups. Hematoxylin-eosin and Masson staining were utilized to evaluate the degree of pathological damage and fibrosis. Meanwhile, a neonatal rat cardiac fibroblast model with GF was established. Western and immunofluorescence were used to find the specific mechanism of myocardial fibrosis caused by GF.

Results Compared with rats in the Con and the DM group, cardiac function in the GF group showed significant impairments. Additionally, the results showed that GF aggravated myocardial fibrosis in vitro and in vivo. Moreover, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) was activated by phosphorylation, prompting an increase in phosphorylation of signal transducer and activator of transcription 3 (Stat3) and induced nuclear translocation. Pretreatment with KN-93 (a CaMKII inhibitor) blocked GF-induced Stat3 activation and significantly suppressed myocardial fibrosis.

Conclusions Glucose fluctuations exacerbate myocardial fibrosis by triggering the CaMKII/Stat3 pathway in type 2 diabetes.

Keywords Glucose fluctuations, Myocardial fibrosis, CaMKII, Stat3

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Introduction

Diabetes is a common chronic disease manifesting as chronic persistent hyperglycemia and glucose fluctuations (GF). Glucose fluctuations, also known as glucose variability, are developing as an emerging indicator of glycemic control [1, 2]. Previous studies have shown that a higher GF is independently associated with heart failure [3], arrhythmias [4], and cardiovascular events [5]. In type 1 diabetes, GF can lead to myocardial fibrosis [6]; however, there is currently limited understanding of whether GF can lead to myocardial fibrosis in type 2 diabetes and the mechanisms involved have not been clarified.

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine-protein kinase, has been identified as a critical factor in various cardiac diseases [7–9]. Many studies have shown that diabetes can increase CaMKII activity in the myocardium [10], resulting in ROS induction [11], necroptosis [12] and ion channel anomalies and so on. Furthermore, Das et al. [13] demonstrated that CaMKII inhibition can prevent chemotherapy-induced cardiac fibrosis. However, the potential effects of CaMKII in myocardial fibrosis resulting from GF are still unknown.

Signal transducer and activator of transcription 3 (Stat3), one of the STAT members, has increasingly gained focused attention due to its significant roles in metabolic diseases [14], cardiac hypertrophy [15, 16], heart failure [17] and arteriosclerosis [18]. Previous studies have verified that inhibition of p-Stat3 attenuated cardiomyopathy caused by type 1 diabetes [19]. Additionally, Unudurthi et al. have found that CaMKII and Stat3 can interact with each other [20]. However, the role of Stat3 activation in myocardial fibrosis induced by GF and upstream events leading to Stat3 activation still needs to be elucidated. Hence, the present study was carried out to unravel the effect of glucose fluctuations on myocardial fibrosis in type 2 diabetes and explore the underlying mechanisms.

Methods

Experimental animals

Male 6–8 weeks Sprague–Dawley (SD) rats were purchased from Changzhou Cavins Biotechnology Company. The rats were housed in a standard environment with 23±1°C, 55–65% humidity and under a 12-h light/12-h dark cycle. All rats were fed with the normal diets and water freely. After one week of adaptive feeding, the rats were randomly divided into two groups, the control (Con) group (n=15) and the type 2 diabetic (T2DM) group. The Con group rats were fed with normal chow. The T2DM model was established by a low dose (35 mg/kg) of streptozotocin (STZ, Sigma-Aldrich, S0130) intraperitoneally after four weeks on a high-fat and high-sugar diet (D12492, Suzhou SPF Biotechnology Co., Ltd, China), according to the method used in previous studies [21]. Blood was extracted from the tail vein after three days and blood glucose>16.7 mmol/L was considered successful in modeling. Then, T2DM rats were divided into the diabetic (DM) group (n=15) and the GF group (n=15). The rats in the DM group continued to be fed with the high-fat diet. The GF model was established based on the previous literature [22]. In brief, the rats in the GF group were injected with insulin subcutaneously at 8:00, 12:00 and 16:00 daily, and 3 g/kg glucose was injected intraperitoneally at 10:00, 14:00 and 18:00 daily for eight weeks. Blood glucose was measured in the tail vein 30 min after each insulin or glucose injection. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals (the revised Animals (Scientific Procedures) Act 1986) and were approved by the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University.

Echocardiography evaluation

After eight weeks, heart function was evaluated using echocardiography (Philip, ie33). After being anesthetized with isoflurane (2%), the hair on the chest of the rats in the three groups (n=6 per group) was shaved and acoustic coupling gel was then applied. M mode of echocardiography was performed to record the following parameters: left ventricular percent ejection fraction (EF), left ventricular fractional shortening (FS), left ventricular internal diameter at end-diastole (LVIDd), and left ventricular internal diameter at end-systole (LVIDs).

Histopathological analysis

After the rats were sacrificed, the left ventricular tissue samples were fixed for 24 h in 4% paraformaldehyde, then paraffin-embedded and sliced into 4-μm sections for hematoxylin-eosin (HE, Beyotime, C0105) and Masson staining (Nanjing Jiancheng Bioengineering Institute, D026). Afterward, the sections were observed under a light microscope (DP73, OLYMPUS).

Primary culture of neonatal rat cardiac fibroblasts

Neonatal rat cardiac fibroblasts (NRCFs) were extracted using the previous method [6]. Neonatal rats born 1–3 days old were selected, and the hearts were quickly removed and digested several times at 37 °C using 0.125% trypsin (Gibco, 25200072) and 0.1% collagenase (Worthington, LS004176). The cell suspension was then seeded in DMEM mediums containing 10% fetal bovine serum (FBS, Gibco, 12664025). After 1 h, remove the unadhered cells and add new mediums. The cells were incubated in a 37 °C incubator with 5% CO_2.

Glucose fluctuations cell model and treatments

NRCFs were divided into three groups as previously reported: the normal glucose (Ctrl) group, the high glucose (HG) group and the glucose fluctuations (GF) group. Cells in the Ctrl group were cultured using DMEM containing 5.5 mmol/L glucose, cells in the HG group were cultured in DMEM containing 33 mmol/L glucose, and cells in the GF group were cultured in DMEM containing 5.5 mmol/L and 33 mmol/L glucose alternately every 12 h for 72 h. In addition, to confirm the effect of CaMKII on myocardial fibrosis, the NRCFs were also treated with KN-93 (a CaMKII inhibitor, 0.5 μmol/L, Medchemexpress, HY-15465) [23].

Western blot analysis

Rat ventricular myocardial tissue or fibroblasts were lysed in lysis buffer containing a cocktail of proteinase/phosphatase inhibitors and then centrifuged at 12000 rpm for 15 min at 4 °C. The proteins were then transferred to PVDF membranes using SDS-PAGE. The PVDF membrane was then incubated with primary antibodies of p-CaMKII (Abcam, ab32678), CaMKII (Santa Cruz, sc100362), p-Stat3 (Cell Signaling Technology, 9145S), Stat3(Cell Signaling Technology, 12640S), Collagen I (Proteintech, 14695-1-AP), Collagen III (Proteintech, 22734-1-AP), TGF-β1 (Abcam, ab179695), β-actin (Abcam, ab6276), β-tubulin(Abcam, ab21058) at 4 °C overnight followed by corresponding secondary antibody incubation. Blot bands were quantified using the Image J software.

Immunofluorescence analysis

Neonatal rat cardiac fibroblasts were washed with phosphate buffered saline (PBS) and fixed using 4% paraformaldehyde for 15 min. Then, the cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich, T9284) and blocked with goat serum (Solarbio, SL038) in PBS for 30 min at 37 °C. After that, NRCFs were incubated at 4 °C overnight with the primary rabbit antibody against p-Stat3, and a fluorescence secondary antibody was added for incubation of 1 h at 37 °C. Afterward, the cells were washed with PBS and counterstained the nucleus with DAPI (Beyotime, C1005) for 10 min and analyzed under a fluorescence microscope.

Statistical analysis

Data were shown as mean±standard error of mean (SEM). Shapiro-Wilk normality test was used to test the data distribution. Non-normally distributed data were analyzed by nonparametric tests. Normally distributed data were analyzed by One-way ANOVA with post hoc LSD. $P<0.05$ was considered statistically significant. All statistics were determined using SPSS 25.0 software.

Results

Glucose fluctuations exacerbated the impairment of cardiac dysfunction

The blood glucose of rats in the three groups 30 min after glucose, insulin injection or normal saline solution was shown in Fig. 1A. Rats in the GF and DM groups had significantly lower body weight than those in the Con group (Fig. 1B). In addition, this study also showed that GF exacerbated cardiac dysfunction by suppressing the EF and increasing the LVIDd and LVIDs (Figures C-G).

Glucose fluctuations aggravated myocardial fibrosis

The HE staining of the three groups showed that the arrangement of myocardial fibers was disordered and there were myocardial fiber breaks in the GF compared to the Con and DM groups. Moreover, the Masson staining showed a significant increase in fibrosis in the ventricular tissue of the GF group rats (Fig. 2A and D). Additionally, GF can significantly increase the protein expression of Collagen I, Collagen III and TGF-β1 in vitro and in vivo (Fig. 2B-C and E-J). Those results indicated that GF can lead to myocardial fibrosis.

Glucose fluctuations promoted the activation of CaMKII and Stat3

To determine whether CaMKII and Stat3 were activated in GF-induced myocardial fibrosis, the relative expressions of total and phosphorylated/activated forms of CaMKII and Stat3 were tested. The results showed upregulation of CaMKII phosphorylation and Stat3 phosphorylation protein expression in the GF rats (Fig. 3A-C) and the cells with fluctuated glucose concentrations (Fig. 3D-F). Immunofluorescent staining showed that GF increased p-Stat3 nuclear accumulation compared to the Con and DM groups (Fig. 4D).

Inhibition of CaMKII reduced myocardial fibrosis

To investigate the role of CaMKII in GF-induced myocardial fibrosis, we used a CaMKII-specific inhibitor, KN-93. As shown in Fig. 5, KN-93 can significantly reduce the expression of Collagen I and Collagen III in HG and GF groups compared with the Con group (Fig. 5A-C). Moreover, the inhibition of CaMKII reversed the upregulation of TGF-β1 in GF groups (Fig. 5A and D). The above results suggested that inhibition of CaMKII can attenuate myocardial fibrosis caused by GF.

Activation of CaMKII promoted GF-induced myocardial fibrosis via activating Stat3

To determine whether Stat3 was the downstream target of CaMKII, we examined the expression of phosphorylated Stat3 in the three groups. The results revealed that inhibition of CaMKII decreased the phosphorylation of Stat3 (Fig. 4A-C). In addition, an immunofluorescence

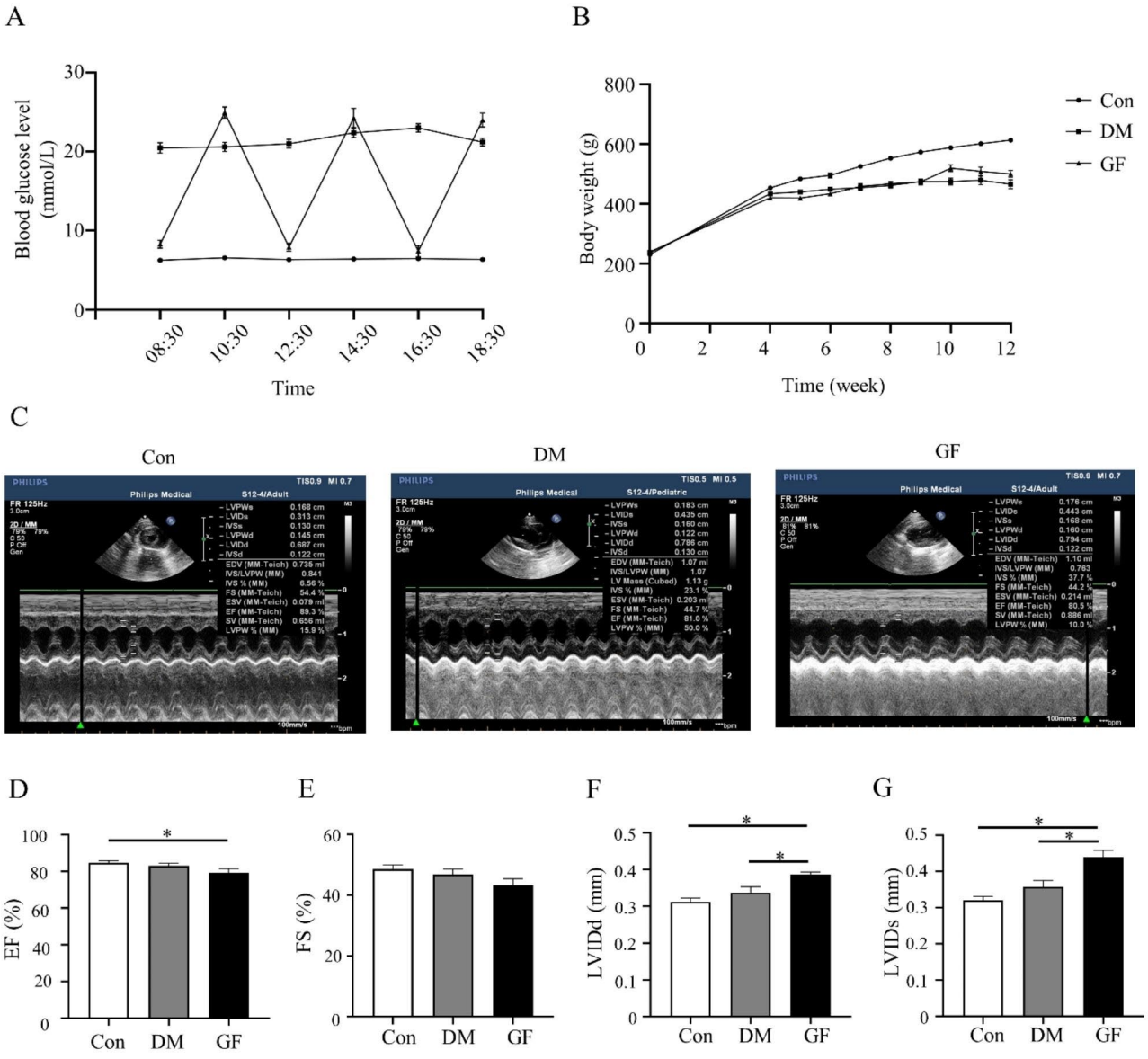

Fig. 1 Glucose fluctuations accelerated cardiac dysfunction. (**A**) Daily blood glucose levels in three groups of rats (n = 15). (**B**) Body weight levels in the three groups (n = 15). (**C**) Representative echocardiographic images of rats. (**D**-**G**) Measurements of ejection fraction (EF), fractional shortening (FS), left ventricular internal diameter at end-diastole (LVIDd) and left ventricular internal diameter at end-systole (LVIDs) (n = 6)

assay was used to evaluate the nuclear translocation of p-Stat3. After the application of KN-93, highly expressed phosphorylated Stat3 in the nucleus of NRCFs in the GF group can be reversed (Fig. 4D).

Discussion

Glycemic management in diabetic patients is now focused not only on effective glucose reduction, but also on how to avoid glucose fluctuations [1]. Our previous studies have shown that glucose fluctuations contribute to the development of many cardiovascular diseases [5, 24]. More importantly, GF in type 1 diabetes can lead to increased myocardial fibrosis [6], but the pathophysiological mechanisms contributing to GF-induced myocardial fibrosis remain elusive, especially in type 2 diabetes. Here, we demonstrated that GF in type 2 diabetes can increase myocardial fibrosis. CaMKII activation played an essential role in this process by activating Stat3, which led to increased myocardial fibrosis (Fig. 6).

As widely reported, CaMKII is a multifunctional serine, threonine protein kinase with four isoforms, α, β, γ and δ. The δ isoform is predominantly expressed in the myocardium and is involved in the development of various cardiovascular diseases, especially in electrical remodeling [9, 10, 25, 26]. However, studies on the role of CaMKII in the development of diabetic myocardial

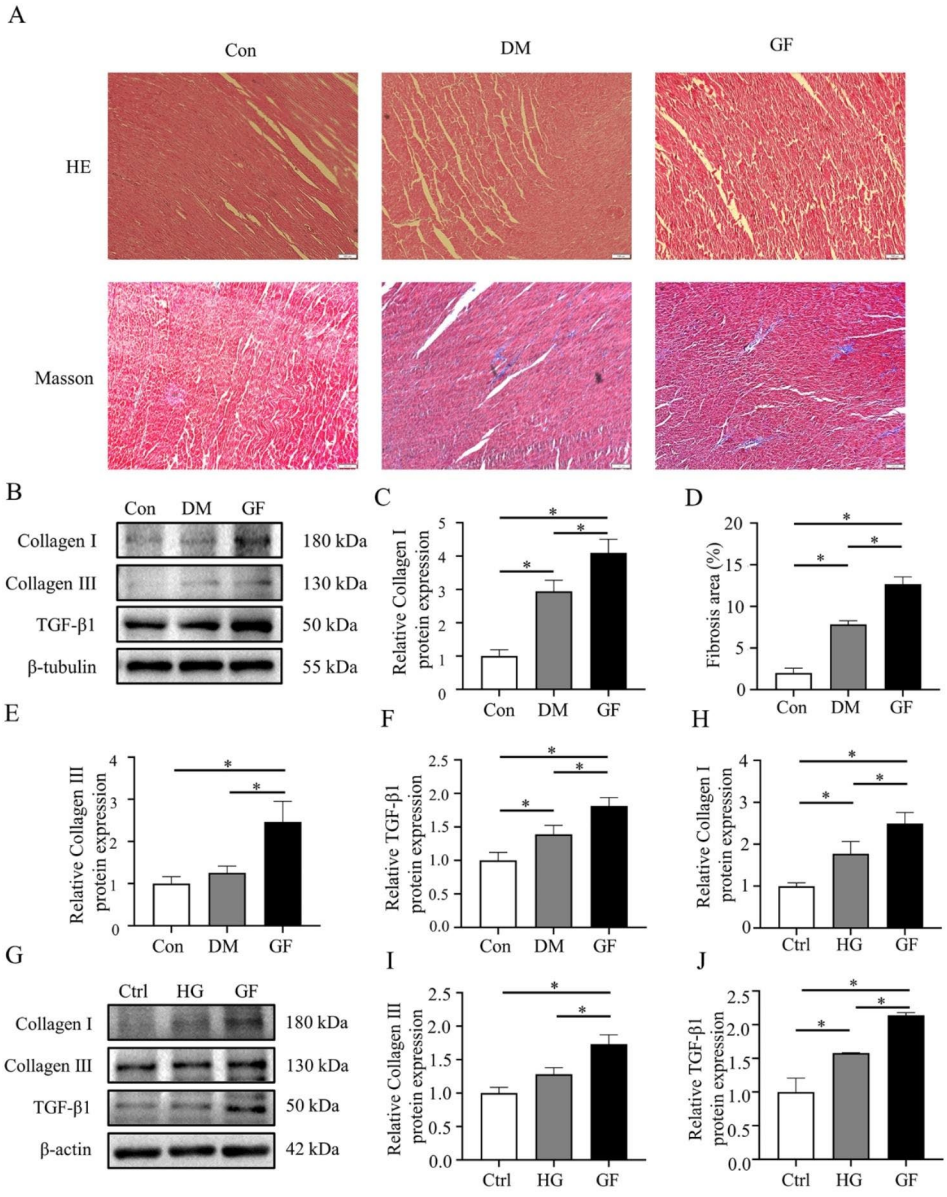

Fig. 2 Glucose fluctuations promoted myocardial fibrosis. (**A**, **D**) HE and Masson staining of ventricular muscle tissue from three groups of rats (n = 3). (**B**, **C**, **E**, **F**) The protein expressions of Collagen I (n = 5), Collagen III (n = 5) and TGF-β1 (n = 6) in rat hearts of the three groups. (**G**-**J**) The protein expressions of Collagen I (n = 6), Collagen III (n = 5) and TGF-β1 (n = 4) in NRCFs of the three groups

fibrosis are limited, especially in diabetic glucose fluctuations. This study showed that GF in type 2 diabetes activated CaMKII, leading to the development of myocardial fibrosis. In contrast, the use of the CaMKII inhibitor KN-93 attenuated myocardial fibrosis. These data demonstrated the vital role of CaMKII in myocardial fibrosis due to GF in type 2 diabetes. Similarly, a previous report has shown that inhibition of CaMKII can attenuate myocardial fibrosis caused by chemotherapy [13]. In addition, upregulation of BACH1 mediated activation of CaMKII was proven to accelerate cardiac hypertrophy and fibrosis [27]. In the current study, it was observed that hesperidin, a specific small-molecule inhibitor of CaMKII-δ, directly bound to CaMKII-δ and specifically blocked its activation in an ATP-competitive manner, may provide a strategy for the joint therapy of cardiovascular disease [28].

Stat3 can be activated through multiple mechanisms and translocated to the nucleus, where it acts as a transcription factor and cofactor [20]. Recent studies have revealed that high glucose can activate Stat3 in fibroblasts, and promote their proliferation and migration [29]. Similarly, high-concentration glucose can induce EGFR-mediated Stat3 phosphorylation, and blocking of Stat3 can repress procollagen gene expressions [19].

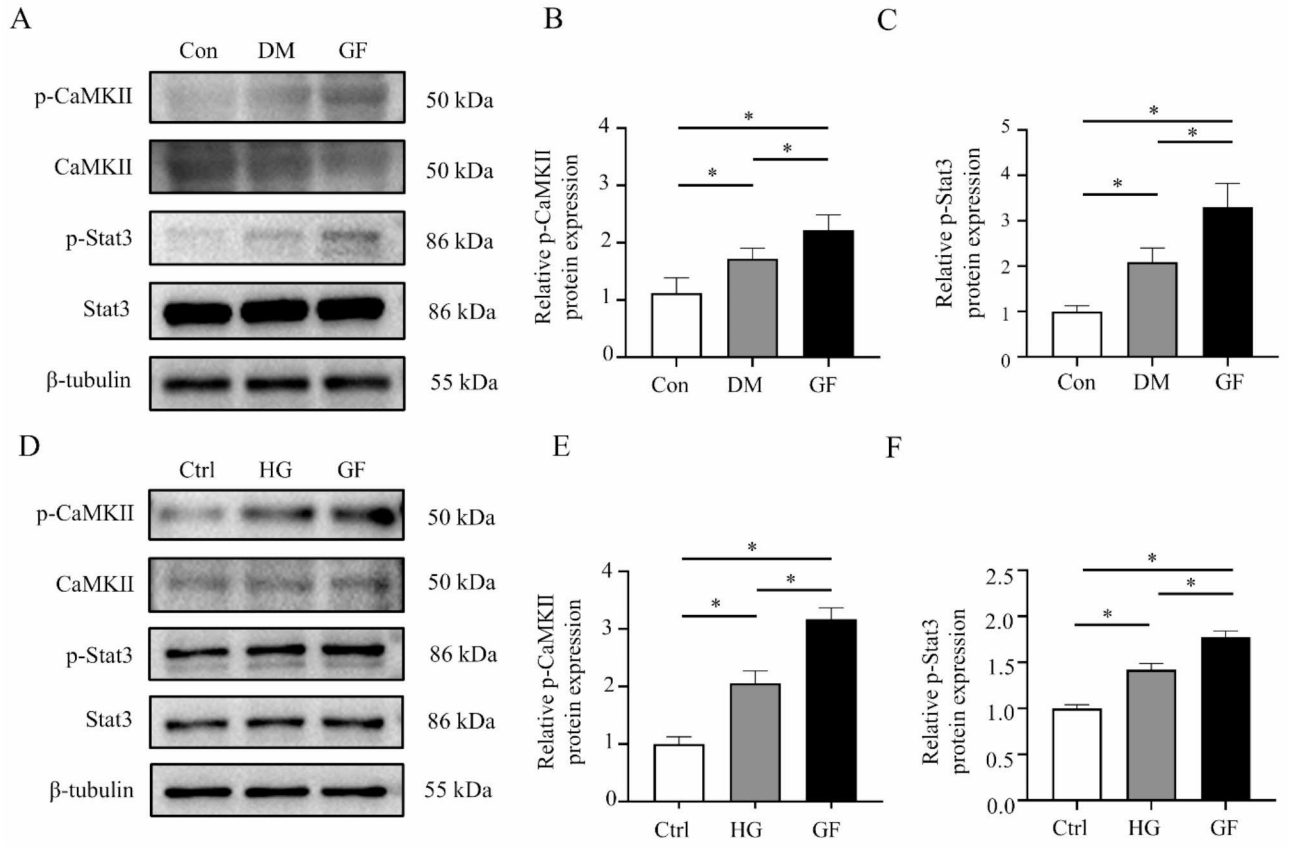

Fig. 3 Glucose fluctuations upregulated the expression of phosphorylated CaMKII and Stat3. (**A**-**C**) The protein expressions of phosphorylated CaMKII (n = 5) and Stat3 (n = 6) in rat hearts of the three groups. (**D**-**F**) The protein expressions of phosphorylated CaMKII (n = 3) and Stat3 (n = 6) in NRCFs of the three groups

Some studies on the mechanism by which Stat3 exacerbates myocardial fibrosis showed that Stat3 is bound with COL1A1 and COL3A1 promoter and activates their transcription [30]. In this study, we demonstrated GF can contribute to increased phosphorylation of Stat3 into the nucleus. Moreover, the ability of the CaMKII inhibitor KN-93 can counteract the fibrotic remodeling induced by GF in type 2 diabetes, underscoring the functional importance of the CaMKII/Stat3 interaction in myocardial fibrosis. Of note, the relationship of CaMKII and Stat3 differs in different diseases. A study showed that KN-93 could down-regulated Stat3 aggravated myocardial microvessel remodelling [31]. Furthermore, cardiomyocyte-specific Stat3 deficiency was also shown to impair cardiac contractility in hypertensive mice [32]. Therefore, the CaMKII/Stat3 pathway may serve distinct roles in different diseases.

In our study, we found that glucose fluctuations in type 2 diabetes exacerbated myocardial fibrosis via the CaMKII/Stat3 pathway. However, there were also some limitations in our study. First, we only investigated the role of CaMKII/Stat3 in regulating myocardial fibrosis with the inhibitors at the cellular level instead of the animal level. Second, we only used the inhibitor KN-93 to explore the underlying mechanisms, and did not use other inhibitors of CaMKII or knockout animals.

Conclusion

In summary, our present study reveals that CaMKII plays a pivotal role in myocardial fibrosis in type 2 diabetes with GF. Targeting the CaMKII-Stat3 pathway may protect the heart from myocardial fibrosis induced by GF in type 2 diabetes.

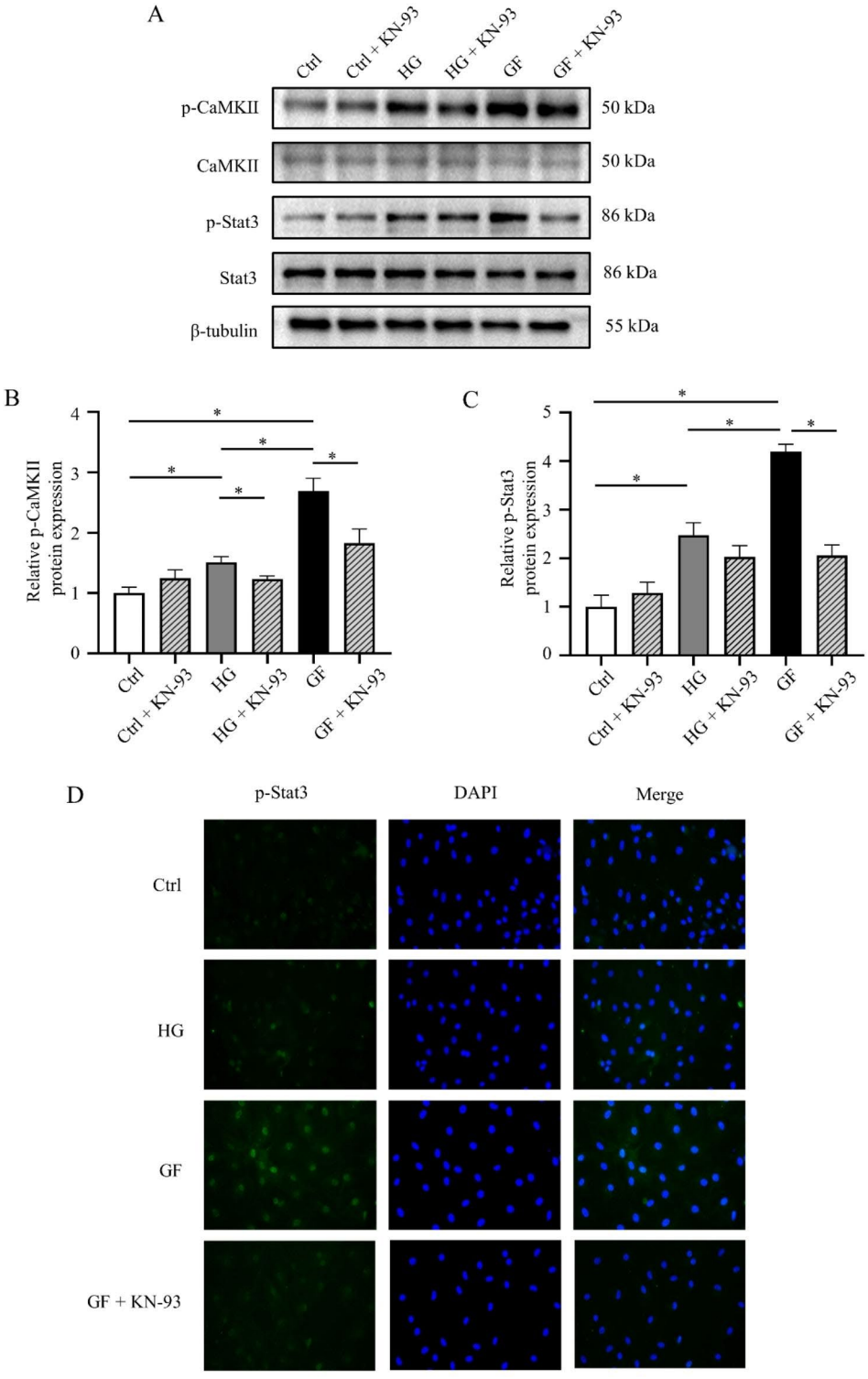

Fig. 4 Inhibition of CaMKII attenuated p-Stat3 elevation due to glucose fluctuations. (**A**) Representative bands of Western blotting of p-Stat3 in NRCFs after using KN-93. (**B**-**C**) Relative levels of p-Stat3 in three groups after using KN-93 (n = 4). (**D**) Immunofluorescence staining of nuclear translocation of p-Stat3 in three groups

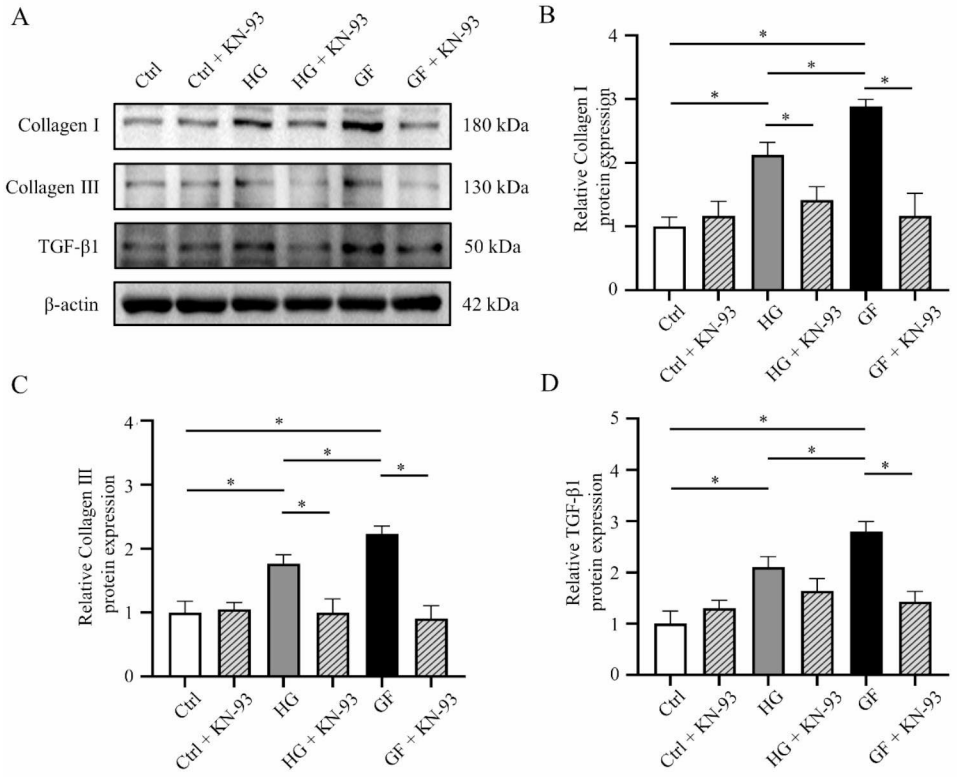

Fig. 5 Role of CaMKII in glucose fluctuation-induced myocardial fibrosis. (**A**) Representative bands of Western blotting of Collagen I, Collagen III and TGF-β1 in NRCFs after using KN-93. (**B**-**D**) Relative levels of Collagen I, Collagen III and TGF-β1 in three groups after using KN-93 (n = 4)

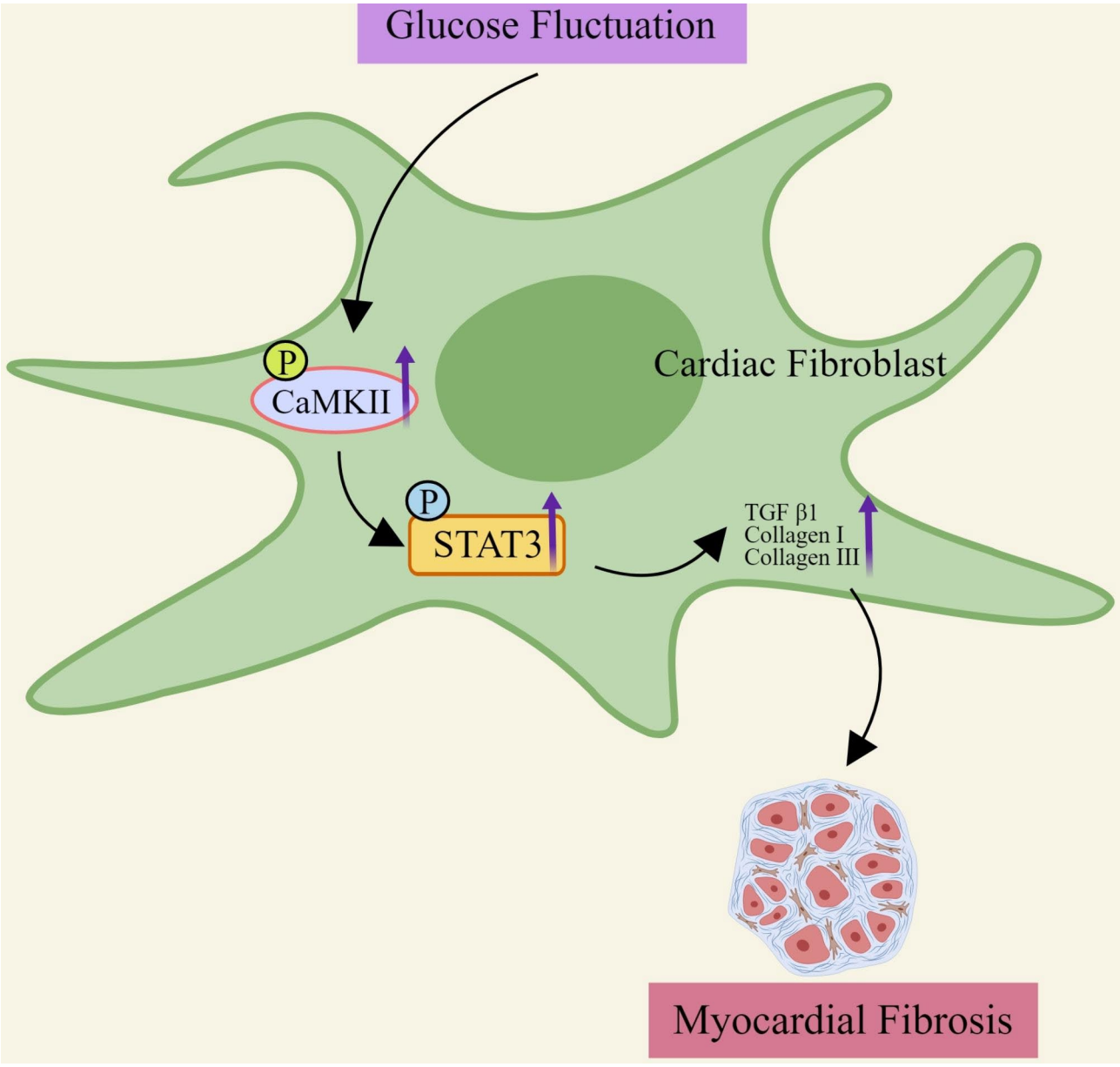

Fig. 6 Role of CaMKII/Stat3 pathway in myocardial fibrosis induced by glucose fluctuation. Glucose fluctuation contributes to the upregulation of CaMKII phosphorylation, leading to Stat3 phosphorylation, and increased expression of fibrosis-related proteins.CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Stat3, Signal transducer and activator of transcription 3

Abbreviations

CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
Stat3	Signal transducer and activator of transcription 3
GF	Glucose fluctuations
SD	Sprague dawley
STZ	Streptozotocin
DM	Diabetes mellitus
T2DM	Type 2 diabetes mellitus
EF	Ejection fraction
FS	Fractional shortening
LVIDd	Left ventricular internal diameter at end-diastole
LVIDs	Left ventricular internal diameter at end-systole
HE	Hematoxylin and eosin
NRCFs	Neonatal rat cardiac fibroblasts
PBS	Phosphate buffered saline
FBS	Fetal bovine serum
HG	High glucose
SEM	Standard error of mean

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13098-023-01197-5.

Supplementary Material 1

Acknowledgements
Not applicable.

Authors' contributions
All authors participated in the design experiment, with R.W. and S.D. as the main leaders. L.Z. and H.L performed the experiments; F.Y., Z.Z and Z.Z participated in animal experiments. X.Z and L.Q analyzed and interpreted the data; L.Z. wrote the paper. All authors read and approved the final manuscript.

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Data Availability
The data used in this research can be made available by the corresponding author upon a reasonable request.

Declarations

Ethics approval and consent to participate
The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and approved by the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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