SGLT2 inhibitor dapagliflozin attenuates cardiac fibrosis and inflammation by reverting the HIF-2α signaling pathway in arrhythmogenic cardiomyopathy

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Abstract
Excessive cardiac fibrosis and inflammation aberrantly contribute to the progressive pathogenesis of arrhythmogenic cardiomyopathy (ACM). Whether sodium-glucose cotransporter-2 inhibitor (SGLT2i), as a new hypoglycemic drug, benefits ACM remains unclear. Cardiomyocyte-specific Dsg2 exon-11 knockout and wild-type (WT) littermate mice were used as the animal model of ACM and controls, respectively. Mice were administered by gavage with either SGLT2i dapagliflozin (DAPA, 1 mg/kg/day) or vehicle alone for 8 weeks. HL-1 cells were treated with DAPA to identify the molecular mechanism in vitro. All mice presented normal glucose homeostasis. DAPA not only significantly ameliorated cardiac dysfunction, adverse remodeling, and ventricular dilation in ACM but also attenuated ACM-associated cardiac fibrofatty replacement, as demonstrated by the echocardiography and histopathological examination. The protein expressions of HIF-2α and HIF-1α were decreased and increased respectively in cardiac tissue of ACM, which were compromised after DAPA treatment. Additionally, NF-κB P65 and IkB

Abbreviations: ACM, arrhythmogenic cardiomyopathy; c-DSG2 KO, cardiomyocyte-specific Dsg2 exon-11 knockout; Collagen I, Type I collagen; Collagen III, Type III collagen; DAPA, dapagliflozin; ECM, extracellular matrix; EF, ejection fraction; FS, fractional shortening; GSK3β, glycogen synthase kinase-3β; GTT, glucose tolerance tests; HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; ICD, implantable cardioverter-defibrillator; IL-1β, interleukin-1β; IL-6, interleukin-6; ITT, insulin tolerance tests; IVS, interventricular septum; IkB, inhibitor of kappa-B; LV, left ventricle; LVAW, left ventricular anterior wall; LVEDD, left ventricular end-diastolic diameter; LVEDV, left ventricular end-diastolic volumes; LVESD, left ventricular end-systolic diameter; LVESV, left ventricular end-systolic volumes; LVPW, left ventricular posterior wall; MMP-13, matrix metalloproteinases-13; NF-κB, nuclear factor kappa-B; RV, right ventricle; SGLT2i, sodium-glucose cotransporter-2 inhibitor; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VT, ventricular tachycardia; WT, wild-type; α-SMA, α-smooth muscle actin.

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phosphorylation, as well as fibrosis indicators (including TGF-β, α-SMA, Collagen I, and Collagen III) were increased in ACM. However, these trends were markedly suppressed by DAPA treatment. Consistent with these results in vitro, DAPA alleviated the IκB phosphorylation and NF-κB p65 transcriptional activity in DSG2-knockdown HL-1 cells. Interestingly, the selective HIF-2α inhibitor PT2399 almost completely blunted the DAPA-mediated downregulation of indicators concerning cardiac fibrosis and inflammation. SGLT2i attenuated the ACM-associated cardiac dysfunction and adverse remodeling in a glucose-independent manner by suppressing cardiac fibrosis and inflammation via reverting the HIF-2α signaling pathway, suggesting that SGLT2i is a novel and available therapy for ACM.

**KEYWORDS**
arrhythmogenic cardiomyopathy, fibrosis, HIF-2α, inflammation, SGLT2 inhibitor

## 1 | INTRODUCTION

Arrhythmogenic cardiomyopathy (ACM) is an inherited cardiac disorder characterized by the risk of life-threatening ventricular tachycardia (VT), cardiac dysfunction, and refractory heart failure. Part of ACM is the dominant familial inheritance with a poor prognosis. The pathological features of ACM include cardiomyocyte loss, fibrofatty replacement, and inflammation. The mutations in cardiac desmosome proteins have been identified to be commonly associated with ACM. Our previous studies have shown that the pathogenic mutation in exon 11 of DSG2, such as the DSG2 p.F531C, has a high incidence in Asians and can easily induce ACM, heart failure, sudden cardiac death, and thromboembolism. Therefore, we need to explore the pathogenic mechanism and treatment strategy of DSG2-related ACM. Current therapeutic approaches to ACM, including implantable cardioverter-defibrillator (ICD) and anti-arrhythmic agents, partially alleviate symptoms but fail to prevent the progression of ACM. Despite the urgent need for pharmacological therapies for ACM targeting underlying mechanisms, surprisingly, there are no commonly used drugs. Only beta-blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers have been elucidated in ACM animal models.

Sodium-glucose cotransporter-2 inhibitors (SGLT2i) promote urinary glucose excretion by avoiding renal reabsorption of filtered glucose to lower blood glucose, which was initially regarded as glucose-lowering agents for patients with type-2 diabetes. Importantly, the clinical application of SGLT2i has expanded far beyond blood-glucose-lowering. Emerging evidence has suggested that SGLT2i may exert cardioprotective effects on patients without diabetes. The well-known clinical trial (dapagliflozin and prevention of adverse outcomes in heart failure, DAPA-HF) showed that the SGLT2i, dapagliflozin (DAPA), reduced the risk of major adverse cardiovascular events and improved the patient prognosis of heart failure with reduced ejection fraction (HFrEF). The concept that SGLT2i reduces cardiac events is widely accepted, but the potential mechanism remains unclear. One reason may be that SGLT2i have pathophysiological roles via sodium and calcium regulation along with cardiac fibrosis and inflammation. Cardiac fibrosis and inflammation are widely considered to be the common pathways involving heart failure progression, resulting in adverse cardiac structural remodeling leading to impaired ventricular compliance and cardiac function. Some experiments performed in animal models, including myocardial infarction and cardiomyopathy, demonstrated that SGLT2i exert cardiac antifibrotic effects via inhibiting collagen synthesis and myofibroblast differentiation. Furthermore, SGLT2i empagliflozin markedly attenuated the fibroblast activation induced by TGF-β and reduced extracellular matrix (ECM) remodeling. Therefore, SGLT2i, attenuating cardiac fibrosis independent of hyperglycemia, may be one of the great therapeutic promises for ACM-associated heart failure. However, the deeper relationship and mechanisms among SGLT2i, antifibrotic effects, and cardiac function are still unclear, especially in ACM-related heart failure. Previous studies have shown that inflammation is the main pathological mechanism promoting early cardiomyopathy progression, especially ACM and dilated cardiomyopathy. Inflammation inhibition may prevent or even reverse the early cardiac remodeling and improve cardiac function. Actually, whether SGLT2i also influences the progression of ACM-related heart failure through inflammatory mechanisms is unclear.

Activation of hypoxia-inducible factor-2α (HIF-2α), primarily acting as a transcription factor in response to hypoxia, has been shown anti-inflammatory and
antifibrotic capacities in diverse organs such as the heart and kidney. Interestingly, SGLT2i are proved to activate HIF-2α and suppress HIF-1α, improving organelle dysfunction, inflammation, and fibrosis in the renal hypoxia model. Based on this evidence, we propose that SGLT2i might be a promising and effective strategy for ACM-associated cardiac fibrosis and inflammation. Therefore, in our study, we intended to explore and evaluate the cardioprotection effects of SGLT2i DAPA on ACM mice and attempted to explain the potential mechanisms.

2 | MATERIAL AND METHODS

2.1 | Generation of animal model and pharmacological intervention

We had successfully generated transgenic mice with a cardiomyocyte-specific Dsg2 exon 11 knockout (c-DSG2 KO) using a targeting Cre-positive recombinase to delete the exon 11 (E11) region of a floxed Dsg2 allele in the heart. A targeting mutant mouse carrying loxP-site and Cre-positive recombinase was knocked out of the Dsg2 E11 region in the heart, which would encode mutant Dsg2 protein of functional loss in cardiac tissues and cell types but not result in a frameshift mutation. We used c-DSG2 KO male mice that developed ACM and heart failure as animal models and age-matched wild-type (WT) Dsg2 littermates controls. Both ACM and WT mice were treated with either SGLT2i dapagliflozin (DAPA, MedChemExpress, USA) or vehicle alone. Mice were randomly assigned into four groups (n = 6 per group): DSG2 WT + vehicle, DSG2 WT + DAPA, ACM + vehicle, and ACM + DAPA. DAPA was administered by gavage daily with 1 mg/kg/day for 8 weeks.

According to standard SPF conditions, they were raised in the Molecular Imaging Center of the Fifth Affiliated Hospital of Sun Yat-sen University. They were fed a rodent diet and day-night rhythm. The mice were sacrificed by cervical dislocation after being anesthetized. The experiments were consistent with the Institutional Animal Care and Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (Ethics issue [2018] No. 00024).

2.2 | Glucose and insulin tolerance tests

After 8 weeks of DAPA intervention, the intraperitoneal glucose tolerance tests (i.p., GTT) and insulin tolerance tests (i.p., ITT) were performed in vivo. Mice were fasted overnight before i.p. GTT and then administered a glucose load (2 g/kg body weight). For i.p. ITT, mice were intraperitoneally injected with recombinant human insulin (0.75 U/kg body weight) after 6 h fasting. Blood glucose levels were measured with a Roch blood glucose monitoring machine in the tail vein blood at 0, 15, 30, 60, 90, and 120 min for i.p. GTT and i.p. ITT.

2.3 | Echocardiography

Cardiac function and structures were measured using a Vevo2100 high-resolution and color imaging scanner system with a SL3116 high-frequency linear array probe and a 22MHz transducer (Visual Sonics, Toronto, Canada). In order to get high-quality and optimum detection conditions of cardiac chambers, mice were acquired under 1.5% (v/v) isoflurane anesthesia and then carefully shaved hair on the surface of the chest, and ultrasound transmission gel was applied. In the long-axis views of the left ventricle next to the sternum, M-mode ultrasound images were obtained at the papillary muscle level of the left ventricle. M-mode recordings were used to acquire some echocardiographic parameters: left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left ventricular anterior wall (LVAW), left ventricular posterior wall (LVPW), left ventricular end-diastolic, and end-systolic volumes (LVEDV and LVESV). All data and parameters were collected for three consecutive cardiac cycles, then the average values were calculated.

2.4 | Histopathology

ACM mice and WT controls were sacrificed by cervical dislocation after anesthetization, and the phenotypes of the heart were macroscopically visible. Next, 4% buffered formaldehyde was used to fix hearts overnight, and then the hearts were dehydrated and embedded in paraffin. Heart tissues were sectioned at 5-μm thick. According to the standard protocol, the prepared tissue sections were stained with hematoxylin and eosin (H&E) dyes. The Olympus BX43 microscope system observed all sections.

2.5 | Cell culture and siRNA transfection

HL-1 cells (a mouse cardiac muscle cell line) were purchased from Sigma–Aldrich Corp (St. Louis, MO, USA). HL-1 cells were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, NY, USA) and 1% penicillin–streptomycin (Gibco, NY, USA) at 5% CO2, 37 °C incubators.
For siRNA-knockdown experiments, HL-1 cells were transfected with 10 nM DSG2 siRNA (ID no. m13511, HanBio Technology, Shanghai, China), HIF-2α siRNA (HanBio Technology, Shanghai, China), or negative control siRNA (HanBio Technology, Shanghai, China). Transfections were performed with GMsiRNA-mate reagent (G04003, Genepharma, Suzhou, China) according to the manufacturer’s protocol. The knockdown efficiency was evaluated by western blot analysis at 72 h after transfection.

After transfection as previously described, HL-1 cells were treated for 24 h with or without DAPA (MedChemExpress, New Jersey, USA) at a concentration of 1, 5, 10 μmol/L. Moreover, to specifically inhibit HIF-2α signaling, a selective HIF-2α inhibitor PT2399 (MedChemExpress, New Jersey, USA) was added to the cultures at a concentration of 1 μmol/L according to the protocol.

To further check what will happen to HIF2-α upon SGLT2 treatment in hypoxia, the well-known hypoxia mimetic CoCl2 was used to induce hypoxia. CoCl2 (Sigma-Aldrich) was dissolved in DMEM and diluted to 200 μM for actual use. Subsequently, DAPA was added to HL-1 cells for treatment in the absence or presence of 200 μM CoCl2 for 48 h prior to cell collection.

### 2.6 Immunofluorescence

Immunofluorescence was performed to investigate the expression and subcellular localization of NF-κB p65 protein in HL-1 cells. After pretreated as previously described, HL-1 cells were transferred to new 12-well plates for immunofluorescence experiments. Cells were fixed with 4% paraformaldehyde for 15 min and subsequently were blocked with 5% normal goat serum/0.1% Triton X-100 for 1 h. Cells were incubated with primary rabbit anti-NF-κB p65 antibody (8242S, CellSignaling Technology, MA, USA) overnight at 4°C, and then with Alexa Fluor 488-conjugated goat anti-rabbit antibody (A0423, Beyotime, Shanghai, China) for 1 h at room temperature. Nuclei were stained using DAPI dye. Cells were washed with PBS between steps. Images were visualized and collected with an inverted fluorescent microscope system (Nikon Instruments), and the microscope settings of all images were kept constant.

### 2.7 Immunoblot analyses

Total Protein Extraction Kit (Keygen Biotech, Jiangsu, China) was used to isolate total protein from heart tissue and HL-1 cells. The extracted supernatants’ concentration was measured using Enhanced BCA Protein Assay Kit (Beyotime) and trimmed with extraction buffer and 5×SDS-PAGE Loading Buffer. The appropriate and equal volume of protein was loaded in SDS-PAGE gel for electrophoresis. Then, the separated proteins were blotted on PVDF membranes, and the membranes were blocked with 5% defatted milk for 1 h at room temperature. Primary antibodies were diluted overnight at 4°C. Primary antibodies included rabbit anti-DSG2 antibody (ab150372, 1:5000, Abcam, Cambridge, MA, UK), rabbit anti-A20/TNFAIP3 antibody (5630S, 1:1000, CellSignaling Technology, MA, USA), mouse anti-IκBα antibody (4814S, 1:1000, CellSignaling Technology), rabbit anti-Phospho-IκBα antibody (2859S, 1:1000, CellSignaling Technology), rabbit anti-NF-κB p65 antibody (8242S, 1:1000, CellSignaling Technology), rabbit anti-Phospho-NF-κB p65 antibody (3033S, 1:1000, CellSignaling Technology), and rabbit anti-β-actin antibody (AC026, 1:5000, Abclonal, Wuhan, China). The membranes were incubated in secondary antibodies (Abclonal) for 1 h. Membranes were washed with TBST between steps. Image J software (National Institutes of Health, Bethesda, MD, USA) was used to measure the density value of the target protein bands.

### 2.8 Immunoblot analyses

Total RNA was isolated with chloroform and Trizol Kit (Takara, Shuzo, Japan). The mixtures including samples and reagents were shaken vigorously, and then placed at room temperature for 5 min and centrifugated at a 12000 g speed at 4°C. The RNA extract was precipitated with isopropyl alcohol at room temperature and was washed three times with 75% ethanol, then air-dried and dissolved in DEPC water (Omega Bio-tek, Georgia, USA). The RNA solution was treated with gDNA Eraser and 5×gDNA Erase Buffer (purchased from Takara) to remove gDNA. The appropriate amount of RNA was used as a template and transcribed into cDNA using PrimeScriptTM reverse RT reagent Kit (Takara) according to the concentration of the total RNA and OD260/280 ratio measured with Thermo Nanodrop 2000. Quantitative real-time PCR (qPCR) was performed using a 2x SYBR Green qPCR master mix (Bimake). The total volume of the qPCR reaction system was 20μl: cDNA as a template, forward and reverse primer (all sequences presented in Table S1), mix, and RNase-free water. There were three replications for each sample. The data were analyzed by the way of ΔΔCt value.

### 2.9 Statistical analysis

All results are presented as the means ± SD. If not otherwise explained, one-way ANOVA was performed to analyze the significance of data using GraphPad Prism 7 (GraphPad Software Inc. San Diego, USA), and P values <0.05 were considered statistical significance.
3 | RESULT

3.1 | DAPA attenuated ACM-induced cardiac dysfunction in glucose independent manner

Both glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed to investigate the effects of SGLT2i DAPA on blood glucose in ACM mice and WT mice. As shown in Figure 1A,B, the blood glucose levels of the four groups were no significant differences throughout the study, indicating that transgenic mice presented normal glucose homeostasis. Next, we observed that ACM mice’s heart/body weight ratio was significantly increased, consistent with the results of developing ventricular dilation and heart failure, which was mitigated to some extent after DAPA administration (Figure 1C). ACM mice showed impaired cardiac function as indicated by the significantly decreased ejection fraction (EF), and fractional shortening (FS) compared to WT mice, which were significantly attenuated by DAPA treatment. However, there were no DAPA-mediated beneficial effects on cardiac function of normal mice under basal conditions (Figure 1D,E). All these data suggested that SGLT2i DAPA attenuated ACM-associated cardiac dysfunction in glucose independent manner in mice.

3.2 | DAPA ameliorated adverse cardiac remodeling and ventricular dilation in ACM mice

Echocardiography was performed, and cardiac parameters were measured to explore whether the SGLT2i DAPA could attenuate adverse left ventricle remodeling in ACM mice. As shown in Figure 2A, M-mode recordings in the short axis of ACM mice exhibited that the thicknesses of the left ventricular anterior wall (LVAW) and left ventricular posterior wall (LVPW) were decreased than those in WT mice, which were significantly attenuated after DAPA administration. In addition, DAPA treatment markedly ameliorated the dilation of heart chambers in ACM mice (Figure 2B).

The heart rates were comparable after DAPA treatment between ACM and WT mice (Figure 2C). The left ventricular end-diastole and end-systole volumes (LVEDV and LVESV), left ventricular end-diastole, and end-systole diameters (LVEDD and LVESD) were significantly increased in ACM mice. The elevated ventricular volumes and diameters indicated cardiac dilation and impaired ventricular compliance, markedly alleviated in ACM mice after DAPA treatment (Figure 2D,E,J,K). In agreement with the adverse cardiac remodeling and impaired cardiac function, the thickness of LVAW at both end-diastole and end-systole intervals was decreased in ACM mice and
FIGURE 2  DAPA ameliorated adverse cardiac remodeling and ventricular dilation in ACM mice. (A) Representative examples of M-mode echocardiography images. (B) Representative examples of short-axis views of the left ventricle. (C) Measurement of heart rate after DAPA intervention. (D–K) Echocardiographic analysis of cardiac parameters of LVAW, LVPW, LVEDV, LVESV, LVEDD and LVESD in four groups. Data are expressed as the mean ± SD. *p < .05 versus DSG2 WT + NS; †p < .05 versus c-DSG2 KO + NS.
markedly attenuated by DAPA treatment (Figure 2F,G). In addition, DAPA treatment attenuated LVPW thickness to some extent, but no significant difference was observed (Figure 2H,I).

### 3.3 DAPA-attenuated ACM-associated cardiac fibrofatty replacement

The pathological hallmark of ACM is progressive cardiomyocyte loss and its replacement by fibrofatty tissue. As shown in Figure 3A, ACM mice showed obvious fibrofatty lesions of various shapes across the atrial and ventricular wall by visual inspection. The white lesions indicated the loss of heart muscle and complete substitution of fibrous tissue, and progression to scarring tissue resulting in ventricular wall thinning. After DAPA treatment, these pathological changes in ACM mice were significantly alleviated. Moreover, consistent with the echocardiography result, enlargement and dilation of heart chambers were also observed in ACM mice according to microscopic appearance, which was attenuated with DAPA treatment (Figure 3B). Histopathological heart sections with hematoxylin and eosin staining presented extensive structural disorganization and inflammatory cell infiltration across cardiac tissues, including left ventricle (LV), interventricular septum (IVS), and right ventricle (RV) in ACM mice. It is worth mentioning that these lesions with fibrosis act as substrates for ventricular arrhythmias through the scar-related mechanism. However, we observed that DAPA administration benefited heart disorganization and inflammatory infiltration (Figure 3C). Collectively, we concluded that DAPA treatment attenuated cardiac fibrofatty replacement in ACM mice.

![Figure 3](image-url)
3.4 | DAPA-alleviated ACM-associated cardiac fibrosis and inflammation through the HIF-2α signaling pathway

Cardiac hypoxia has played an important role in the progression of chronic heart failure. Maintaining the balance of hypoxia-inducible factors (HIFs) has been proven anti-inflammatory and antifibrotic capacities in the heart. Our study detected the HIF-2α and HIF-1α expression in the heart tissue of both ACM and WT mice. The western blotting results revealed the decreased HIF-2α and increased HIF-1α expression in the ACM mice, compared to the WT mice. After an 8-week DAPA treatment in the ACM mice, the expressions of HIF-2α and HIF-1α were restored and reduced (Figure 4A–C), respectively.

The activation of NF-κB signaling has been proved in ACM, resulting in excessive cardiac inflammation, apoptosis, and fibrosis. As shown in Figure 4A,D, protein abundances of total nuclear factor kappa-B (NF-κB) and inhibitor of NF-κB (IκB) seemed unchanged between groups. Still, the abundances of NF-κB P65 and IκB phosphorylation were significantly increased in ACM mice, indicating activation of the NF-κB pathway in the heart of ACM mice. Interestingly, these effects were markedly suppressed in the presence of DAPA. Moreover, TNFAIP3 (also known as A20), an important protein to negatively regulate inflammation, was decreased in the ACM heart (Figure 4A,D). After DAPA treatment, A20 was slightly increased in ACM mice.

The synthesis of extracellular matrix (ECM), including collagen and fibronectin, plays a critical role in cardiac fibrosis. We observed that the abundances of the protein associated with ECM synthesis were significantly increased in the cardiac tissue of ACM mice, including transforming growth factor-β (TGF-β), α-smooth muscle actin (α-SMA), type I and type III collagen (Collagen I and Collagen III). After DAPA treatment, these indicators were significantly attenuated (Figure 4E,G).

All these results suggested that the cardioprotective effects of DAPA on cardiac fibrosis and inflammation may involve a HIF-2α-dependent mechanism in ACM mice.

3.5 | HIF-2α inhibition blunted DAPA-reduced cardiac fibrosis induced by DSG2 knockdown in vitro

To determine whether HIF-2α is responsible for DAPA-mediated protective effects in vitro, we administered DAPA to HL-1 cells after DSG2 knockdown via siRNA-DSG2. The western blots showed that HIF-2α and HIF-1α expressions were markedly decreased and increased, respectively, in DSG2-knockdown HL-1 cells, which were significantly compromised after DAPA administration in a dose-dependent manner (Figure 5A,B). The mRNA expressions of HIF-2α, VEGF, and GLUT1 were down-regulated in DSG2 knockdown HL-1 cells, but the effects were markedly restored in the presence of DAPA. When treated with PT2399, a selective HIF-2α inhibitor, DAPA-mediated upregulation of HIF-2α was blunted (Figure 5C).

We further explored the role of HIF-2α signaling in DAPA that prevented c-DSG2-KO-induced cardiac fibrosis. As shown in Figure 5D,E, DAPA administration in DSG2-knockdown HL-1 cells induced the obvious decrease in the protein expressions (TGF-β, α-SMA, Collagen I, and Collagen III) related to cardiac fibrosis in a dose-dependent manner. However, inhibition of HIF-2α with PT2399 or HIF-2α knockdown via siRNA- HIF-2α (Figure S2E,F) almost completely blunted DAPA-mediated down-regulation of α-SMA, Collagen I, and Collagen III. These results suggested that DAPA reduced cardiac fibrosis in DSG2-knockdown HL-1 cells via the HIF-2α signaling pathway.

3.6 | HIF-2α was responsible for DAPA-attenuated cardiac inflammation induced by DSG2 knockdown in vitro

To further elucidate whether DAPA exerted protective effects on excessive cardiac inflammation through the
HIF-2α signaling pathway, we investigated the activation and subcellular localization of NF-κB p65 protein in HL-1 cells. The results revealed that DAPA could alleviate cardiac inflammation in vitro through the HIF-2α signaling pathway. Consistent with the data in vivo, DAPA administration significantly alleviated the phosphorylation
of IκB and nuclear translocation of NF-κB p65 together with increased NF-κB p65 transcriptional activity in DSG2 knockdown HL-1 cells (Figure 6A–C). Remarkably, the cardioprotective effects of DAPA were abolished when cells were administered with PT2399 or HIF-1α knockdown via siRNA- HIF-2α (Figure S2A–D), which caused an increment in inflammation again in cells. It must also be mentioned that DAPA pre-treatment significantly decreased the phosphorylation of IκB and NF-κB p65 transcriptional activity in DSG2 knockdown HL-1 cells, but the effects of DAPA were also abolished after a 1 or 2-day washout (Figure 6D,E). Similarly, washout response almost played the same role in abolishing the DAPA-mediated decrease of fibrosis (Figure 6F,G).

4 | DISCUSSION

In this study, we first determined that SGLT2i DAPA attenuated ACM-associated cardiac fibrosis and excessive inflammation. DAPA exerted a cardioprotective effect on cardiac dysfunction and significantly ameliorated adverse cardiac remodeling and ventricular dilation through HIF-2α/NF-κB/TGF-β signaling pathway in a blood-glucose-independent manner in ACM mice. DAPA restored HIF-2α expression to reduce cardiac fibrosis and inflammation in the ACM heart. HIF-2α inhibition with PT2399 almost completely blunted DAPA-mediated cardioprotective effects in DSG2 knockdown HL-1 cells. Therefore, we first illustrated that SGLT2i is a promising...
**FIGURE 6** HIF-2α inhibition blunted DAPA-attenuated cardiac inflammation in vitro. (A) Effects of DAPA administration with indicated concentrations or PT2399 on the expression of NF-κB P65 and IκB in HL-1 cells by western blotting. (B) The relative ratio of phosphorylation IκB over total IκB and phosphorylation NF-κB P65 over total NF-κB P65 in cells by densitometric analysis, respectively. (C) Immunofluorescence showing NF-κB p65 protein expression and localization in HL-1 cells after DAPA treatment (Scale bar 50 μm). NF-κB p65 expression: green labeling, nuclei: blue labeling. (D) Effects of DAPA pre-treatment and post-washout experiments on the expression of NF-κB P65 and IκB in HL-1 cells by western blotting. (E) The relative ratio of phosphorylation IκB over total IκB and phosphorylation NF-κB P65 over total NF-κB P65. (F) Effects of DAPA pre-treatment and post-washout experiments on the expression of collagen I, α-SMA and TGF-β. (G) The relative ratio of collagen I, α-SMA and TGF-β over β-actin respectively. Data are expressed as the mean ± SD. *p < .05 versus CTL; **p < .05 versus si-RNA DSG2; ***p < .05 versus si-RNA DSG2 + DAPA.
treatment against ACM progression through the HIF-2α signaling pathway.

ACM is one of the main reasons for sudden cardiac death, especially among young individuals and athletes. At present, ACM treatment includes angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and beta-blockers. When patients with ACM are accompanied by heart failure, diuretics can be added. Even so, the treatment effect of many patients is still limited, and the ACM is still progressing and deteriorating. ICD is only a palliative treatment to terminate ventricular tachycardia and ventricular fibrillation. ICD repeating discharge defibrillation may aggravate the ACM progression, and some patients still die. Thus, a promising treatment based on the pathophysiological mechanism for ACM is urgently required to be addressed.

Cardiac fibrosis has been implicated in the pathogenesis of the progression of ACM. It used to be accepted that a reparative process, including the formation of fibrous tissue and the collagen-based scar, was triggered in response to the loss of a significant number of cardiomyocytes in ACM. However, recent evidence indicates that cardiac fibrosis is associated with the pathogenesis of genetic cardiomyopathies, such as hypertrophic cardiomyopathy. The profibrotic state preceded the development of cardiac dysfunction in cardiomyopathies. The cardiac fibrotic conditions related to genetic cardiomyopathies may not be only an epiphenomenon of the reparative process in response to cardiomyocyte injury but also may directly participate in the disease-specific pathophysiologic process of cardiac dysfunction. Moreover, cardiac fibrosis in ACM contributes to the development of life-threatening ventricular arrhythmias by electrical conduction disturbances. Thus, inhibition of cardiac fibrosis may be the important pharmacological therapy for ACM. Previous experimental studies have confirmed that SGLT2i DAPA exerts the non-hypoglycemic antifibrotic effect on the angiotensin II-infused rat cardiac fibrotic model. They have found that DAPA decreased angiotensin II-induced collagen synthesis in primary cardiac fibroblasts and suppressed the expression of TGF-β1 and the phosphorylation of Smad3 and Smad2 in angiotensin II-infused rats. However, a detailed mechanism of how DAPA positively regulates angiotensin II-induced cardiac fibrosis needs further elucidation. Previous studies demonstrated that DAPA improved hypertensive, diabetic, and doxorubicin-induced heart failure.

In our research, DAPA administration significantly prevented ACM-associated cardiac fibrosis. It suppressed the expression of TGF-β, α-SMA, collagen I, and collagen III both in vivo and in vitro, and DAPA attenuated cardiac dysfunction in response to DSG2 knockdown. SGLT2i has been widely used to treat patients with ejection fraction reduced heart failure without adverse effects, which exerts the cardiovascular protective effects in a non-glucose-lowering dependent manner. In addition, emerging studies have demonstrated that TGF-β elevation abnormally contributes to cardiac fibrosis in heart diseases, including cardiomyopathies, valvular diseases, and arrhythmias. TGF-β acts as a central cytokine in the activation of fibrosis cascades and is a potent regulatory for the α-SMA expression in the heart tissue. TGF-β is considered a prognostic marker and target for prevention due to its protein level correlated with heart dysfunction or cardiac fibrosis. Interestingly, compared with the control group in our study, 8-week DAPA treatment markedly reduced the TGF-β expression in ACM mice, which suggested that DAPA would be a promising therapeutic agent for ACM-associated cardiac fibrosis.

Inflammation is an important part of the initiation and development of ACM. The pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) significantly increased in plasma of ACM patients. These pro-inflammatory cytokines in plasma, just as an important role in viral myocarditis, trigger the expression of inducible nitric oxide synthase (iNOS). The iNOS induction results in strand breaks and cell apoptosis, which aggravates inflammation in turn. In three ACM models, the inflammation promotes cardiac damage induced by cytokine cascades mediated by activating NF-κB signaling. Bay 11–7082, as a small-molecule inhibitor of NF-κB signaling, showed beneficial effects on cardiac function and prevented the development of major features of the ACM phenotype. The NF-κB pathway has been considered to be an important regulator of inflammation and is closely related to glycogen synthase kinase-3β (GSK3β). The GSK3β activation aggravates inflammation through the NF-κB pathway. Previous evidence suggested that GSK3β inhibition has increased survival in a zebrafish ACM model. Accordingly, mechanism-based targeting cardiac inflammation drug therapy may be an effective strategy for ACM patients. Our study found that DAPA attenuated excessive cardiac inflammation by targeting NF-κB signaling in ACM mice. Further research illustrated that DAPA significantly mitigated the phosphorylation of IκB and transcriptional activity of NF-κB p65 in DSG2-knockdown HL-1 cells. Therefore, DAPA is a therapeutic promise for attenuating cardiac inflammation by suppressing the NF-κB signaling pathway in ACM.

The hypoxia-inducible factors (HIFs) pathway is increasingly recognized in chronic cardiovascular diseases and adipose tissues. HIF-2α and HIF-1α exert mutual antagonistic effects on anti-fibrosis and inflammation in the heart. We found that HIF-2α expression was markedly decreased, while HIF-1α expression was increased in ACM mice and DSG2-knockdown HL-1 cells. HIF-2α not only suppresses the activation of iNOS, which is considered a potent
pro-inflammatory regulator, and reduces M1 macrophage polarization, yet both effects are opposed to HIF-1α. In addition, HIF-2α enhances collagen matrix degradation by upregulating matrix metalloproteinases-13 (MMP-13), but HIF-1α activates the profibrotic chemokines and collagen deposition. Previous studies demonstrated that SGLT2 enhancement directly involves inflammation, oxidative stress, DNA damage, mitochondrial dysfunction, and cell apoptosis in a non-glucose-lowering dependent manner. SGLT2i attenuates these adverse effects on cellular homeostasis in diabetic kidney injury models. Interestingly, we found that DAPA alleviated cardiac fibrosis and excessive inflammation by preventing the HIF-2α downregulation in the heart tissue of ACM mice. Our further research showed that pharmacological inhibition of HIF-2α almost completely blunted the beneficial effects on cardiac fibrosis and inflammation, suggesting that targeting HIF-2α signaling plays a critical role in alleviating cardiac dysfunction and subsequent heart failure by regulating cardiac fibrosis and inflammation in ACM mice.

5 | CONCLUSIONS

As demonstrated in c-DSG2-E11 knockout ACM mice and DSG2-knockdown HL-1 cells, our study firstly indicated that SGLT2i DAPA exerted antifibrosis and anti-inflammation through HIF-2α/NF-κB/TGF-β signaling pathway in the glucose-independent manner, which therefore attenuated cardiac dysfunction, adverse remodeling and ventricular dilation in ACM. More importantly, our study suggested that SGLT2i may be a novel and available pharmacological approach to managing ACM.

6 | LIMITATIONS

ACM is often caused by gene mutations in the desmosomal complex and a minority in non-desmosomal proteins. The clinical manifestations are different based on various pathogenic gene mutations. Our study observed that SGLT2i DAPA attenuates ACM-associated cardiac fibrosis and inflammation in the example of Dsg2 mutation. However, we still need further clinical studies to confirm whether SGLT2i DAPA benefits ACM patients. The beneficial effects for those with the rare and heterogeneous presentation of SGLT2i on the heart remain unclear. We selected Dsg2 exon-11 knockout transgenic mice as ACM animal models. The mutant protein might present a fragmented or light band by analysis of western blotting. Surprisingly, the results of western blots showed that Dsg2 protein completely disappeared. The reason we speculate is the change of protein antigenicity after knockout.

AUTHOR CONTRIBUTIONS

Animal and molecular biology experiments and writing: Zhe Yang, Yubi Lin, and Tengling Li; establishment of experimental animal model: Jia Chen; echocardiography: Jianzhong Xian; quality control of data and figure: Qin Zhang, Yin Huang, and Xiufang Lin; research design: Hongyun Lu and Yubi Lin. All authors reviewed the manuscript.

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DISCLOSURES

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data used during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL

The experiments were consistent with the Institutional Animal Care and Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-sen University (Ethics issue [2018] No. 00024).

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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