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GYY4137 protects against type 2 diabetes mellitus-associated myocardial autophagy by suppressing FOXO1 signal pathway

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ABSTRACT

Purpose: Diabetic cardiomyopathy (DCM) is a major complication of type 2 diabetes mellitus (T2DM), but its effective prevention and treatment are still limited. We investigated the effects of GYY4137, a slow-releasing hydrogen sulfide donor, and its downstream mediator forkhead box protein O1 (FOXO1) on T2DM-associated DCM. **Methods:** *In vivo*, T2DM mice were induced by a high-fat diet coupled with streptozotocin injection. Intragastric administration of GYY4137 was also performed. *In vitro*, AC16 cardiomyocytes were treated with glucose and palmitate to mimic high-glucose and high-fat (HGHF) conditions, in which GYY4137 or a FOXO1 inhibitor (AS1842856) was also introduced. Bioinformatics analysis was performed using public GEO datasets. **Results**: GYY4137 demonstrated a protective effect against cardiac dysfunction, fibrosis, and autophagy in cardiac tissues of T2DM mice. Moreover, GYY4137 alleviated cell injury and lipid accumulation in HGHF-treated AC16 cells. In both *in vivo* and *in vitro* models, hyperactivation of autophagy was dampened by GYY4137. Bioinformatic analysis revealed the potential role of the FOXO pathway and autophagy in DCM. Further experiments showed that GYY4137 rescued diabetes-induced overexpression of FOXO1. AS1842856 displayed a notable capacity to shield cardiomyocytes against diabetes-induced injury similar to that achieved by GYY4137. **Conclusion:** GYY4137 protected against cardiac dysfunction and fibrosis in T2DM mice, and the mechanism might involve suppression of FOXO1-induced autophagy.

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Diabetic cardiomyopathy; hydrogen sulfide; FOXO1; autophagy

Introduction

Diabetic cardiomyopathy (DCM) arises as a consequence of the disrupted glucose and lipid metabolism inherent in diabetes mellitus and is intricately intertwined with cardiac dysfunction. Notably, individuals with type 2 diabetes mellitus (T2DM) experience a significantly elevated risk of heart failure, with a 2.4-fold increase in men and 5.1-fold increase in women compared to those without diabetes (Dauriz et al. 2017). Furthermore, the prevalence of cardiac dysfunction among T2DM patients stands at 35%, a markedly higher figure than that observed in individuals with type 1 diabetes mellitus (T1DM) (Tan et al. 2020). Hence, delving into the mechanisms and identifying therapeutic targets for DCM holds promise for mitigating the morbidity and mortality associated with heart failure in patients with diabetes, particularly in those with T2DM.

Over the last decade, a growing body of preclinical and clinical studies has focused on elucidating the pathogenesis and clinical characteristics of T2DM-associated cardiomyopathy. These efforts have revealed a complex interplay between mitochondrial dysfunction, oxidative stress, and lipid accumulation, collectively fostering inflammation and death of cardiomyocytes (Tan et al. 2020). Autophagy, a conserved catabolic process involving the degradation of abnormal intracellular metabolites and damaged organelles, has been suggested to play a key role in diabetic hearts (Dewanjee et al. 2021). Hyperactivation of cardiac autophagy can be induced by impairment of insulin signaling in diabetic rats (Guo et al. 2020) and further causes self-digestion and enhanced reactive oxygen species (ROS) production in cardiomyocytes (Xu et al. 2019). The inhibition of hyperactivated autophagy significantly reversed cardiac

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dysfunction and protected against DCM (Madonna et al. 2023).

Forkhead box protein O1 (FOXO1), a member of the FOXOs family that functions as a nuclear transcription factor, is critical in the insulin cascade. It can be activated by inhibition of insulin signaling and is associated with several pathological pathways, including autophagy and disturbance of glucose metabolism in DCM (Kandula et al. 2016). FOXO1 ablation resulted in significant cardioprotection in mice with T2DM (Battiprolu et al. 2012).

Despite profound insights into DCM pathogenesis, effective strategies for the prevention and treatment of T2DM-associated cardiomyopathy remain limited. Hydrogen sulfide (H_2S), a smelly gasotransmitter, protects against insulin secretion and tissue repair in diabetes (Cheng and Kishore 2020; Zhang et al. 2021). In our previous study, we revealed the effects of H_2S on high-glucose-induced inflammation and cytotoxicity in cardiomyocytes (Huang et al. 2016). Nonetheless, it is worth noting that the majority of H_2S donors currently available are characterized by rapid release kinetics and susceptibility to toxic doses, which limits their *in vivo* applications. In recent years, GYY4137, a novel slow-releasing H_2S donor with high safety and efficacy, has attracted increasing attention. In this study, we introduced GYY4137 into T2DM mice and explored the molecular pathways underlying the cardioprotective effects of GYY4137 in human cardiomyocytes. We hypothesized that GYY4137 protects against cardiac dysfunction and fibrosis in T2DM mice, mechanism of which involves the suppression of FOXO1-induced myocardial autophagy.

Materials and methods

Establishment of in vivo *model*

Male mice (C57BL/6, six weeks old) were used in this study. All mice were housed in a room at a controlled temperature (23 ± 2°C) with a 12-hour light–dark cycle and had free access to water and diet. The mice were divided into three groups: control ($n = 6$) received standard chow for 15 weeks; the others received a five-week high-fat diet (HFD; 20% protein, 20% carbohydrate, and 60% fat), followed by prepandial intraperitoneal injections of streptozotocin (40 mg/kg body weight) for three days to induce diabetes. The control mice were injected with sodium citrate buffer at the same time. After confirming diabetes by fasting blood glucose levels four weeks later, the diabetic mice were split randomly into DCM and DCM + GYY groups ($n = 6$ each). The DCM + GYY group received GYY4137 (2 mg/kg body weight) via gastric lavage daily for six weeks. Mice in the DCM and control groups received the corresponding doses of saline simultaneously. At the end of the study, the mice underwent echocardiography (anesthetized with 2% inhaled isoflurane) and blood collection (retro-orbital plexus after anesthesia). Then, the mice received an intracardiac injection of KCL solution (60 mmol/L, 5 mL per mouse) to arrest the hearts in diastole. Murine hearts were further divided into three parts for histological analysis (fixed in 4% paraformaldehyde), qRT-PCR, and western blot analysis (both stored in liquid nitrogen). The experiments were approved by the Ethics Committee of Guangdong Provincial People's Hospital (approved number: KY-Z-2020-123-02).

Echocardiography

The ejection fraction, fractional shortening, end-systolic diameter, and end-systolic volume of the left ventricle were determined using two-dimensional M-mode echocardiography with a Vevo2100 high-resolution imaging system (VisualSonics, Toronto, Canada).

Masson's trichrome staining of cardiac tissue sections

Cardiac samples were fixed in 4% paraformaldehyde at room temperature, and then cardiac sections of 4μm were obtained and subjected to Masson's trichrome staining (BASO Diagnostics, Zhuhai, China, Cat.No: BA4079B) according to the manufacturer's instructions.

Bioinformatics

Two GEO datasets utilized in the present study (GSE241166 and GSE202418) were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). These are the expression profiles of cardiac tissues in T2DM mice. The T2DM model applied by GSE241166 included HFD and streptozotocin injections, similar to that in our study. In GSE202418, db/db mice were used as a T2DM model. Bioinformatics analysis was performed using R language (version 3.4.4). Differentially expressed genes (DEGs) were analyzed by R package 'limma' (Ritchie et al. 2015), where absolute value of fold change (FC) ≥ 2 and adjusted $p < 0.05$ was considered significant. Gene annotation analysis was performed using the Metascape online tool (Zhou et al. 2019). The lollipop plots were drawn by R package 'ggplot2' (Wickham 2016).

Cell culture

The AC16 human ventricular cardiomyocyte line was cultured in DMEM (Gibco, NY, USA, Cat No: 11885084)

supplemented with 10% fetal bovine serum (FBS). Co-treatment with 25 mM glucose and 100μM palmitate for 24 h was used to establish the high-glucose and high-fat (HGHF) model, simulating the *in vivo* environment of T2DM. 100 μM GYY4137 (Sigma-Aldrich, St. Louis, MO, USA, Cat. No: SML2470) pretreatment for 24 h was used to evaluate its effect on the AC16 cells. 200 nM FOXO1 inhibitor (AS1842856, Selleck, Shanghai, China, Cat. No: S8222) was used to inhibit the function of FOXO1.

RNA isolation and qRT-PCR

Total RNA was isolated from the heart using the TRIzol reagent. Reverse transcription into cDNA was performed using the HiScipt IIQRT SuperMix kit (Vazyme, Nanjing, China, Cat.No: R223-01). The real-time quantitative PCR amplification was done by ChanmQ SYBR qPCR Master Mix (Vazyme, Nanjing, China, Cat.No: Q311-02,). The primers used are listed in Table S1. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blot analyses

Total protein was extracted, and the samples were separated by 10% SDS-PAGE and then transferred onto nitrocellulose membranes, which were blocked with 5% nonfat milk dissolved in PBS for 1 h at room temperature. Membranes were probed with primary antibodies against FOXO1 (1:500, Signalway Antibody, MD, USA, Cat No:40934), phosphorylated FOXO1 (p-FOXO1, 1:500; Signalway Antibody, MD, USA, Cat No:12198), LC3B (1:1000, Cell Signaling Technology, MA,USA, Cat No: 83506), SQSTM1/p62 (1:1000, Cell Signaling Technology, MA,USA, Cat No: 23214), and β-actin (1:20000, Proteintech, Wuhan, China, Cat.No:66009-1) at 4°C overnight. The membranes were then washed thrice with TBST and incubated with the corresponding secondary antibodies (1:5000) for 1 h at room temperature. β-actin was used as an internal control. Western blot bands were imaged using a Tanon 5200 imaging system (Tanon, Shanghai, China) and analyzed using the ImageJ software (NIH, USA).

Cell viability assay

Cell viability was determined using the CCK-8 assay. AC16 cells were incubated in 96-well plates at a concentration of 3×10^4 cells/ml at 37°C. After the indicated treatments, cells were washed twice with PBS. Then the cells were incubated with 10 μL CCK-8 test solution (Selleck, Shanghai, China, Cat. No: B34304) and 90 μL DMEM at 37°C for 2 h. The optical density (OD) was determined by measuring the absorbance value at the 450 nm wavelength using a microplate reader (Thermo Fisher, MA, USA). The mean OD of three wells in each group was used to calculate the percentage of cellular activity according to the following formula: Cell viability (%) = (OD treatment group/OD control group) × 100%.

Measurement of ROS

AC16 cells with indicated treatment were incubated with 10 μM solution of H_2DCFDA (Beyotime, Shanghai, China, Cat No: S0033) in DMEM for 20 min at 37°C and then washed thrice with PBS. Fluorescence was measured using a fluorescence microscope (Olympus, Tokyo, Japan) and quantified using the ImageJ software (NIH, USA).

Detection of mitochondrial damage

Mitochondrial damage was detected by Mito-Tracker Red CMXRos following the manufacturer's instruction (Beyotime, Shanghai, China, Cat. No: C1035). The cells were incubated with Mito-Tracker Red CMXRos solution for 20 min in the dark at 37°C and then washed twice with PBS. Fluorescence was measured using a fluorescence microscope (Olympus, Tokyo, Japan) and quantified using the ImageJ software (NIH, USA). In healthy cells with intact mitochondrial membranes, Mito-Tracker Red CMXRos aggregates (red fluorescence). In cells with disrupted mitochondrial membranes, Mito-Tracker Red CMXRos cannot bind to mitochondria. Mitochondrial damage was evaluated by measuring the density of the red fluorescence.

Detection of lipid accumulation

Cellular lipid accumulation was detected by BODIPY 493/503 (Glpbio, California, USA, Cat. No: GC42959). Briefly, after individual treatment, cells were incubated with BODIPY 493/503 staining solution for 15 min at 37°C. The cells were then washed thrice with PBS. Fluorescence was measured using a fluorescence microscope (Olympus, Tokyo, Japan) and quantified using the ImageJ software (NIH, USA).

Hoechst 33342 staining

AC16 cells were double-stained with Hoechst 33342 to visualize nuclei. After the above fluorescent staining, cells were stained with Hoechst 33342 reagent (Beyotime, Shanghai, China, Cat.No: C1022) for 10 mins at room temperature. The cells were then washed thrice

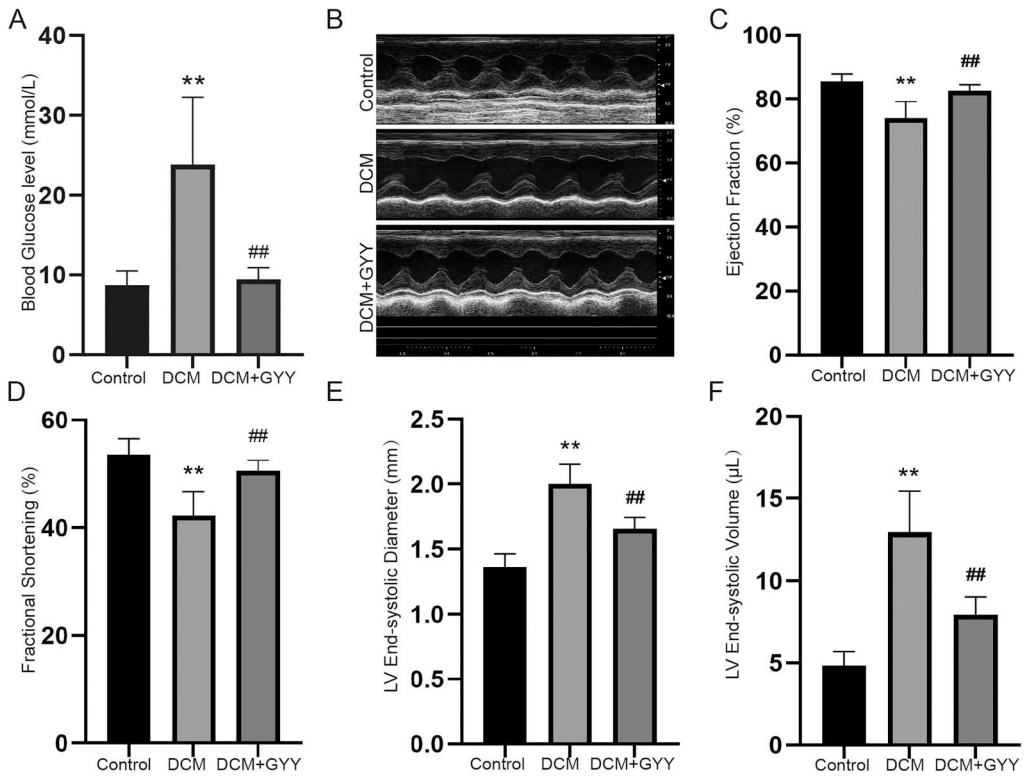

Figure 1. GYY4137 attenuates cardiac dysfunction in T2DM mice. After individual treatment, blood glucose levels were monitored (A). Noninvasive echocardiographic assessments were performed to evaluate cardiac function, including left ventricular M-mode echocardiographic tracings (B), quantification of left ventricular ejection fraction (C), fractional shortening (D), end-systolic diameter (E) and end-systolic volume (F). **, $p < 0.01$ vs. Control group; ##, $p < 0.01$ vs. DCM group. GYY, GYY4137.

with PBS. The fluorescence was measured using a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

In *in vivo* studies, there were six animals in each group. In *in vitro* studies, we ran each experiment thrice. For quantitative analysis of fluorescent images, five high-magnification (×200) fields were randomly chosen for each group. All data are expressed as the mean ± SD. Differences between groups were determined by one-way analysis of variance (ANOVA) using the SPSS software (version 21.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant throughout the analysis.

Result

Protective effect of GYY4137 against DCM in T2DM mice

In T2DM mice, oral supplementation with GYY4137 for six weeks significantly reduced fasting blood glucose levels (Figure 1A). M-mode echocardiographic tracing (Figure 1B) revealed that diabetes-induced elevation of the left ventricular internal diameter was prominently relieved by GYY4137. Moreover, parameters of murine cardiac function, including left ventricular ejection fraction (Figure 1C), fractional shortening (Figure 1D), end-systolic diameter (Figure 1E), and end-systolic volume (Figure 1F), were all improved by GYY4137. Masson's trichrome staining of the cardiac sections showed marked collagen deposition in T2DM mice, which was dampened by GYY4137 (Figure 2A). Two representative parameters of autophagy, LC3B (I and II) and SQSTM1/p62, were detected using western blot analysis. The protein levels of LC3B-II, an indicator of activated autophagosomes, were significantly higher in the myocardium of T2DM mice, whereas those of SQSTM1/p62 were lower, suggesting overactive cardiac autophagy in T2DM (Figure 2B). However, these abnormalities were reversed by GYY4137 treatment.

Protection of GYY4137 in HGHF treated cardiomyocytes

In our study, we employed the human ventricular cardiomyocyte line AC16. Our initial observations revealed that GYY4137 exhibited dose-dependent capacity to safeguard the viability of HGHF-treated AC16 cells

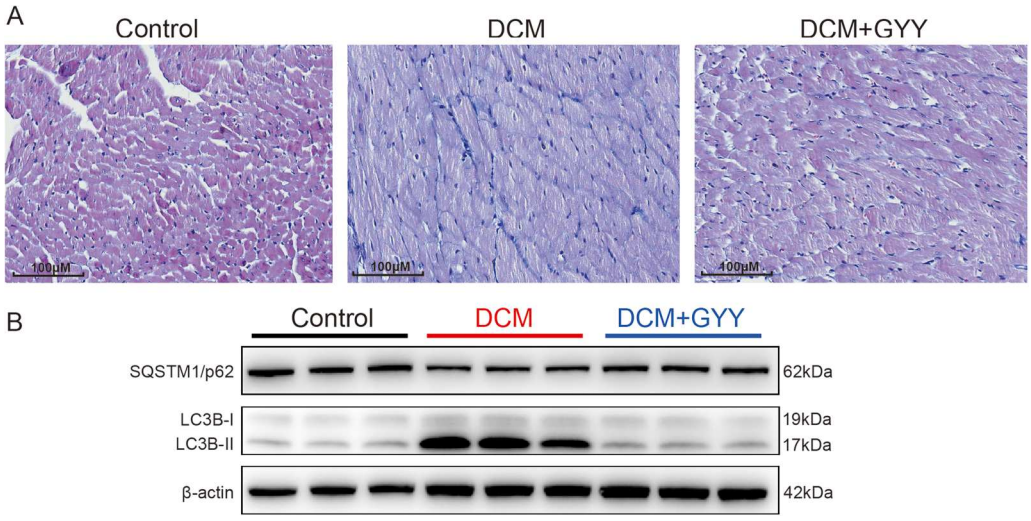

Figure 2. GYY4137 protects against cardiac fibrosis and autophagy in T2DM mice. After individual treatment, cardiac sections were assessed by Masson staining (A). Western blots of LC3B and p62 in cardiac tissue were applied to analyze cardiac autophagy (B). GYY, GYY4137.

(Figure 3D). Moreover, GYY4137 alleviated the HGHF-induced ROS generation (Figure 3A and S1) and mitochondrial damage (Figure 3B and S2). Similar to the *in vivo* study, overactivated autophagy in HGHF-treated cardiomyocytes was also alleviated by GYY4137 pretreatment (Figure 3E), presenting dampened expression of LC3B-II and increased level of SQSTM1/p62. HGHF treatment caused severe lipid accumulation in cardiomyocytes (Figure 3C and S3), which was largely reversed by treatment with GYY4137.

Bioinformatic analysis reveals the potential role of FOXO signal pathway in T2DM-associated DCM

To unravel the underlying mechanism of GYY4137's protective effect against DCM, we used two GEO datasets of murine diabetic hearts (GSE241166 and GSE202418) for bioinformatics analysis. We identified 711 DEGs in GSE241166 (292 upregulated and 419 downregulated) and 402 DEGs in GSE202418 (244 upregulated and 158 downregulated) that were related to T2DM-associated DCM (Figure S4). We subsequently conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses to gain insights from the two DEG lists. Notably, DEGs in both datasets indicated significant enrichment of the FOXO signaling pathway in the cardiac tissues of diabetic mice (Figure 4A and B). Autophagy, oxidative damage response, and lipid metabolism were also enriched in the diabetic hearts (Figure 4A and B), which is consistent with our *in vivo* and *in vitro* findings. Joint enrichment analysis of the aforementioned datasets showed that the FOXO signaling pathway and autophagy were the predominant pathways (Figure 4C and D). These results suggest that the FOXO signaling pathway and autophagy may play an important role in T2DM-associated myocardial injury.

GYY4137 rescues diabetes-induced overexpression of FOXO1

Next, we confirmed the mRNA and protein levels of FOXO1, a major contributor to the FOXO signaling pathway, in both *in vivo* and *in vitro* diabetic models. In diabetic mice, FOXO1 was significantly upregulated in cardiac tissue, which was effectively mitigated by GYY4137 treatment (Figure 5A and B). In HGHF-treated AC16 cells, pretreatment with 100 μM GYY4137 for 24 h also reduced diabetes-induced overexpression of FOXO1, as evident at both mRNA and protein levels (Figure 5C and D). Moreover, GYY4137 induced the phosphorylation of FOXO1, thereby leading to its inactivation, resulting in increased levels of phosphorylated FOXO1 in both the murine diabetic model and *in vitro* myocardiocytes (Figure 5B and D). Taken together, GYY4137 rescued diabetes-induced overexpression of FOXO1 by influencing its transcription and phosphorylation.

FOXO1 is required for HGHF-induced cellular injury in cardiomyocytes

To further explore the potential role of FOXO1 in GYY4137-induced cardioprotection, we introduced the FOXO1 inhibitor, AS1842856, into AC16 cells. First, we confirmed that AS1842856 at concentrations between 50 and 200 nM did no significantly affect cell viability (Figure 6D), and 200 nM AS1842856 was chosen for further study. Remarkably, 200 nM AS1842856 treatment led to a substantial increase the viability of HGHF-treated AC16

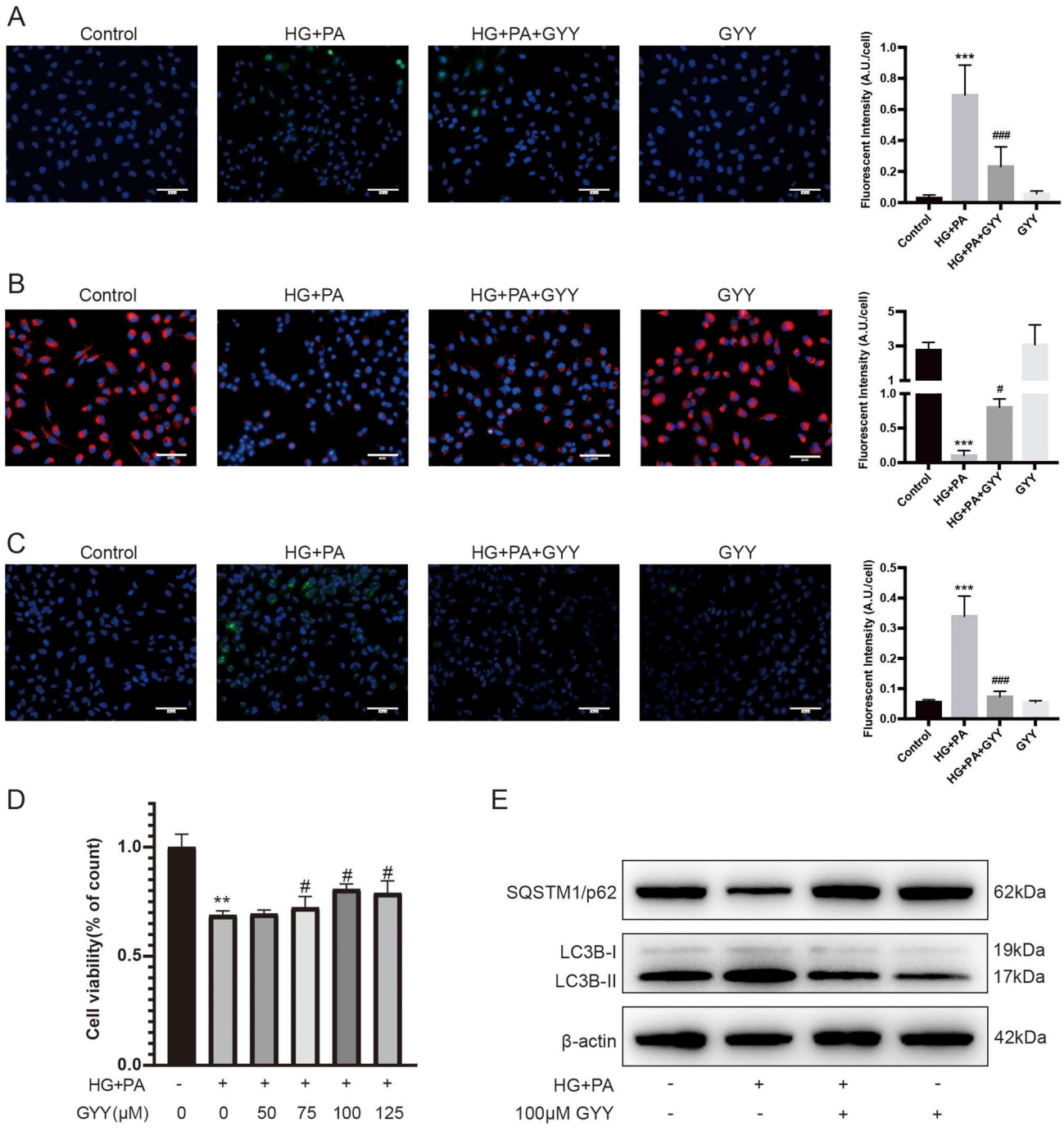

Figure 3. Protective effect of GYY4137 in HGHF treated cardiomyocytes. After individual treatment, AC16 cells were harvested. Cellular oxidative stress was detected by ROS fluorescence staining (A). Mitochondrial function was analyzed by Mito-tracker red CMXRos staining (B). The cellular lipid accumulation was evaluated by BODIPY 493/503 fluorescence staining (C). Fluorescent intensities were evaluated and presented as bar charts, and Hoechst staining was also applied to present nuclei. The cell viability was detected by CCK-8 assay (D). Autophagy of cardiomyocytes was detected by western blot of LC3B and p62 (E). **, $p < 0.01$ vs. Control group; ***, $p < 0.001$ vs. Control group; #, $p < 0.05$ vs. HG + PA group; ###, $p < 0.001$ vs. HG + PA group. GYY, GYY4137; HG, high glucose; PA, palmitate. The scale bar is 100 μM.

cells (Figure 6E). Additionally, AS1842856 was effective in reducing ROS generation (Figure 6A and S5), ameliorating mitochondrial damage (Figure 6B and S6) and dampening lipid accumulation (Figure 6C and S7) in cardiomyocytes exposed to HGHF. Furthermore, the expressions of SQSTM1/p62 and LC3B-II (Figure 6F) were markedly reduced by AS1842856 treatment, indicating that FOXO1 inhibition acts as a safeguard against diabetes-associated autophagy. Collectively, these findings highlight the pivotal role of FOXO1 in diabetes-induced cardiac injury.

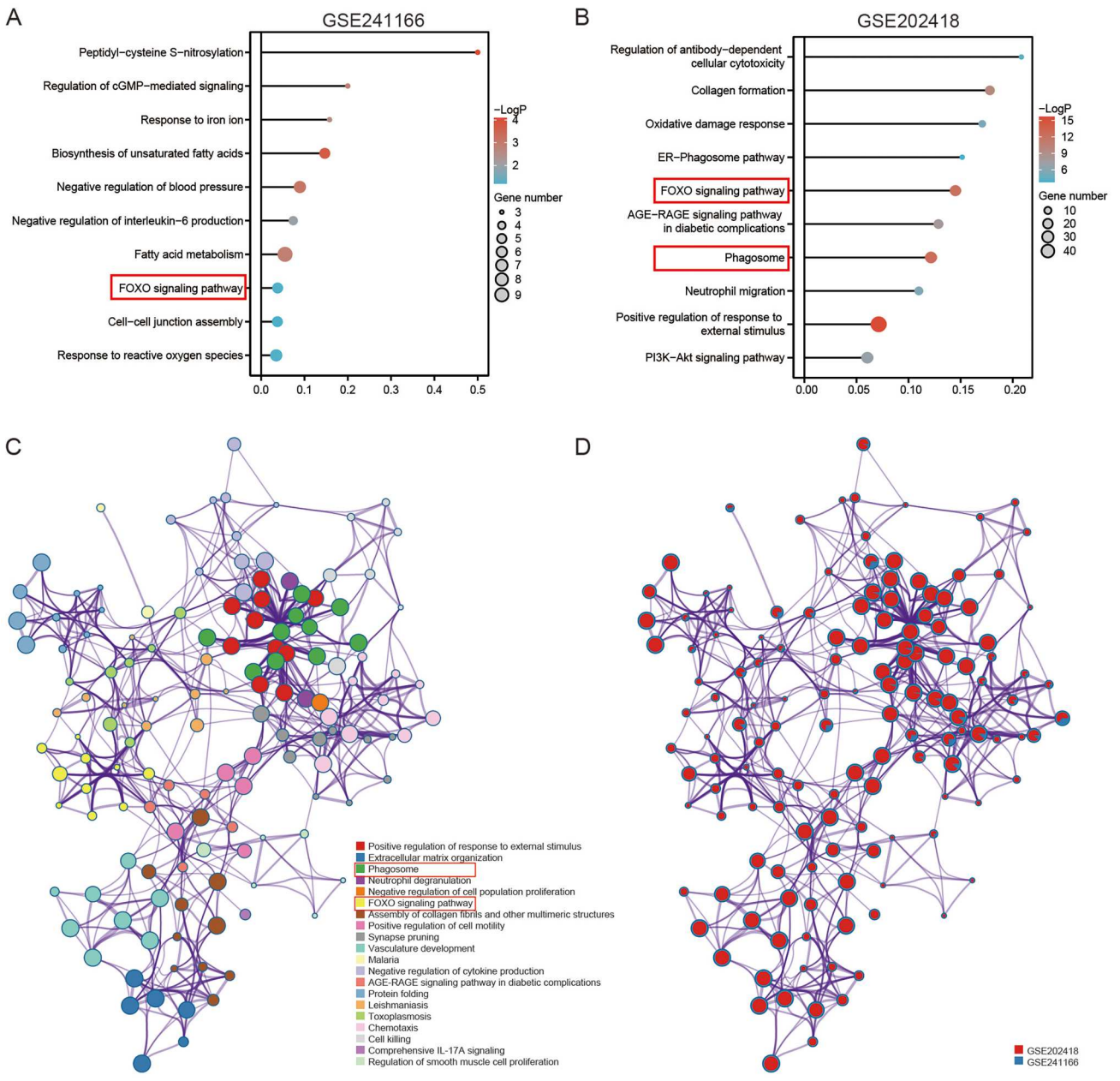

Figure 4. Bioinformatic analysis reveals the potential role of FOXO signal pathway in DCM. Two GEO datasets were used (GSE241166 and GSE202418). Functional annotations of DEGs were carried out. The representative KEGG pathways were presented as lollipop plots (A, B). The dot size stands for gene number enriched in individual term, the dot color stands for *p* value of individual term, and the dot deposition stand for normalized enrichment score in individual term. A joint enrichment analysis of the above two datasets was also applied and presented as a network of clusters. (C) Each term is represented by a circle node, where its size is proportional to the number of genes that all into an individual term, and its color represents its cluster identity. (D) Each term is represented by a pie, where the pie sector is proportional to the number of genes from an individual group.

Given that GYY4137 can reverse the overexpression of FOXO1 in diabetes, GYY4137 may be cardioprotective by FOXO1 in T2DM mice.

Discussion

In the present study, we harnessed GYY4137, a slow-releasing H_2S donor, in T2DM mice and demonstrated its efficacy in safeguarding diabetes-associated cardiac dysfunction. Through bioinformatic analysis of diabetic mice, we revealed the potential significance of the FOXO signaling pathway in DCM. Subsequently, we verified the modulatory effect of GYY4137 on FOXO1 expression and assessed the role of FOXO1 in diabetes-induced overactive cardiac autophagy, oxidative stress, mitochondrial damage, and lipid accumulation.

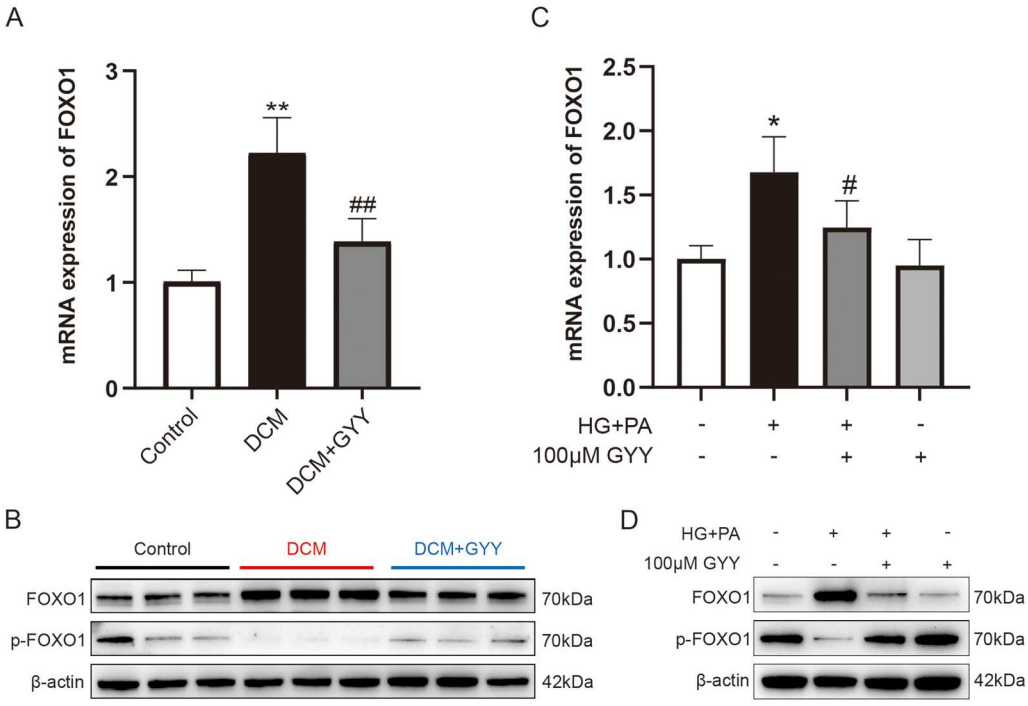

Figure 5. GYY4137 rescues diabetes-induced overexpression of FOXO1. In murine cardiac tissue, the expression of FOXO1 mRNA was detected by qRT-PCR (A), and the level of FOXO1 protein was detected by western blot (B). The mRNA (C) and protein (D) levels of FOXO1 in AC16 cells were also presented. *, $p < 0.05$ vs. Control group; **, $p < 0.01$ vs. Control group; #, $p < 0.05$ vs. HG + PA group; ##, $p < 0.01$ vs. HG + PA group. GYY, GYY4137; HG, high glucose; PA, palmitate.

Based on these results, it is reasonable to infer that GYY4137 may mitigate T2DM-associated cardiomyopathy by targeting FOXO1-induced autophagy.

H_2S, a well-known endogenous gasotransmitter, plays an important role in the pathogenesis and progression of T2DM (Zhang et al. 2021). Impaired biosynthesis of H_2S has been observed in patients with T2DM and in diabetic animals (Cheng and Kishore 2020). More specifically, the myocardial level of H_2S was also decreased in T2DM mice (Gong et al. 2022). These findings highlight the critical role of endogenous H_2S dysfunction in DCM pathogenesis. Given its potential to enhance insulin sensitivity and confer anti-oxidant, anti-inflammatory, and anti-autophagic effects, restoring the H_2S system has emerged as a promising strategy to combat diabetes (Piragine and Calderone 2021). Notably, H_2S exerts its biological effects in a biphasic dose–response manner, transitioning from cytoprotective at lower concentrations to cytotoxic at higher concentrations. Therefore, an ideal H_2S donor must generate H_2S gradually. In our study, we utilized a water-soluble, slow-releasing donor of H_2S, GYY4137, and revealed its robust protection in DCM. In cardiomyocytes, GYY4137 significantly ameliorated diabetes-associated oxidative stress, mitochondrial dysfunction, and autophagy. Typical myocardial lipid accumulation in T2DM was also relieved by GYY4137. Based on these molecular mechanisms, GYY4137 was found to successfully restore cardiac function in T2DM mice.

Recently, several studies were consistent with our findings and suggested that the prominent cardiac protection of GYY4137 may lie in its role of regulating endoplasmic reticulum-mitochondria crosstalk (Yang et al. 2017), inhibiting autophagy and oxidative stress-associated signaling pathways (Li et al. 2019), and eliciting local and systemic anti-inflammation effect (Rodrigues et al. 2017). Furthermore, H_2S has been found to exert pleiotropic functions in cell signaling. It modulates the activity of various proteins through the post-translational modification of sulfhydration, which can either enhance or diminish protein function (Mustafa et al. 2009; Cirino et al. 2023). Additionally, H_2S is known to influence biological responses by engaging with reactive oxygen species and metalloproteins (Murphy et al. 2019).

FOXO1, FOXO3, FOXO4, and FOXO6 are the critical transcription factors in mammals. They serve as key effectors within the insulin pathway, acting as nutrient sensors that regulate blood glucose levels, primarily by diminishing gluconeogenesis in the liver and enhancing glucose uptake in the skeletal muscle and adipocytes (Orea-Soufi et al. 2022). In particular, FOXO1 has emerged as a significant mediator of pathological cardiac hypertrophy and remodeling in metabolic

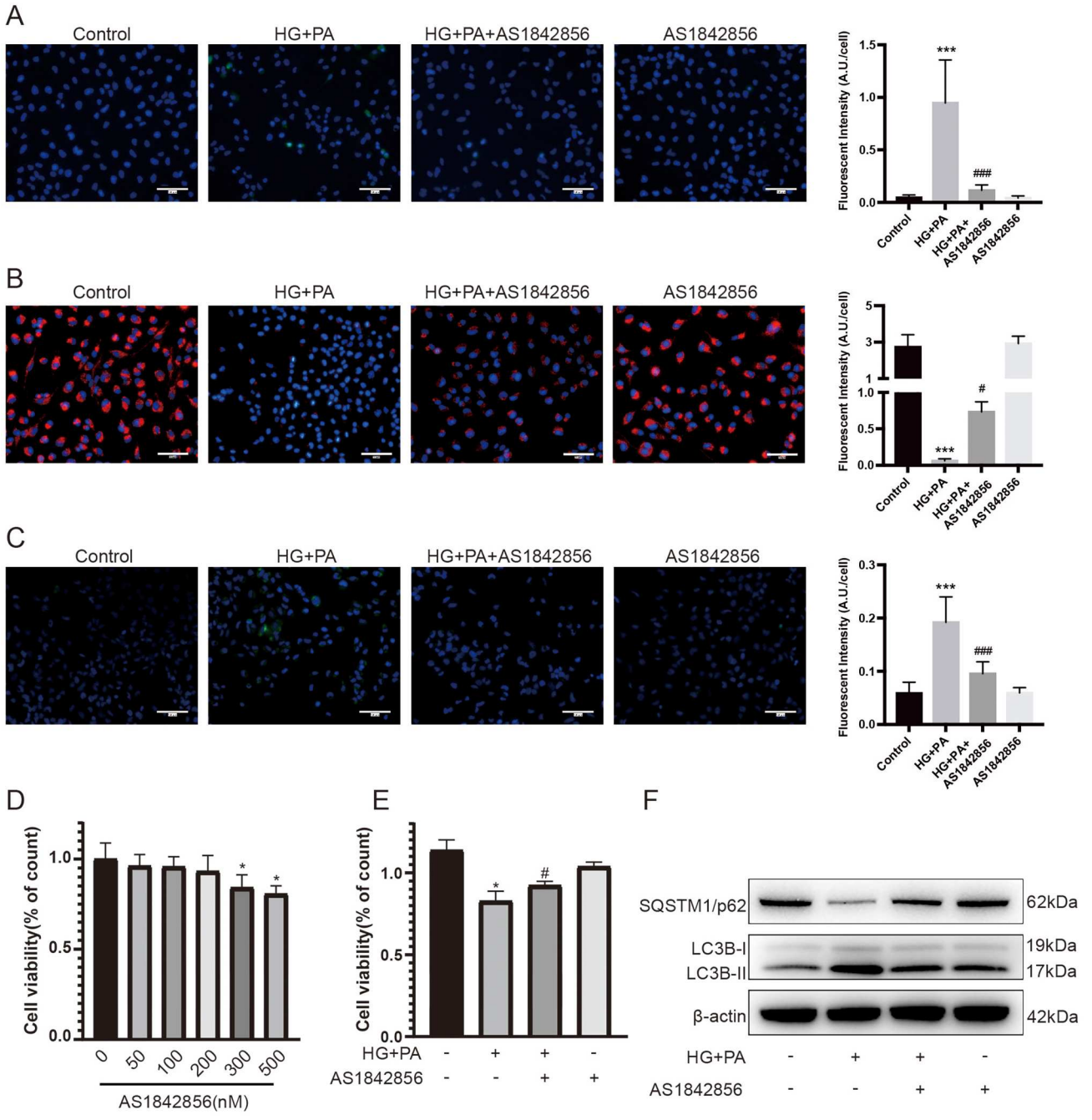

Figure 6. FOXO1 is required for HGHF-induced cellular injury in cardiomyocytes. FOXO1 inhibitor (AS1842856) was applied in AC16 cells. Cellular oxidative stress was detected by ROS fluorescence staining (A). Mitochondrial function was analyzed by Mitotracker red CMXRos staining (B). The cellular lipid accumulation was evaluated by BODIPY 493/503 fluorescence staining (C). Fluorescent intensities were evaluated and presented as bar charts, and Hoechst staining was also applied to present nuclei. The cell viability was detected by CCK-8 assay (D and E). Autophagy of cardiomyocytes was detected by western blot of LC3B and p62 (F). *, $p < 0.05$ vs. Control group; ***, $p < 0.001$ vs. Control group; #, $p < 0.05$ vs. HG + PA group; ###, $p < 0.001$ vs. HG + PA group. The scale bar is 100 μM. HG, high glucose; PA,palmitate.

stress-induced cardiac pathologies including DCM (Pfleger et al. 2020). Our findings provide further support for the theory that the overexpression of myocardial FOXO1 directly contributes to the pathology of T2DM-associated DCM. Both cardiac tissues of T2DM mice and HGHF-treated cardiomyocytes displayed elevated transcription and expression of FOXO1. Inhibition of FOXO1 signaling by AS1842856 effectively reversed diabetes-induced myocardial autophagy and dysfunction, including ROS generation, mitochondrial damage, and cell death. These observations align with the finding that inhibition of insulin signaling in T2DM

activates FOXO1 and triggers several pathological pathways, including autophagy, inflammation, and glucose metabolism, all contributing to DCM pathogenesis (Kyriazis et al. 2021). Moreover, myocardial lipid accumulation, a hallmark feature of hyperlipidemia with a higher prevalence in T2DM than in T1DM (Goldberg 2022), was also ameliorated by FOXO1 inhibition in our study. The critical effect of FOXO1 on T2DM-associated myocardial lipid accumulation may be attributed to the direct binding of FOXO1 to Peroxisome Proliferator Activated Receptor (PPAR)α promoter, subsequently mediating the expression of this cardiac lipid metabolic regulator (Kyriazis et al. 2021).

Given the significant role of FOXO1 in DCM, targeting FOXO1 has emerged as a crucial focus in the development of antidiabetic therapeutic strategies. A recent study demonstrated that the oral administration of AS1842856 significantly increased glucose oxidation rates and improved diastolic function in diabetic mice (Gopal et al. 2021). However, this selective FOXO inhibitor failed to reduce cardiac fibrosis in T2DM mice. In our study, GYY4137 offered convincing protection to maintain systemic glucose homeostasis, improve cardiac function, and alleviate cardiac collagen deposition, mainly by inhibiting the FOXO1 signaling pathway. GYY4137 has been shown to functionally inactivate FOXO1 by nuclear exclusion of FOXO1 from the cytoplasm after its phosphorylation (Majumder et al. 2019), which may explain the beneficial effect of GYY4137 against FOXO1 overexpression. Therefore, GYY4137 is a potential pharmacological inhibitor of FOXO1 in T2DM-associated DCM.

The current study had several limitations. First, since DCM primarily affects individuals with T2DM, our study focused on T2DM mice established via a high-fat diet combined with streptozotocin induction. However, the effects of GYY4137 on T1DM-associated DCM warrant further investigation. Second, we did not employ gene knockout or transgenic mice, and it is essential to recognize the relative selectivity of the pharmacological inhibitors. Finally, our study only involved male mice to mitigate the potential influence of estrogen on insulin resistance. However, the impact of sex on our findings should be assessed in future studies.

Conclusion

GYY4137 protected against cardiac dysfunction and fibrosis in T2DM mice, and the mechanism might involve suppression of FOXO1-induced myocardial autophagy, oxidative stress, mitochondrial damage, and lipid accumulation.

Author contributions

Gaofeng Zhu: analyzed data; Xiaoyong Li: performed experiments; Qinyuan Gao: interpreted results of experiments; Yuanjun Wang: performed experiments; Jiajie Li: performed experiments; Zena Huang: drafted manuscript; Yan Lin: edited and revised manuscript. All the authors have read and approved the final manuscript.

Data sharing statement

The datasets analyzed during current study are available at the GEO website.

Disclosure statement

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Ethics approval

All animal experiments were approved by the Ethics Committee of Guangdong Provincial People's Hospital (approved number: KY-Z-202-123-02).

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