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ARTICLE

Glibenclamide alleviates β adrenergic receptor activation-induced cardiac inflammation

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β-Adrenergic receptor (β-AR) overactivation is a major pathological factor associated with cardiac diseases and mediates cardiac inflammatory injury. Glibenclamide has shown anti-inflammatory effects in previous research. However, it is unclear whether and how glibenclamide can alleviate cardiac inflammatory injury induced by β-AR overactivation. In the present study, male C57BL/6J mice were treated with or without the β-AR agonist isoprenaline (ISO) with or without glibenclamide pretreatment. The results indicated that glibenclamide alleviated ISO-induced macrophage infiltration in the heart, as determined by Mac-3 staining. Consistent with this finding, glibenclamide also inhibited ISO-induced chemokines and proinflammatory cytokines expression in the heart. Moreover, glibenclamide inhibited ISO-induced cardiac fibrosis and dysfunction in mice. To reveal the protective mechanism of glibenclamide, the NLRP3 inflammasome was further analysed. ISO activated the NLRP3 inflammasome in both cardiomyocytes and mouse hearts, but this effect was alleviated by glibenclamide pretreatment. Furthermore, in cardiomyocytes, ISO increased the efflux of potassium and the generation of ROS, which are recognized as activators of the NLRP3 inflammasome. The ISO-induced increases in these processes were inhibited by glibenclamide pretreatment. Moreover, glibenclamide inhibited the cAMP/PKA signalling pathway, which is downstream of β-AR, by increasing phosphodiesterase activity in mouse hearts and cardiomyocytes. In conclusion, glibenclamide alleviates β-AR overactivation-induced cardiac inflammation by inhibiting the NLRP3 inflammasome. The underlying mechanism involves glibenclamide-mediated suppression of potassium efflux and ROS generation by inhibiting the cAMP/PKA pathway.

Keywords: glibenclamide; cardiac inflammation; isoprenaline; sympathetic stress; NLRP3 inflammasome; adrenergic receptor

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INTRODUCTION

Overactivation of the sympathoadrenergic system is a major pathological factor in cardiac diseases. It results in cardiac β-adrenergic receptor (β-AR) overactivation and causes cardiac inflammatory injury [1], which is central to the development of cardiac diseases [2]. Glibenclamide is a sulfonylurea that is used as a hypoglycaemic agent for patients with diabetes [3], and it exerts anti-inflammatory effects on asthmatic inflammation and infectious diseases [4, 5]. However, it is unclear whether and how glibenclamide inhibits cardiac inflammatory injury induced by β-AR overactivation.

Our previous study indicated that β-AR overactivation in cardiomyocytes initiates sterile cardiac inflammation by activating the NLR family pyrin domain-containing 3 (NLRP3) inflammasome [6, 7]. The NLRP3 inflammasome is a multiprotein complex that triggers the activation of caspase-1 and the maturation of interleukin (IL)-1β and IL-18 [8, 9]. The inflammasome can be activated by reactive oxygen species (ROS) and potassium efflux. Glibenclamide is a classic ATP-sensitive potassium channel (K_{ATP}) blocker that inhibits potassium efflux [9]. However, it is unknown whether glibenclamide inhibits inflammasome activation induced by β-AR overactivation. Moreover, our previous research revealed that isoprenaline (ISO), a β-AR agonist, induces ROS generation in mouse cardiomyocytes by activating the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway [10]. The effect of glibenclamide on ROS and the cAMP/PKA pathway is also unclear and needs to be elucidated.

In the present study, we investigated the effect of glibenclamide on β-AR overactivation-induced cardiac inflammation, fibrosis, dysfunction and NLRP3 inflammasome activation in vivo and in vitro. We further determined whether potassium efflux and ROS were involved in the protective effect of glibenclamide.

MATERIALS AND METHODS

Antibodies and reagents

The Mac-3 antibody was purchased from BD Biosciences (San Jose, CA, USA). NLRP3, glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH), pro-caspase-1, pro-IL-18, and phospho-PKA substrate antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). The caspase-1 (P20) antibody was purchased from Sigma (St. Louis, MO, USA). The cleaved IL-18 antibody was purchased from MBL (Nagoya Aichi, Japan). IL-6, tumour necrosis factor-α (TNF-α), macrophage chemokine protein-5 (MCP-5) and C-X3-C motif chemokine ligand 1 (CX3CL-1) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Incorporated (Minneapolis, MN, USA). Glibenclamide, glimepiride and ISO were purchased from Sigma. Glibenclamide was dissolved in dimethylsulfoxide (Sigma) and diluted to the desired concentrations with saline for the animal experiments. The PKA inhibitor 14–22 amide (PKI) was purchased from Calbiochem (Billerica, MA, USA). ROS generation was determined by dihydroethidium (DHE, Invitrogen Molecular Probes, Eugene, OR, USA) staining. The cAMP-Glo Max assay kit was purchased from Promega (Madison, WI, USA).

Animals

All animal experimental protocols were approved by the Committee of Peking University on the Ethics of Animal Experiments (LA2016-018). The study was conducted in accordance with the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and the guidelines of the Peking University Health Science Centre. Male C57BL/6 J mice were purchased from the Department of Laboratory Animal Science, Peking University. The mice were used for experiments at 10 weeks of age and were housed in a specific pathogen-free environment (temperature: 20–24 °C; relative humidity: 30%–70%) under a 12 h/12 h light-dark cycle and fed a rodent diet ad libitum.

The mice were randomly divided into 4 groups. Glibenclamide (1 mg/kg body weight, sc) was injected into the mice once per day beginning 3 days before ISO treatment (5 mg/kg body weight, sc). Heart tissues were collected on day 1 after ISO treatment to analyse inflammasome activation, on day 3 to analyse macrophage infiltration, and on day 7 for cardiac fibrosis analysis. All animals were fasted for 12 h before euthanasia, and fasting blood glucose was measured. Blood glucose levels in tail blood were measured 60 min before euthanasia by using glucose test strips (Boehringer, Mannheim, Germany). Finally, the mice were euthanized by an intraperitoneal injection with an overdose of sodium pentobarbital. Cardiac samples were collected in lysis buffer (10 mmol/L Tris-HCl, pH 7.4; 100 mmol/L NaCl; 1 mmol/L EDTA; 1 mM EGTA; 1 mmol/L NaF; 20 mmol/L $Na_4P_2O_7$; 2 mmol/L Na_3VO_4; 1% Triton X-100; 10% glycerol; 0.1% sodium dodecyl sulphate (SDS); 1% deoxycholic acid; 1 mmol/L PMSF; and 1 g/mL aprotinin).

Isolation and culture of primary neonatal mouse cardiomyocytes (NMCMs)

Cardiomyocytes were isolated from 1- to 3-day-old neonatal C57BL/6 J mice and cultured as described previously [11]. In brief, cardiomyocytes were collected using trypsin and collagenase type II (Gibco, Carlsbad, CA, USA). Dissociated cells were plated on 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum and incubated for 2 h. The non-attached cardiomyocyte-rich fraction was plated on plastic dishes (5×10^5 cells/dish), and 100 μmol/L bromodeoxyuridine (Sigma) was added to prevent fibroblast proliferation. The cells were incubated in serum-free medium for 3 h prior to drug or mock treatments.

Histochemistry and immunohistochemistry

The hearts were harvested from the mice, washed with cold phosphate-buffered saline, fixed with 4% paraformaldehyde for 8 h, and embedded in paraffin. Serial sections (5 μm thick) were stained with picrosirius red to detect collagen deposition as described previously [6, 12]. For immunohistochemistry, heart sections were incubated with antibodies against the macrophage marker Mac-3 (1:200 dilution, BD Biosciences, San Jose, CA, USA). The sections were imaged with a NanoZoomer-SQ digital slide scanner (Hamamatsu Photonics, Shizuoka, Japan). To evaluate cardiac fibrosis and macrophage infiltration, 10 fields were randomly selected from each section, and the ratio of the positively stained area to the total myocardial area was calculated with Image-Pro Plus 6.0 (Media Cybernetics, MD, USA).

Echocardiography

Echocardiography was used to evaluate cardiac function on the 7th day after ISO administration as previously described [6]. A VisualSonics high-resolution Vevo 2100 system (VisualSonics Inc., Toronto, Canada) was used. In brief, the mice were anaesthetized with 1.0% isoflurane until the heart rate was stabilized at 400–500 bpm. An apical four-chamber view was acquired, and the peak flow velocities during early diastole (E wave) were measured across the mitral valve. Early-diastolic peak velocity (*E'* wave) of the mitral valve ring was also measured in this view, and the *E/E'* was calculated to determine left ventricular diastolic function.

ELISA

The levels of MCP-1, CX3CL-1, IL-6, and TNF-α in mouse heart tissue were measured with ELISA kits (R&D) as described previously [13]. Briefly, mouse heart tissue was harvested, immediately frozen in liquid nitrogen and then homogenized in lysis buffer. The procedures were conducted according to the manufacturer's instructions, and the absorbances were measured at 450 nm with a Multiskan GO instrument (Thermo Fisher Scientific Cellomics, Pittsburgh, PA, USA). All values were in the linear range and were calculated based on known protein concentrations.

Western blot analysis

The levels of NLRP3, caspase-1 (pro-caspase-1), caspase-1 (p20), pro-IL-18, cleaved IL-18, phospho-PKA substrate and GAPDH were examined by Western blotting. Protein samples (30–40 μg) were separated by electrophoresis on 12% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies for at least 8 h at 4 °C. After the membranes were incubated with the corresponding HRP-conjugated secondary antibodies (ZSGB-BIO, Beijing, China), the protein bands were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, St. Burlington, MA, USA). Blot images were obtained using a Syngene GeneGnome-XRQ-NPC imager (Gene Company, Shanghai, China), and the proteins were quantified by calculating the greyscale value of each band using ImageJ (version 1.52) software.

Measurement of intracellular potassium

To assess potassium efflux, potassium-binding benzofuran isophthalate-acetoxymethyl ester (PBFI-AM) (Sigma-Aldrich) was administered to NMCMs in white polystyrene 96-well microplates for fluorometric quantification of potassium ions, as described previously [14]. Briefly, NMCMs were plated in white polystyrene 96-well microplates at a density of 1×10^3 cells/well. The cells were washed with Hank's buffer, equilibrated with 50 μL of 10 μmol/L PBFI-AM, and incubated at 37 °C in a humidified 5% CO_2 atmosphere in the dark for 2 h. Excess PBFI-AM was removed by washing the cells twice with Hank's buffer. Measurements were performed using a Tecan spectrofluorometer (Infinite 200 PRO). The fluorescence intensity of PBFI (excitation wavelengths: 340 and 380 nm, emission wavelength: 510 nm) was recorded alternately. The ratio of the fluorescence intensity obtained by exciting PBFI at 340 nm to that obtained by exciting PBFI at 380 nm was used to determine intracellular potassium efflux.

Measurement of ROS levels

ROS levels in NMCMs were determined by DHE staining. Briefly, serum-starved NMCMs were incubated with 5 mmol/L DHE for 15 min at 37 °C and 5% CO_2/95% air. The cardiomyocytes were also stained with Hoechst 33342 (Invitrogen Molecular Probes, Eugene,

OR, USA) to visualize the nuclei. Fluorescence intensity was measured and analysed with a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher Scientific Cellomics, Pittsburgh, PA, USA) with the Morphology Explorer Bio Application. Images of the cells were acquired using excitation wavelengths of 386 and 535 nm with a 300 ms exposure time. The level of ROS is expressed as the mean fluorescence intensity [10].

Bioluminescent detection of cAMP

The levels of cAMP in NMCMs and mouse heart tissue were measured with a cAMP-Glo Max Assay (Promega) as described previously [15]. The procedures were performed according to the manufacturer's instructions. Briefly, 1×10^6 NMCMs were pretreated with 100 μmol/L glibenclamide and then treated with or without 10 μmol/L ISO in complete induction buffer (500 μmol/L 3-isobutyl-1- methylxanthine [IBMX; Sigma], 100 μmol/L Ro 20-1724 [Ro; Sigma] and 20 mmol/L $MgCl_2$ in DMEM). After the indicated volume of cAMP detection solution was added to all wells, the plate was incubated at room temperature for 20 min. For mouse heart tissues, 10 mg of cardiac tissue was lysed in 1 mL of complete induction buffer (lysis buffer with 500 μmol/L IBMX and 100 μmol/L Ro), and the lysate (2 μL/well) was added to a 96-well plate. Then, we added the indicated volume of room-temperature Kinase-Glo® reagent to all wells and incubated the plate at room temperature for 10 min. The luminescence was measured with a plate-reading luminometer (GloMax, Promega). All values were in the linear range and were calculated based on known cAMP concentrations.

Measurement of cAMP phosphodiesterase activity

The activity of cAMP phosphodiesterases (cPDEs) in NMCMs and mouse heart tissue was measured with a cPDE activity assay kit (Abcam, Cambridge, UK). The procedures were conducted according to the manufacturer's instructions. Briefly, NMCMs (1×10^6 cells) and mouse heart tissue (10 mg) were rapidly homogenized in 100 μL of cold cPDE assay buffer and then centrifuged at 4 °C. The supernatant was added to the wells of a white, flat bottom 96-well plate. All samples were adjusted to 50 μL/well with cPDE assay buffer. Reaction Mix (50 μL/well) was added to the wells and incubated for 30 min at 37 °C. The fluorescence of each well was measured at Ex/Em = 535/587 nm with a Tecan spectrofluorometer (Infinite 200 PRO). All values were in the linear range, and cPDE activity was calculated based on the standard curve.

Statistical analysis

The data are expressed as the means ± SEMs. All the samples were independent. For parametric analysis, one-way ANOVA with a two-sided Tukey's *post hoc* test was used to analyse the differences among groups if the data were determined to follow a normal distribution and have equal variances. For nonparametric analysis, a Kruskal-Wallis ANOVA combined with a two-sided Dunn's multiple comparison post hoc test was performed when more than two groups were evaluated. The data were analysed using GraphPad Prism software (version 8.0, GraphPad Software Inc., San Diego, CA, USA). A value of $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Glibenclamide alleviated ISO-induced cardiac inflammation, fibrosis and dysfunction in mice

As revealed by immunohistochemical staining of the macrophage marker Mac-3, macrophage infiltration was increased in mouse hearts on day 3 following ISO treatment (5 mg/kg body weight, sc, Fig. 1a). However, macrophage infiltration was inhibited by pretreatment with glibenclamide for 3 days (1 mg·kg^{-1}·day^{-1} body weight, sc) (Fig. 1a). Consistent with this finding, glibenclamide pretreatment also inhibited ISO-induced increases in the expression of macrophage chemokines (MCP-1 and CX3CL-1) and proinflammatory cytokines (IL-6 and TNF-α) (Fig. 1b). Cardiac fibrosis and cardiac diastolic dysfunction on day 7 after ISO treatment were also alleviated by glibenclamide pretreatment (Fig. 1c,d). ISO treatment and glibenclamide pretreatment did not cause significant changes in fasting blood glucose in this experimental model (supplementary Fig. S1).

Glibenclamide inhibited ISO-induced NLRP3 inflammasome activation in vivo and in vitro

To clarify the underlying mechanism of the anti-inflammatory effect of glibenclamide, cardiac NLRP3 inflammasome activation was examined. In NMCMs, treatment with 10 μmol/L ISO for 1 h activated the NLRP3 inflammasome, as indicated by the increased expression of NLRP3, cleaved caspase-1 (p20) and cleaved IL-18 and the decreased expression of pro-caspase-1 and pro-IL-18. Glibenclamide pretreatment for 30 min inhibited inflammasome activation in a dose-dependent manner (1–100 μmol/L). However, glibenclamide per se did not change the expression levels of NLRP3, cleaved caspase-1 (p20), cleaved IL-18, pro-caspase-1 or pro-IL-18 (Fig. 2a,b).

In vivo, ISO induced NLRP3 inflammasome activation in mouse hearts at 1 h and 24 h after treatment. Glibenclamide pretreatment (1 mg·kg^{-1}·day^{-1} body weight, 3 days, sc) inhibited NLRP3 inflammasome activation in mouse hearts induced by ISO treatment for 1 h and 24 h (Fig. 2c,d). We further determined the effect of glibenclamide administration time on ISO-induced macrophage infiltration. Pretreatment and simultaneous treatment with glibenclamide inhibited ISO-induced macrophage infiltration (Fig. 1a and Fig. S2). However, treatment with glibenclamide 1 h after ISO injection, when the NLRP3 inflammasome was already activated, failed to alleviate macrophage infiltration (Fig. S2).

Glibenclamide inhibited ISO-induced NLRP3 inflammasome activation by inhibiting potassium efflux and ROS generation

To clarify how glibenclamide inhibits cardiac NLRP3 inflammasome activation, potassium efflux and ROS generation in NMCMs were examined. The intracellular levels of potassium ions in NMCMs were measured with the PBFI-AM fluorescence indicator (Schematic diagram, Fig. 3a). The levels of intracellular potassium ions were lower in NMCMs treated with ISO (10 μmol/L) for 30 min than in untreated cells (Fig. 3b), indicating the occurrence of potassium efflux. Pretreatment with glibenclamide (100 μmol/L) blocked the ISO-induced decreases in intracellular potassium ion levels (Fig. 3b). To clarify its role in NLRP3 inflammasome activation, potassium efflux was blocked with the K_{ATP} channel blocker glimepiride or a high concentration of potassium chloride (KCl, 130 mmol/L). Both glimepiride and KCl suppressed ISO-induced NLRP3 inflammasome activation (Fig. 3c, Fig S3), mimicking the inhibitory effect of glibenclamide and thus supporting the idea that glibenclamide targets potassium efflux to prevent inflammasome activation. We also found that the decrease in intracellular potassium was inhibited when the PKA signalling pathway was blocked with the PKA inhibitor PKI (0.1 μmol/L) (Fig. 3b).

ROS levels in NMCMs were measured by DHE staining. ISO induced ROS generation in NMCMs after 30 min and 120 min of treatment (Fig. 4a). Glibenclamide pretreatment inhibited ROS generation induced by 30 min of ISO treatment but not that induced by 120 min of ISO treatment (Fig. 4a). The inhibition of ROS generation by glibenclamide at 30 min after ISO treatment was dose-dependent (1–100 μmol/L) (Fig. 4b).

Glibenclamide inhibited ISO-induced activation of the cAMP/PKA signalling pathway in vivo and in vitro

To clarify whether glibenclamide inhibits the β-AR-mediated downstream signalling pathway, the accumulation of cAMP and

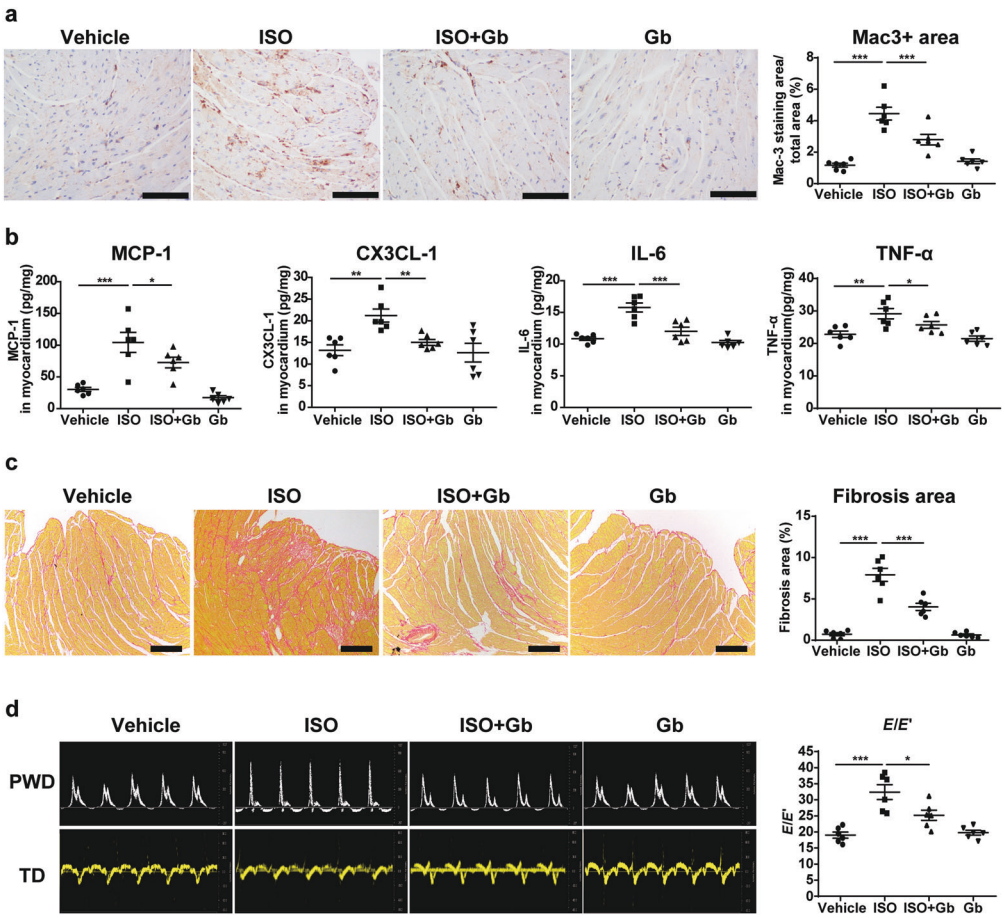

Fig. 1 Glibenclamide alleviated ISO-induced cardiac inflammation, fibrosis and dysfunction in mice. Glibenclamide (1 mg·kg^{-1}·day^{-1} body weight, sc) was administered for 3 days before ISO (5 mg/kg body weight, sc) treatment. **a** Representative images and quantification of immunostaining for Mac-3 (a macrophage marker) in the heart on the 3rd day after ISO treatment ($n = 6$; scale bars: 100 μm). **b** The concentrations of the indicated chemokines (MCP-1, CX3CL-1) and proinflammatory cytokines (IL-6 and TNF-α) on the 1st day after ISO treatment were measured by ELISA ($n = 6$). **c** Representative images and quantification of picrosirius red-stained collagen in the heart on the 7th day after ISO treatment ($n = 6$; scale bars: 200 μm). **d** Representative images of pulsed wave Doppler (PWD) across the mitral flow and tissue Doppler (TD) of the mitral valve ring and *E/E'* values on the 7th day after ISO treatment. ($n = 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Gb Glibenclamide, ISO isoprenaline. One-way ANOVA with Tukey's *post hoc* test.

the activation of PKA were examined. The cAMP-Glo™ Max Assay revealed that cAMP concentrations were increased in NMCMs at 30 min after ISO treatment, but this effect was dose-dependently blocked by glibenclamide (1–100 μmol/L) (Fig. 5a). The phosphorylation of PKA substrates, which reflects PKA activation, was induced at 5 min and 30 min after ISO treatment (Fig. 5b). Glibenclamide pretreatment attenuated ISO-induced PKA activation (Fig. 5b). In vivo, glibenclamide pretreatment also inhibited ISO-induced cardiac accumulation of cAMP and activation of PKA (Fig. 5c,d). Intracellular cAMP is degraded by cPDEs. Glibenclamide pretreatment increased cPDE activity in cardiomyocytes and heart tissues stimulated with or without ISO (Fig. 5e,g).

DISCUSSION

In this study, we found that glibenclamide suppressed β-AR overactivation-induced cardiac inflammation, fibrosis and dysfunction by inhibiting NLRP3 inflammasome activation during sympathetic stress both in vivo and in vitro. Glibenclamide inhibited the cAMP/PKA signalling pathway, which is downstream of β-AR, and suppressed both potassium efflux and ROS production, which activated the NLRP3 inflammasome in the heart (Fig. 6).

Acute sympathetic stress-induced cardiac injury is accompanied by inflammatory responses, such as inflammatory cell infiltration and inflammatory cytokine/chemokine release [6]. Therapeutic modulation of inflammatory responses may hold promise for preventing sympathetic stress-induced cardiac injuries [16]. The anti-inflammatory effects of glibenclamide have been reported in previous studies [17]. For example, glibenclamide inhibits neutrophil and macrophage influx in bronchopulmonary dysplasia [18] and inhibits the expression of IL-1β in hearts in response to lipopolysaccharide challenge in streptozotocin-induced diabetic mice [19]. In the present study, we investigated the effect of glibenclamide on sympathetic stress-induced cardiac inflammation and demonstrated that glibenclamide pretreatment could inhibit ISO-induced cardiac inflammation, supporting its future application in the treatment of cardiac inflammatory injuries. Although sulfonylureas have been reported to be associated with an increased risk of adverse cardiovascular outcomes [20], some studies have reported that glibenclamide has a protective effect on patients with diabetes [21–23]. The inhibitory effect on the inflammasome revealed by the present study may contribute to the protective effect of glibenclamide on hearts.

Although persistent hyperglycaemia activates inflammatory responses [24], interestingly, in the present study, glibenclamide did not significantly change the glucose levels of ISO-treated mice, suggesting that the anti-inflammatory effects of glibenclamide are independent of its hypoglycaemic effect in the context of ISO-

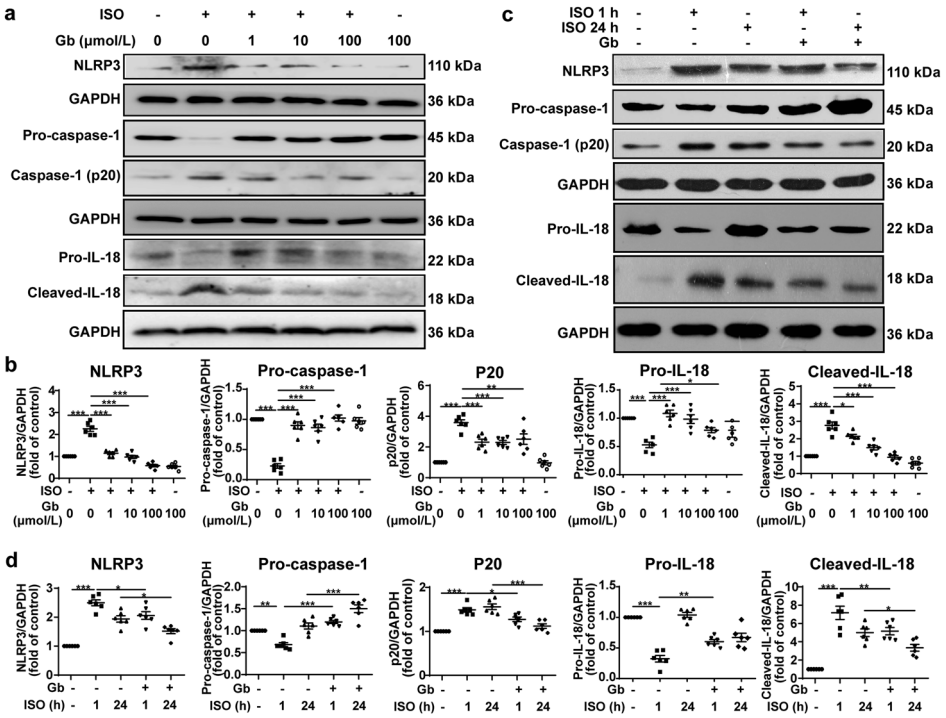

Fig. 2 Glibenclamide inhibited ISO-induced activation of the NLRP3 inflammasome in the mouse myocardium. a, **b** Protein levels of NLRP3, pro-caspase-1, caspase-1 (p20), pro-IL-18 and cleaved IL-18 in mouse cardiomyocytes at 1 h after ISO treatment with 1–100 μmol/L glibenclamide pretreatment for 30 min. **c**, **d** Protein levels of NLRP3, pro-caspase-1, caspase-1 (p20), pro-IL-18 and cleaved IL-18 in mouse hearts at 1 or 24 h after ISO treatment with or without glibenclamide (1 mg·kg^{-1}·day^{-1} body weight, sc) pretreatment. $n = 6$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Gb glibenclamide, ISO isoprenaline. Kruskal–Wallis ANOVA with Dunn's multiple comparison *post hoc* test

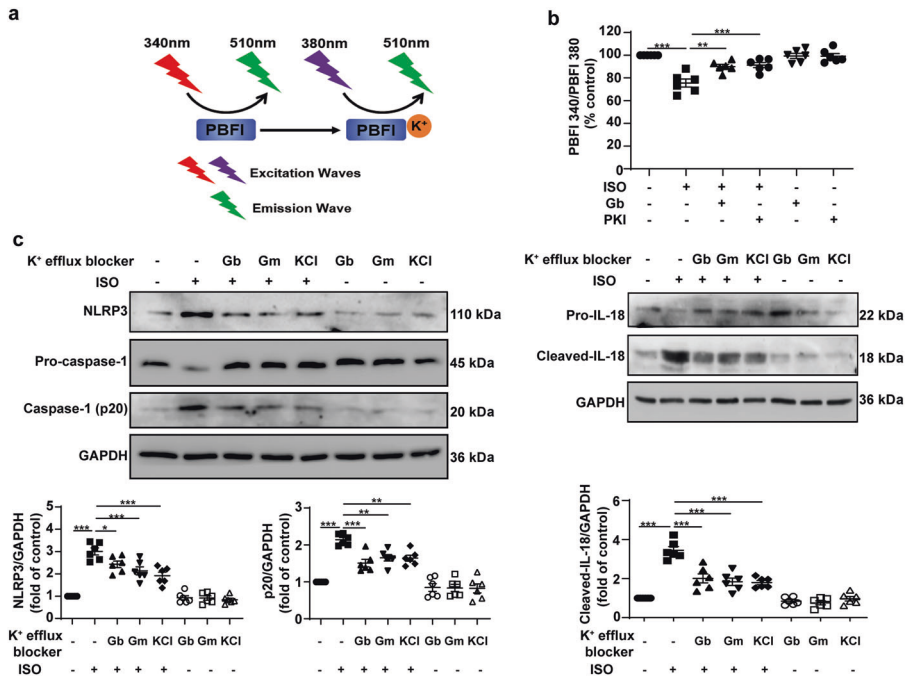

Fig. 3 Glibenclamide inhibited ISO-induced NLRP3 inflammasome activation in cardiomyocytes by inhibiting potassium efflux. a Schematic diagram of the K^+-sensitive fluorescent indicator PBFI-AM. **b** NMCMs were exposed to ISO (10 μmol/L) for 30 min with or without glibenclamide (100 μmol/L)/PKI (0.1 μmol/L) pretreatment for 30 min. Intracellular K^+ levels were estimated by loading the cells with PBFI-AM. **c** Protein levels of NLRP3, Caspase-1 (p20) and cleaved-IL-18 at 1 h after ISO (10 μmol/L) treatment in mouse cardiomyocytes that were pretreated with the K^+ efflux blocker glibenclamide (Gb, 100 μmol/L), glimepiride (Gm, 100 μmol/L) or a high concentration of KCl (130 mmol/L) for 30 min. ISO, isoprenaline. $n = 6$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Kruskal-Wallis ANOVA with Dunn's multiple comparison *post hoc* test

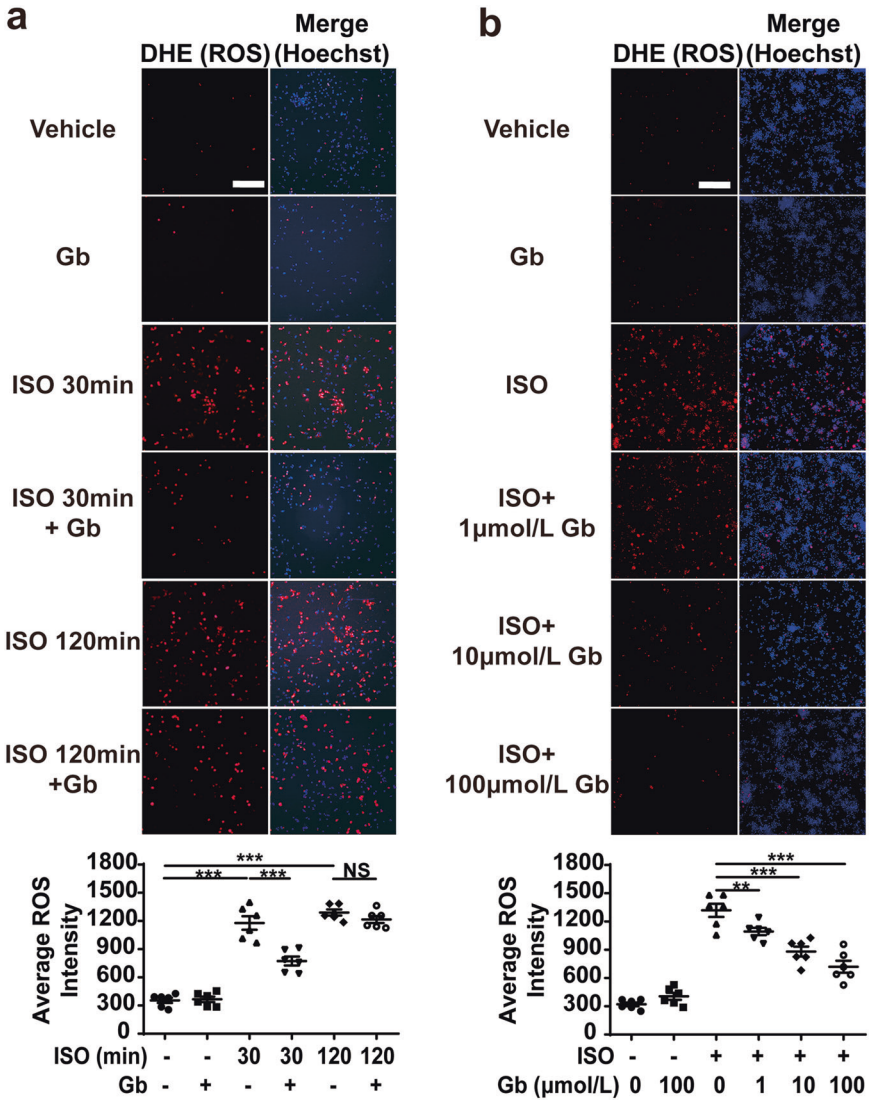

Fig. 4 Glibenclamide decreased ISO-induced ROS generation in cardiomyocytes. a Mouse cardiomyocytes were pretreated with glibenclamide (100 μmol/L) for 30 min and then treated with ISO for 30 or 120 min. The average ROS signal intensity was measured after DHE staining. **b** Mouse cardiomyocytes were treated with ISO for 30 min after 1–100 μmol/L glibenclamide pretreatment for 30 min. The average ROS signal intensity was measured after DHE staining. Gb, glibenclamide; ISO, isoprenaline. $n = 6$; scale bars: 100 μm; $^{**}P < 0.01$; $^{***}P < 0.001$. One-way ANOVA with Tukey's *post hoc* test

induced cardiac inflammation. Glibenclamide suppressed NLRP3 inflammasome activation in isolated cardiomyocytes, suggesting that its anti-inflammatory effects are mediated by direct targeting of the NLRP3 inflammasome.

The NLRP3 inflammasome is critical in ISO-induced cardiac inflammation, promoting the cleavage and activation of IL-18 and resulting in the initiation of cardiac inflammation, as revealed in our previous research [6]. In the present study, glibenclamide inhibited ISO-induced cardiac NLRP3 inflammasome activation both in vitro and in vivo and alleviated subsequent cardiac inflammation. This finding is consistent with the findings of previous studies on other organs, which suggested that glibenclamide inhibits NLRP3 inflammasome activation in the kidneys of adenine diet-induced rats [25] and the bladders of cyclophosphamide-treated rats [26]. These findings suggest that glibenclamide might be a promising cardioprotective drug that exerts its beneficial effects by blocking NLRP3 inflammasome activation.

Many stimuli have been shown to activate the NLRP3 inflammasome, including potassium efflux and ROS production [27]. In our previous research, ISO induced cardiac inflammasome activation by promoting ROS, and inflammasome activation could be inhibited by ROS scavenger pretreatment [6]. The present study further showed that ISO promoted potassium efflux in cardiomyocytes. This result is consistent with a previous study in which ISO was shown to promote potassium efflux in beating guinea pig atria [28]. In addition, the blockade of potassium efflux suppressed ISO-induced inflammasome activation in this study. Therefore, both ROS production and potassium efflux are involved in ISO-induced inflammasome activation in the heart during sympathetic stress.

Glibenclamide is a classic K_{ATP} channel blocker that inhibits potassium efflux, and the K_{ATP} channel is expressed in the heart [29]. In ISO-induced potassium efflux in the heart, one of the mechanisms may involve PKA-mediated phosphorylation of Ser1387 of the SUR2B subunit of the K_{ATP} channel and the subsequent promotion of channel activation [30]. We found that glibenclamide inhibited ISO-induced activation of the cAMP/PKA signalling pathway in vivo and in vitro. The inhibitory effect of glibenclamide on cAMP may occur by targeting cPDEs, as the activity of cPDEs can be promoted by glibenclamide in

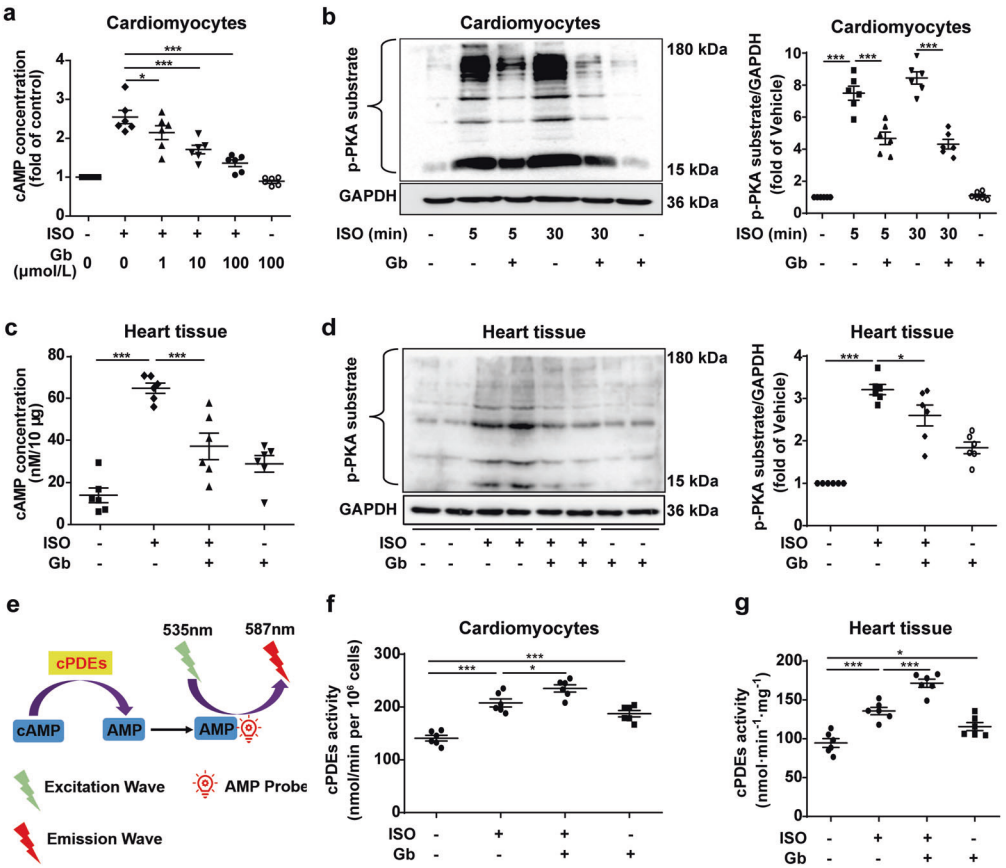

Fig. 5 Glibenclamide inhibited cAMP/PKA signalling in ISO-induced cardiomyocytes in vivo and in vitro. a Mouse cardiomyocytes were pretreated for 30 min with 1–100 μmol/L glibenclamide and then treated with ISO for 30 min. The cAMP levels in cardiomyocytes were measured with a cAMP ELISA kit. One-way ANOVA with Tukey's *post hoc* test. **b** Protein levels of p-PKA substrate in mouse cardiomyocytes that were pretreated with 100 μmol/L glibenclamide for 30 min and then treated with ISO for 5 or 30 min. A Kruskal-Wallis ANOVA combined with Dunn's multiple comparison *post hoc* test. **c** Glibenclamide (1 $mg \cdot kg^{-1} \cdot day^{-1}$ body weight, sc) was administered for 3 days before ISO (5 mg/kg body weight) treatment for 1 h. cAMP levels in mouse hearts were measured with a cAMP ELISA kit. One-way ANOVA with Tukey's *post hoc* test. **d** Protein levels of p-PKA substrate in mouse hearts. A Kruskal-Wallis ANOVA combined with Dunn's multiple comparison *post hoc* test. **e** Schematic diagram of the cPDE activity assay. **f** cPDE activity in mouse cardiomyocytes that were pretreated with 100 μmol/L glibenclamide for 30 min before ISO treatment for 30 min. One-way ANOVA with Tukey's *post hoc* test. **g** cPDE activity in the heart tissues of mice that were pretreated with glibenclamide (1 $mg \cdot kg^{-1} \cdot day^{-1}$ body weight, sc) for 3 days before ISO (5 mg/kg body weight) treatment for 1 h. One-way ANOVA with Tukey's *post hoc* test. Gb, glibenclamide; ISO, isoprenaline. cPDEs, cAMP phosphodiesterases. $n = 6$; $^{*}P < 0.05$; $^{***}P < 0.001$

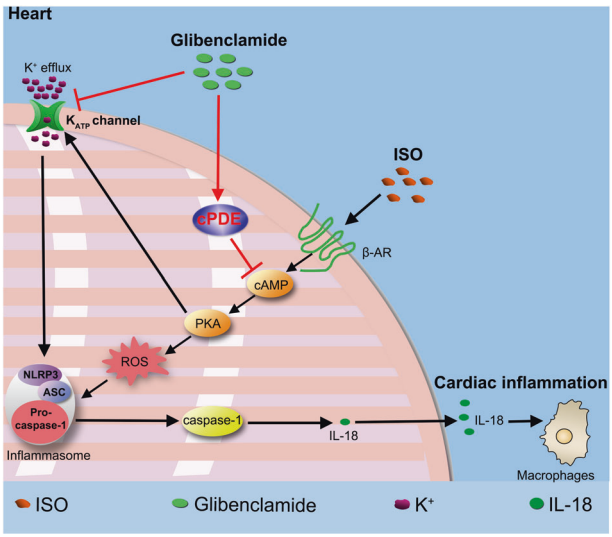

Fig. 6 The schematic diagram of this study. Working model showing how glibenclamide alleviates ISO-induced cardiac inflammation by inhibiting the NLRP3 inflammasome

cardiomyocytes and heart tissue. This finding is consistent with a previous study in which glibenclamide increased the activity of PDEs and promoted the degradation of cAMP in adipocytes [31]. Importantly, glibenclamide further increased cPDE activity in ISO-treated cardiomyocytes and heart tissues, suggesting that glibenclamide-mediated inhibition of the cAMP/PKA pathway is due to elevated cPDE activity. Thus, glibenclamide inhibits potassium efflux in hearts by not only blocking K_{ATP} channels but also inhibiting PKA-induced K_{ATP} channel activation through elevated cPDE activity. Upon acute β-AR stimulation in NMCMs, the classical cAMP/PKA pathway is also responsible for rapid ROS generation (15–60 min) but not slow ROS generation (1–2 h) [10]. Interestingly, glibenclamide inhibited ISO-induced ROS generation in NMCMs at 30 min but not 120 min in this study, suggesting that glibenclamide specifically inhibited PKA pathway-mediated ROS generation following ISO treatment. Therefore, these findings indicate that glibenclamide inhibits both potassium efflux and ROS generation in NMCMs through multiple targets, including the K_{ATP} channel and the cAMP/PKA signalling pathway.

In conclusion, glibenclamide attenuates ISO-induced NLRP3 inflammasome activation and cardiac inflammation in mouse hearts. The underlying mechanism involves the suppression of both potassium efflux and ROS production by inhibiting the cAMP/PKA signalling pathway. Our study reveals that glibenclamide exerts a

multitarget protective effect to inhibit cardiac inflammation induced by β-AR activation and has potential therapeutic significance for patients with cardiovascular risks under sympathetic stress.

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AUTHOR CONTRIBUTIONS

HX, YYZ and EDD conceived the project and designed the study. NC, JJW, JMW, WLX, RW, XDC, YNF and WWC performed the experiments. NC and JJW analysed the data. NC and JMW wrote the manuscript.

ADDITIONAL INFORMATION

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