Original Article
Cardioprotection of an I_{K1} channel agonist on L-thyroxine induced rat ventricular remodeling

Qing-Hua Liu[1*], Li-Jun Zhang[1*], Jin Wang[2], Bo-Wei Wu[2], Ji-Min Cao[2]

[1]Department of Pathophysiology, Shanxi Medical University, Taiyuan, China; [2]Key Laboratory of Cellular Physiology (Shanxi Medical University), Ministry of Education, The Department of Physiology, Shanxi Medical University, Taiyuan, China. [*]Equal contributors.

Abstract: Downregulation of inward rectifier potassium (I_{K1}) channel is a hallmark in cardiac hypertrophy and failure. The cardioprotection of zacopride (a selective I_{K1} agonist) and underlying mechanisms were investigated in L-thyroxine (T4) or Triiodothyronine (T3)-induced cardiac remodeling. In the *in vivo* study, adult male Sprague-Dawley (SD) rats were randomly divided into control, L-thyroxine, L-thy+zacopride, and L-thy+zacopride+chloroquine (an I_{K1} antagonist) groups. Echocardiography, histopathology, TUNEL assay, western blotting and confocal imaging for intracellular Ca^{2+} fluorescence were performed. In the *in vitro* study, zacopride and nifedipine (a LTCC blocker) were used to compare their effects on Kir2.1, SAP97, autophagy, and $[Ca^{2+}]_i$ in H9C2 (2-1) cardiomyocytes. Zacopride treatment attenuated L-thyroxine- or T3 induced cardiac remodeling and dysfunction which manifested as cardiac hypertrophy and collagen deposition, dilated ventricle, decreased ejection fraction (EF), increased cardiomyocytes apoptosis, hyper-activation of CaMKII and PI3K/Akt/mTOR signaling, decreased cardiac autophagy, and increased expression of integrin β3. The cardioprotection of zacopride is strongly associated with the upregulation of I_{K1}, SAP97, and $[Ca^{2+}]_i$ homeostasis in cardiomyocytes. I_{K1} antagonist chloroquine or $BaCl_2$ reversed these effects. Nifedipine could attenuate intracellular Ca^{2+} overload with no significant effects on I_{K1}, SAP97, and autophagy. This study showed that zacopride could improve cardiac remodeling via facilitating Kir2.1 forward trafficking, and negatively regulating calcium-activated and PI3K/Akt/mTOR signalings, in an I_{K1}-dependent manner.

Keywords: Inward rectifier potassium channel, L-thyroxine, cardiac remodeling, calcium overload, PI3K, autophagy

Introduction

Hyperthyroidism is a form of thyrotoxicosis that is caused by an excessive generation of thyroid hormones (TH). Long-term TH overload induces the overexpression of TH-responsive cardiac genes and resultant cardiac hypertrophy [1-3]. Some hyperthyroid patients develop congestive heart failure (HF) [4], dilated cardiomyopathy, pulmonary hypertension or right HF [5]. Thyroid dysfunctions concomitant with symptomatic HF and an ejection fraction (EF) < 35% are high risk factors for sudden death [6]. In parallel with the treatment of primary hyperthyroidism, the preventions of ventricular remodeling and HF are important issues to improve the outcomes and all-cause mortality.

Cardiac I_{K1} channels comprise Kir2.x isoforms and are expressed in all ventricular and atrial myocytes. These channels are critical in maintaining the resting potential (RP), establishing the excitation threshold, and modulating the final repolarization phase of the action potential (AP) [7, 8]. The downregulation of I_{K1} is a hallmark of cardiac remodeling in hypertrophy and HF [9]. Activation of the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway is a common mechanism of cardiomyocyte growth in TH-induced hypertrophy [10, 11]. A series of studies from our laboratory showed that enhancing I_{K1} attenuated isoproterenol- and myocardial infarction (MI)-induced ventricular remodeling depending on the strength of compensation for I_{K1} deficit [12, 13]. Facilitation of intracellular calcium homeostasis and inhibition of the mTOR/70-kDa ribosomal protein S6 kinase (p70S6K) signaling pathway were involved in the cardioprotective effects of I_{K1} activation. Therefore, the present study aimed to confirm the effects of the I_{K1} agonism on cal-

cium dyshomeostasis and PI3K/Akt/mTOR signaling in thyrotoxicosis-induced cardiac remodeling.

Synapse-associated protein-97 (SAP97) is a membrane-associated guanylate kinase (MAGUK) scaffolding protein and ubiquitously expressed in the heart [14]. Emerging evidence unraveled that the protein SAP97 contributes to the function, localization, and forward (towards the plasma membrane) trafficking behavior of Kir2.1 channel [15-17]. Data from Vaidyanathan et al. showed that SAP97 silencing downregulated the surface expression of channels underlying I_{K1}, probably by decreasing the number of functional I_{K1} channels in the membrane [18]. In the present study, attempt was made to clarify whether SAP97 is associated with the surface expression of Kir2.1 channel in cardiac remodeling.

Four pharmacological tools were used to identify the I_{K1} channel as a target for cardiac remodeling: zacopride, a moderate I_{K1} channel agonist/opener [19]; chloroquine and $BaCl_2$, nonspecific I_{K1} channel blockers [20]; and L-thyroxine, a synthetic form of thyroxine (T4).

Materials and methods

Animals

Male Sprague-Dawley (SD) rats weighing 220-250 g were provided by the Laboratory Animal Center of Shanxi Medical University, China. The investigation conformed to the guidelines for the Care and Use of Laboratory Animals (NIH, revised 2011) and the Bioethical Committee of Shanxi Medical University. The Bioethical Committee of Shanxi Medical University approved the study protocols (No. SYDL2021004). Adult rats were housed under standard conditions at room temperature 20-24°C, humidity 40-60%, and 12: 12-h light-dark (LD) cycles with a light intensity up to 200 lux. Rats received standard chow and water *ad libitum*. Rats were randomly assigned into six groups. Cardiac remodeling was induced via daily intragastric administrations of 1 mg/kg/d L-T4 (Euthyrox, 50 mg per tablet, Merck, German) for 10 d. Treatment groups were injected with 15 µg/kg/d zacopride (Zac, Tocris, England) into the peritoneal cavity (i.p.). Chloroquine (CQ, Sigma, USA) was administered i.p. at 7.5 µg/kg/d in combination with L-thyroxine and zacopride. Control rats were injected with normal saline. The doses of zacopride or chloroquine were based on our previous studies [12, 13] and a pilot study of the optimal dose. We previously showed that zacopride (15 µg/kg/d) and chloroquine (7.5 µg/kg/d) had no significant effects on cardiac structure or function [12]. Therefore, the present study did not include a zacopride (15 µg/kg/d) or chloroquine alone group.

Echocardiographic examination

The GE Vivid 7 Pro ultrasound system (10 S probe, probe frequency of 8.0 MHz, equipped with 2D strain imaging software and Echo PAC workstation) was used in M-mode for rodent hearts. The following parameters were used: approximate exploration angle of 15-30°, depth at 2-3 cm, frame rate > 250/s, and maximum frame rate up to 400/s. The positioning criterion was the left long axis section. Left ventricle (LV) dimensions at end diastole (LVIDd) and end systole (LVIDs), interventricular septum thickness at end diastole (IVSd) and end systole (IVSs), LV posterior wall thickness at end diastole (LVPWd) and end systole (LVPWs), LV ejection fraction (EF) and LV short axis fractional shortening (FS) were monitored and compared.

Histopathological examination

Formalin-fixed hearts were subjected to routine histopathological processing. Transverse or longitudinal ventricular sections (~5 µm thick) were cut using a microtome. Hematoxylin and eosin (H&E) staining was performed to evaluate necrosis and myofiber size. Fibrosis was observed using Masson's trichrome staining, and the collagen deposition in the extracellular matrix was estimated from image analyses in each group. Total collagen area was quantified as percent of the total ventricular area [21].

TUNEL staining for apoptosis examination

The effect of zacopride treatment on apoptosis was further evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay following the manufacturer's protocol (Roche, Germany). Ventricular tissue was fixed in formalin, dried in ethanol, cut into 5 µm-thick slices, and prepared for TUNEL assay. TUNEL-positive cells were identified by brown staining of nuclei which were counted

and compared across treatment groups. Six microscopic field-of-views from 3 hearts were manually quantified and used for statistical comparisons.

Triiodothyronine (T3) infusion in H9C2 (2-1) cardiomyocytes

The thyroid hormones (TH) are mainly composed of two forms, thyroxine (T4) and triiodothyronine (T3). At target tissues, deiodinases convert T4 to T3. T3 has higher activity and affinity for TH receptors. So, in the present study, T3 (Sigma, USA) was used to establish *in vitro* remodeling in H9C2 (2-1) cells. H9C2 (2-1) cardiomyocytes were derived from rat embryonic heart and purchased from National Collection of Authenticated Cell Cultures (Shanghai, China). In the *in vivo* study, chloroquine was used as an I_{K1} blocker. But it is an inhibitor of autophagy too [22], which challenges its certification on autophagy in view of blocking I_{K1}. Therefore, we also applied $BaCl_2$, another I_{K1} blocker to clarify the effect of I_{K1} regulation on cardiomyocyte autophagy. The cells were randomly divided into 11 groups: normal control, T3 infusion (100 nmol/L), T3+Zac (1 µmol/L), T3+Zac+$BaCl_2$ (1 µmol/L), T3+Zac+CQ (0.3 µmol/L), T3+nifedipine (Nife, 10 µmol/L), T3+Nife+Zac, Zac, $BaCl_2$, CQ and Nife. After 48 h of incubation, cells were collected for Western blotting. Besides, cells were seeded in a 24-well plate and treated with above mentioned agents for 48 h. The resting $[Ca^{2+}]_i$ of cardiomyocytes was detected by laser confocal microscopy.

Western blotting

Proteins from samples of LV tissue or H9C2 (2-1) cells were loaded (40 µg per lane) on 5-15% acrylamide gels. After electrophoretic transfer, the nitrocellulose membranes were incubated overnight at 4°C with primary antibodies against Kir2.1 (rabbit monoclonal, Abcam), SAP97 (DLG1, rabbit polyclonal, Abclonal), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII, mouse monoclonal, Abcam), phosphorylated CaMKII (rabbit polyclonal, Abcam), PI3K (p85) and phosphorylated PI3K (p-p85) (rabbit monoclonal, Cell signaling Technology), Akt and phosphorylated Akt (rabbit polyclonal, Abcam), mTOR and phosphorylated mTOR (rabbit monoclonal, Cell Signaling Technology), LC3B (rabbit monoclonal, Abcam),

P62 (rabbit monoclonal, Cell Signaling Technology), integrin β3 (rabbit monoclonal, Abcam), total caspase 3 and cleaved caspase-3 (rabbit monoclonal, Cell Signaling Technology). GAPDH (rabbit monoclonal, Cell Signaling Technology) was used as the loading control in each case. The secondary antibody is goat anti-rabbit or goat anti-mouse IgG (Abclonal). Quantification was performed using ImageJ or Image Lab.

Confocal imaging

The fluorescent Ca^{2+} indicator Fluo-4 AM (Dojindo Laboratories, Japan) was used for the measurement of intracellular Ca^{2+} concentrations in native cardiomyocytes or cultured H9C2 (2-1) cells. Native ventricular myocytes isolated from different groups were subjected to gradient recalcification using a modified Tyrode's solution (containing 0.5% BSA and 1 mM $CaCl_2$). The myocytes were incubated with 5 µM Fluo-4 AM in the dark at 37°C for 0.5 h. Unincorporated Fluo-4 AM was removed by washing the myocytes twice in PBS. The surface area and the $[Ca^{2+}]_i$ of cardiomyocytes were recorded using an FV1000 laser scanning confocal microscope (Olympus).

Statistical analysis

Quantitative data were presented as the means ± SEM and analyzed using the least significant difference (LSD) and Games-Howell tests of ANOVA (analysis of variance). Statistical differences were considered significant when the *P* value was less than 0.05.

Results

The effect of zacopride on cardiac hypertrophy indexes

Cardiac hypertrophy was assessed via the measurement of whole cardiac hypertrophic index (heart weight to body weight ratio) and the left ventricular hypertrophic index (LV weight to body weight ratio), which were calculated in mg/g. The hypertrophy indexes in L-thy infusion rats were significantly increased compared to the controls (*P* < 0.01), which indicates that hypertrophy was successfully established. Zacopride (15 µg/kg/d) prevented myocardial hypertrophy in a dose-dependent manner. Low-dose chloroquine (7.5 µg/kg/d) largely antago-

Table 1. Effects of zacopride on L-thy-induced ventricular remodeling in rats (mean ± SEM)

	n	BW (g)	HW/BW (mg/g)	LVW/BW (mg/g)
Control	8	240.5 ± 7.2	2.70 ± 0.05	1.49 ± 0.11
L-thy	8	243.2 ± 5.0	4.77 ± 0.11[**]	3.26 ± 0.14[**]
L-thy+Zac 15 μg/kg	8	249.0 ± 3.1	3.93 ± 0.09[**,##]	2.68 ± 0.08[**,##]
L-thy+Zac 15 μg/kg+CQ	8	231.5 ± 10.5	4.72 ± 0.12[**,ΔΔ]	3.10 ± 0.10[**,Δ]

L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. [**]$P < 0.01$ vs. control. [##]$P < 0.01$ vs. L-thy. [Δ]$P < 0.05$, [ΔΔ]$P < 0.01$ vs. L-thy+Zac 15 μg/kg.

Table 2. Echocardiographic parameters in hypertrophic rats (mean ± SEM)

	Control	L-thy	L-thy+Zac	L-thy+Zac+CQ
LVIDd (mm/m^2)	4.27 ± 0.22	6.57 ± 0.56[**,#]	4.77 ± 0.31	5.93 ± 0.60[*]
LVIDs (mm/m^2)	2.61 ± 0.10	4.76 ± 0.55[**,##]	2.94 ± 0.22	4.35 ± 0.42[**,#]
IVSd (mm/m^2)	1.60 ± 0.06	1.69 ± 0.13	1.56 ± 0.10	1.77 ± 0.12
IVSs (mm/m^2)	2.09 ± 0.11	2.46 ± 0.25	2.13 ± 0.12	2.14 ± 0.11
LVPWd (mm/m^2)	1.81 ± 0.22	1.75 ± 0.31	1.95 ± 0.13	1.67 ± 0.09
LVPWs (mm/m^2)	2.32 ± 0.31	2.29 ± 0.33	2.45 ± 0.13	2.38 ± 0.05
EF (%)	75.6 ± 1.4	59.0 ± 3.5[**,##]	75.2 ± 1.9	61.4 ± 2.3[**,##]
FS (%)	38.8 ± 1.3	27.4 ± 1.9[**,##]	38.2 ± 1.6	28.8 ± 1.4[**,##]

L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. The dose of zacopride is 15 μg/kg. LVIDd, left ventricular dimension in end diastole; LVIDs, left ventricular dimension in end systole; IVSd, interventricular septum end-diastolic thickness. IVSs, interventricular septum end-systolic thickness; EF, ejection fraction; FS, fractional shortening. N = 5 in each group. [*]$P < 0.05$, [**]$P < 0.01$ vs. control; [#]$P < 0.05$, [##]$P < 0.01$ vs. L-thy+Zac.

nized the effects of zacopride treatment ($P < 0.01$ or $P < 0.05$). **Table 1** lists additional details.

Echocardiographic data

The echocardiographic parameters are represented in **Table 2** and **Figure 1A**. Compared to normal control, hearts in L-thyroxine infusion groups showed increased left ventricular diastolic and systolic dimensions ($P < 0.01$) and reduced EF and FS ($P < 0.01$), which indicated cardiac dilation and dysfunction. LVID values were decreased ($P < 0.05$ or $P < 0.01$) in the zacopride (15 μg/kg/d) treatment group, which is consistent with the improved EF and FS ($P < 0.01$). Chloroquine (7.5 μg/kg/d) reversed the anti-remodeling effects of zacopride ($P < 0.05$ or $P < 0.01$).

Histopathological observations

HE staining showed uniform and neatly arranged myofibers in the control group, with little inflammatory cell infiltration (**Figure 1B**). Myofibers in the L-thyroxine group were misarranged with wider gaps, hemorrhage and neutrophil granulocyte infiltration. Consistent with the changes in hypertrophy index, the cross-sectional area (CSA) of myocardial fibers in the L-thyroxine group (717.0 ± 34.0 μm^2) was approximately 4 times larger than the control group (179.2 ± 8.0 μm^2) (**Figure 1C**, $P < 0.01$). Zacopride treatment significantly decreased the CSA (187.1 ± 10.0 μm^2) ($P < 0.01$) and attenuated the tissue injury. Co-administration of chloroquine (669.7 ± 28.6 μm^2) significantly inhibited these effects ($P < 0.01$). Compared to control hearts, thyrotoxic hearts also exhibited significant fibrosis with increased collagen deposition (**Figure 1D** and **1E**, $P < 0.01$). Zacopride treatment dramatically alleviated cardiac fibrosis ($P < 0.01$), and chloroquine co-administration blocked these effects ($P < 0.01$).

TUNEL assay

Under the light microscope, TUNEL-positive cells were identified by brown staining of nuclei and intact membrane. The nuclei of the negative cells were stained in blue. TUNEL assay illustrated increased apoptotic cell population after L-thyroxine infusion (**Figure 2**, $P < 0.01$, compared with control). Zacopride treatment significantly reduced cell apoptosis, and the effect was reversed by chloroquine ($P < 0.01$).
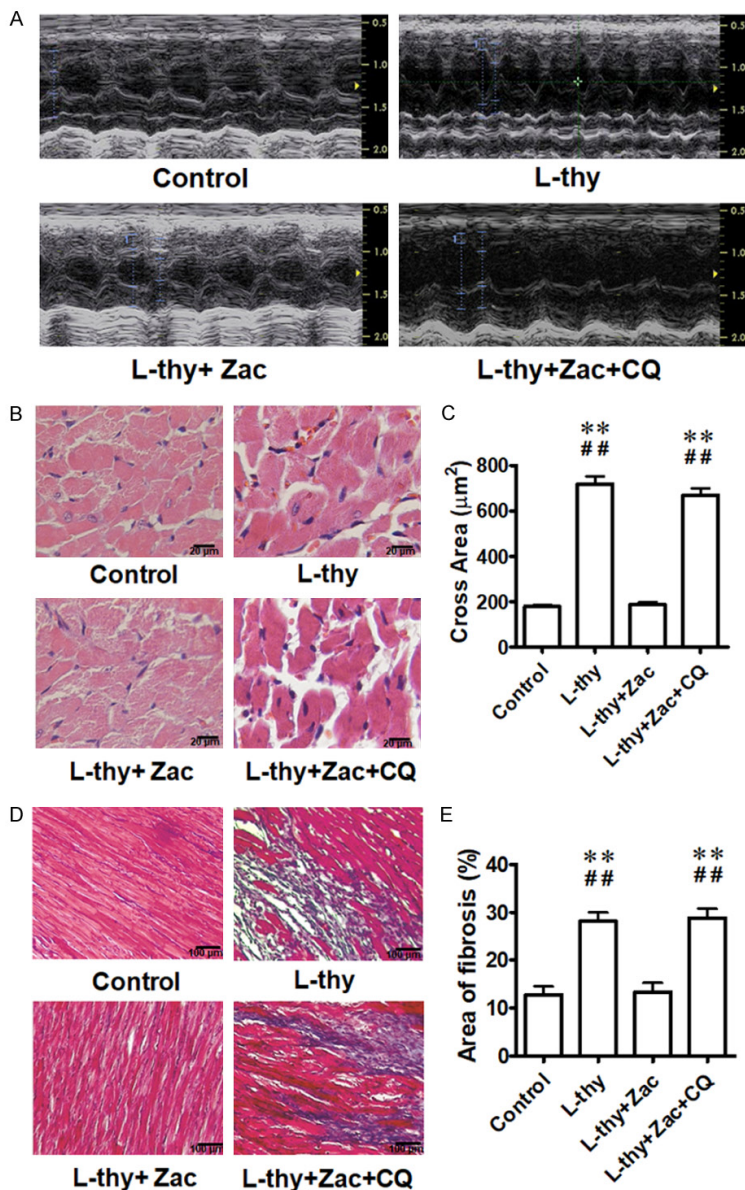
Figure 1. The effect of zacopride on L-thy-induced cardiac hypertrophy and dysfunction. A. The representative echocardiographic images from L-thyroxine infusion rats. B. Representative images for HE staining showing L-thyroxine induced cardiac hypertrophy and injury. Magnification: 400×. Bars represent 20 µm. In the control group, the myofibers were uniform and arranged neatly, little inflammatory cell infiltration was present. In the hyperthyroid group, myofibers were hypertrophic, misarranged with the wider gaps, hemorrhage, and neutrophil granulocyte infiltration. Zacopride treatment significantly decreased the cell size and reduced the tissue injury. These effects were reversed by co-application of chloroquine. C. Myocyte cross section areas (CSAs) in each group were measured morphometrically at high-powered field (n = 100 fibers in each group). D. Representative images for Masson's trichrome staining showing L-thyroxine induced LV fibrosis. Cardiomyocytes and collagen fibers were stained as red and blue, respectively. Magnification: 100×. Bars represent 100 µm. E. Fibrotic area was calculated as percentage of total area in each field (n = 5 images in each group). Values are presented as the mean ± SEM. L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. **$P < 0.01$ vs. control, ##$P < 0.01$ vs. L-thy+Zac.

Specific cardiac protein expression in rats' heart after L-thyroxine infusion

Native I_{K1} channels in the ventricles predominantly comprise Kir2.1 (KCNJ2) subunits. Zacopride is a selective Kir2.1 channel agonist [23]. In L-thyroxine-infused hearts, Kir2.1 was downregulated, in line with the downregulation of SAP97 (**Figure 3A** and **3B**, $P < 0.01$). Calcium-activated CaMKII (**Figure 3C**, $P < 0.01$) and caspase-3 (**Figure 3D**, $P < 0.05$) were upregulated. Zacopride administration restored the expression of Kir2.1 ($P < 0.01$) and SAP97, and reduced the activity of CaMKII ($P < 0.01$) and caspase-3 ($P < 0.05$). Chloroquine eliminated these effects ($P < 0.01$ or $P < 0.05$). The phosphorylation levels of PI3K (**Figure 4A** and **4B**), Akt (**Figure 4A** and **4C**), and mTOR (**Figure 4A** and **4D**) indicated hyperactivation in thyrotoxic rat hearts compared to normal controls ($P < 0.01$ or $P < 0.05$). The decreased LC3BII/I ratio and increased P62 expression indicated the inhibition of cell autophagy ($P < 0.01$). Zacopride treatment attenuated the hyperphosphorylation of PI3K/Akt/mTOR and promoted autophagy, which was shown by the increased LC3BII/I ration and decreased expression of P62 (**Figure 4G** and **4H**, $P < 0.01$ or $P < 0.05$). The I_{K1} antagonist chloroquine reversed these effects. Notably, total PI3K and Akt proteins were downregulated in thyrotoxic ventricles (**Figure 4E** and **4F**, $P < 0.01$ and $P < 0.05$, respectively), which contrasted the hyperphosphorylation of PI3K and Akt. Zacopride treatment preserved the expression of total PI3K and Akt ($P < 0.01$ and $P < 0.05$,
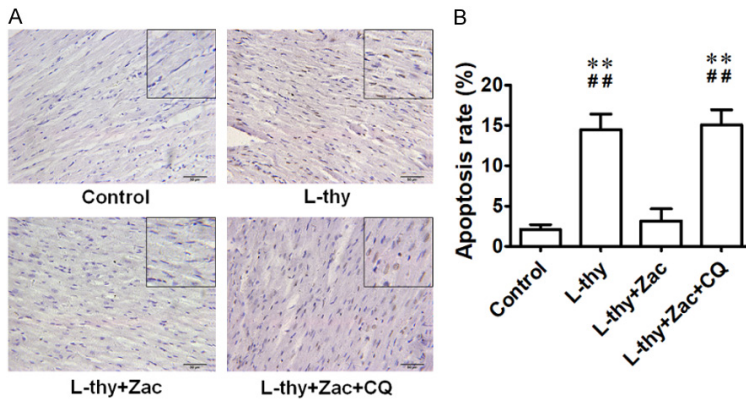
Figure 2. The effect of zacopride on L-thy-induced cardiac apoptosis. A. Representative TUNEL assay images of rat left ventricle post L-thy infusion. Magnification: 400×. Bars represent 50 μm. B. Quantitative analyses of TUNEL assay. Values are presented as the mean ± SEM (n = 6 microscopic field-of-views from 3 hearts). L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. **$P < 0.01$ vs. control, ##$P < 0.01$ vs. L-thy+Zac.
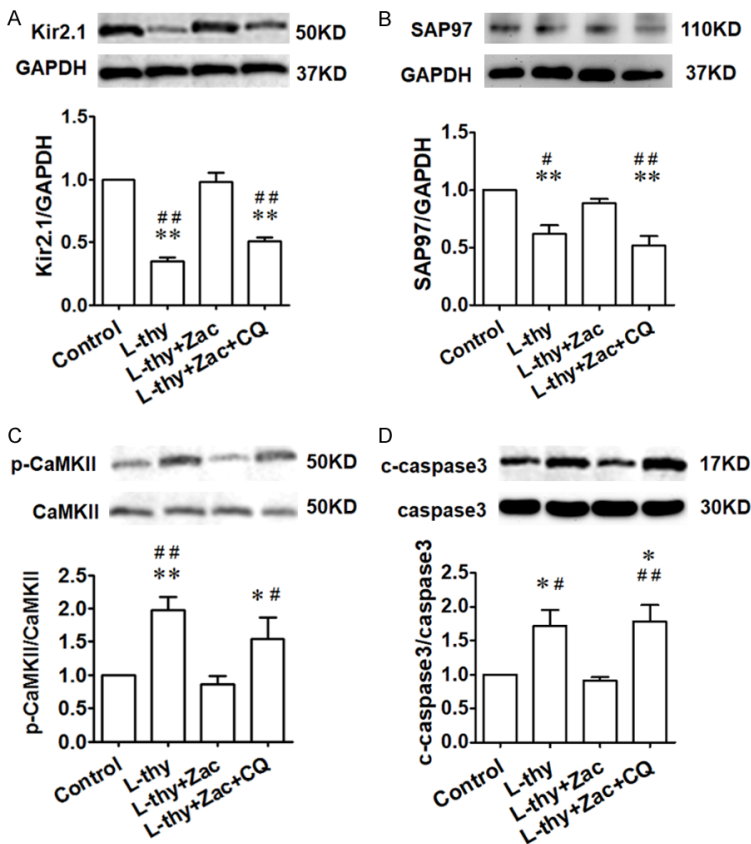
respectively), and chloroquine partially reversed these effects. These results suggest that long-term thyroxine infusion might exhaust the reserves of pro-survival molecules PI3K and Akt. Enhancing I_{K1} preserved total PI3K/Akt and promoted autophagy via inhibition of the PI3K/Akt/mTOR signaling pathway, which exerted a negative regulatory role in cardiac growth and hypertrophy.

Figure 5 shows that the fibrosis indicator, integrin β3, was up-regulated in L-thyroxine-infused hearts ($P < 0.01$). Zacopride decreased the expression of integrin β3 ($P < 0.01$), and chloroquine reversed this effect ($P < 0.01$).

Zacopride attenuated intracellular Ca^{2+} overload in rat cardiomyocytes

Figure 6 shows that the L-thyroxine-induced cardiac hypertrophy was characterized by increased $[Ca^{2+}]_i$ compared to the controls ($P < 0.01$). Zacopride significantly attenuated calcium overload ($P < 0.01$), and chloroquine largely reversed this effect ($P < 0.01$).

Zacopride attenuated T3-induced remodeling in H9C2 (2-1) cardiomyocytes

In H9C2 (2-1) cardiomyocytes, T3 significantly downregulated the expression of SAP97 and Kir2.1 (**Figure 7A**, $P < 0.01$). Zacopride treatment enhanced the expression of SAP97 and Kir2.1 ($P < 0.01$ or $P < 0.05$), and the effects were largely reversed by $BaCl_2$ or chloroquine ($P < 0.01$). Nifedipine had no significant effects on SAP97 or Kir2.1. $BaCl_2$ (1 μmol/L) could downregulate the expression of SAP97 and

Figure 3. Effects of zacopride on the expression of Kir2.1, SAP97, (p-) CaMKII and (cleaved) caspase-3 in rat left ventricles post L-thy infusion. Zacopride restored the expression of Kir2.1 (A) and SAP97 (B), and reduced the activities of CaMKII (C) and caspase 3 (D). These effects could be eliminated by chloroquine. All data were normalized to control. Values are presented as the mean ± SEM (n = 3 hearts). L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. L-thy+Zac.
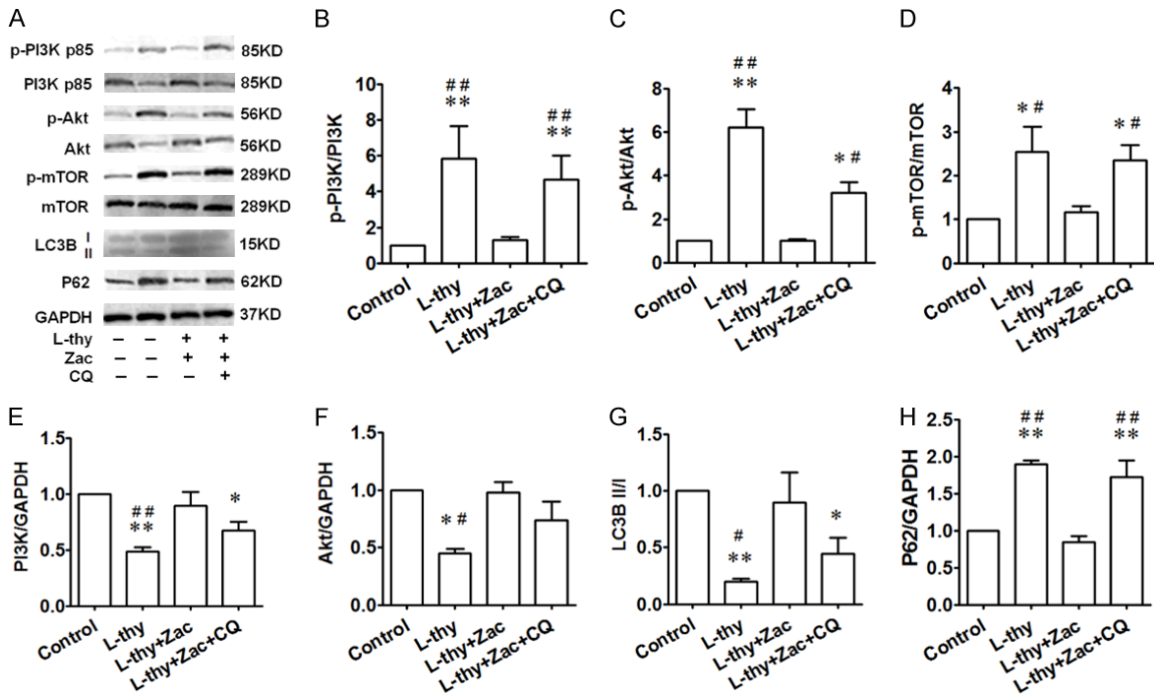
Figure 4. Zacopride promoted autophagy via negative regulation of PI3K/Akt/m-TOR signaling pathway. (A) Representative western blot bands of PI3K, Akt, m-TOR, LC3B and P62. GAPDH was used as a protein loading control. Zacopride treatment decreased the activities of PI3K (B), Akt (C) and mTOR (D), preserved the total PI3K (E) and Akt (F), promoted autophagy illustrated by inclined LC3B II/I ratio (G), and declined P62 (H). These effects were eliminated by chloroquine. All data were normalized to control. Values are presented as the mean ± SEM (n = 3 hearts). L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. L-thy+Zac.
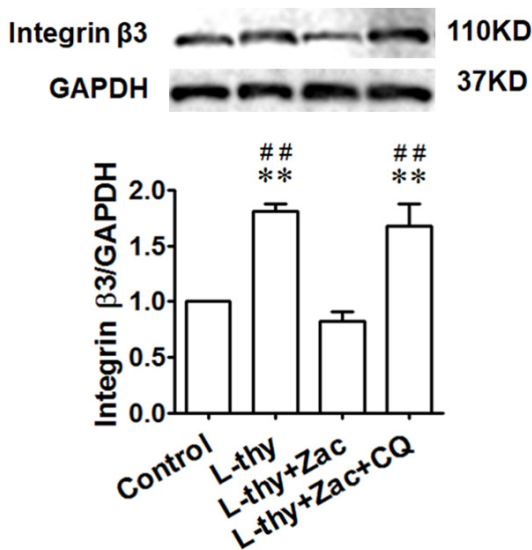

Figure 5. Zacopride decreased the expression of cardiac integrin β3 in thyrotoxic rat hearts. Values are presented as the mean ± SEM (n = 3 hearts). L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. **$P < 0.01$ vs. control, ##$P < 0.01$ vs. L-thy+Zac.

Kir2.1 in normal cardiomyocytes. Chloroquine (0.3 μmol/L) downregulated the expression of SAP97 ($P < 0.01$).

As shown in **Figure 7B**, T3 infusion induced the decrease of autophagy, as illustrated by decreased LCBII/I ratio and increased P62 expression ($P < 0.01$). Zacopride treatment promoted cell autophagy, and the effects could be largely reversed by BaCl$_2$ or chloroquine. Nifedipine had no significant effects on autophagy no matter in normal or T3-treated cardiomyocytes. In the present study, the dosage of chloroquine was 0.3 μmol/L, which was roughly equivalent to that in plasma after injection of chloroquine at 7.5 μg/kg/d for 10 d in *in vivo* study. At 0.3 μmol/L, chloroquine per se had no significant effects on autophagy. All these data convinced that zacopride improved cell autophagy via enhancing I_{K1}.

The confocal imaging data (**Figure 8A** and **8B**) show that zacopride alleviated T3-induced
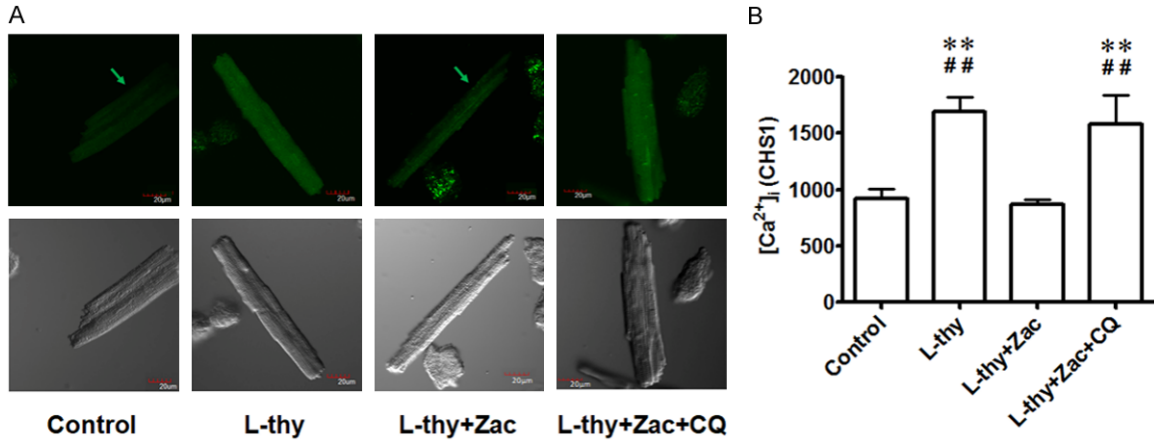
Figure 6. Effects of zacopride on intracellular calcium in hypertrophic rat cardiomyocytes. The isolated rat ventricular myocytes from diverse groups underwent confocal scanning by labeling with the fluorescent Ca^{2+} indicator Fluo-4 AM. A. Representative confocal images. Up panel, dark-field images. Low panel, bright-field images. Magnification (oil lens): 600×. Bars represent 20 μm. B. Zacopride treatment prevented L-thyroxine-induced intracellular Ca^{2+} accumulation, and the effect was eliminated by chloroquine. L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. Values are presented as the mean ± SEM (n = 6 cells from 2 rats in each group). **$P < 0.01$ vs. control, ##$P < 0.01$ vs. L-thy+Zac.
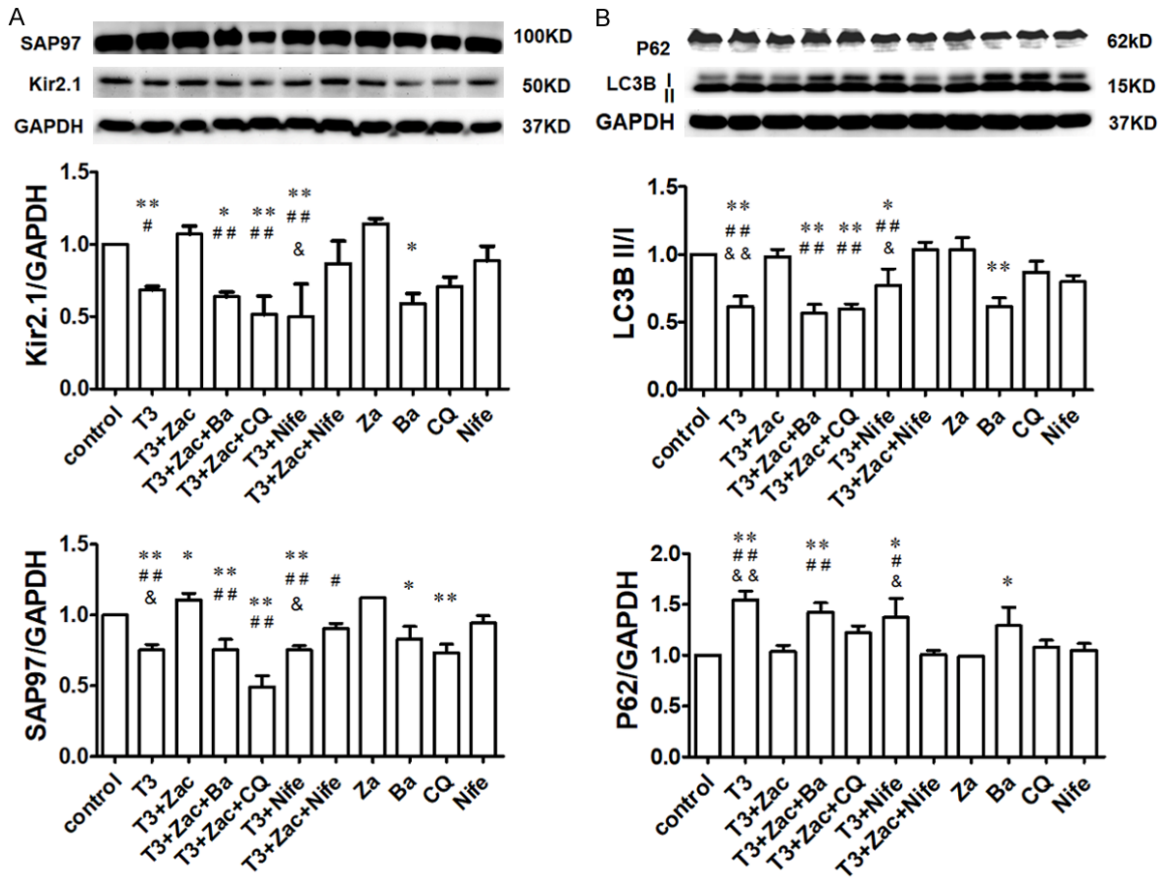

Figure 7. Effects of zacopride on Kir2.1, SAP97 and autophagy in H9C2 (2-1) cells post T3-infusion. A. Effects of Zacopride on Kir2.1 and SAP97. B. Effects of Zacopride on LC3B II/I ratio and P62. T3, Triiodothyronine. Zac, zacopride. CQ, chloroquine. Ba, BaCl$_2$. Nife, nifedipine. Values are presented as the mean ± SEM (n = 3). *$P < 0.05$, **$P < 0.01$ vs. control; #$P < 0.05$, ##$P < 0.01$ vs. T3+Zac; &$P < 0.05$ vs. T3+Zac+Nife.
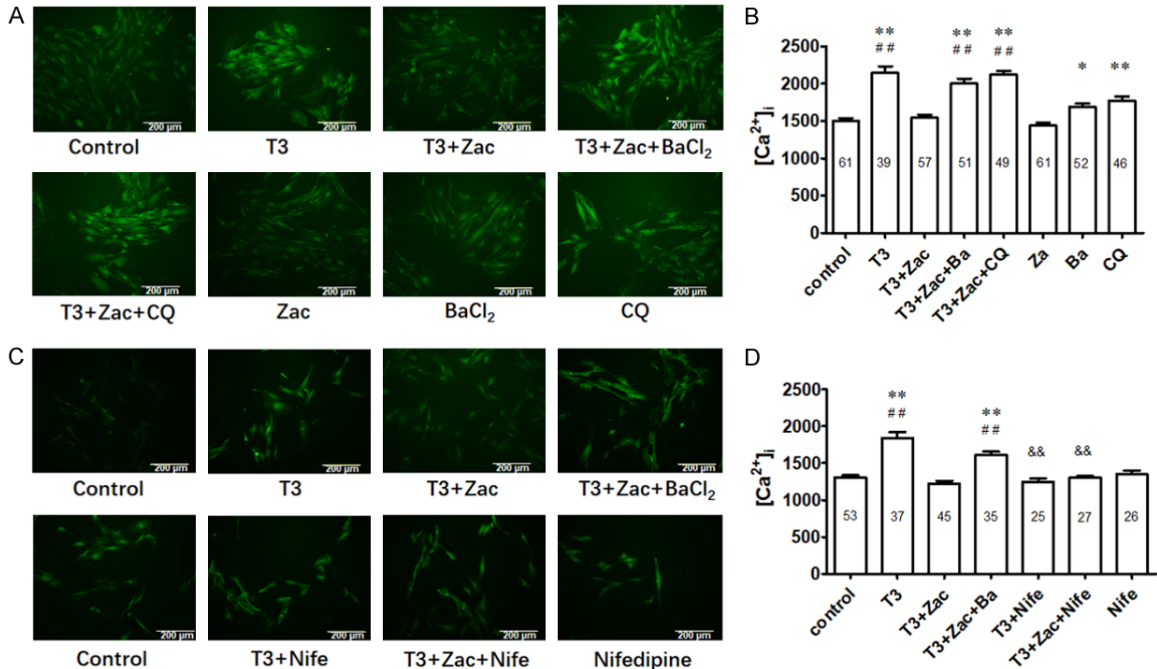
Figure 8. Effects of zacopride and nifedipine on T3-induced $[Ca^{2+}]_i$ overload in H9C2 (2-1) cells. Magnification: 400×. Bars represent 200 μm. T3, Triiodothyronine. Zac, zacopride. CQ, chloroquine. Ba, $BaCl_2$. Nife, nifedipine. Values are presented as the mean ± SEM. N, numbers embedded in the columns. *P < 0.05, **P < 0.01 vs. control; ##P < 0.01 vs. T3+Zac; &&P < 0.01 vs. T3.

$[Ca^{2+}]_i$ overload in H9C2 (2-1) cells (P < 0.01), and the effect could be reversed either by $BaCl_2$ or chloroquine (P < 0.01). As a blocker of voltage-dependent calcium channel (LTCC, I_{Ca-L}), nifedipine also significantly suppressed T3-induced calcium overload (**Figure 8C** and **8D**, P < 0.01). But the combination of nifedipine and zacopride did not exhibit better effect than application of two agents alone. Zacopride or nifedipine had no significant effect on $[Ca^{2+}]_i$ in normal cardiomyocytes. $BaCl_2$ and chloroquine alone slightly increased intracellular $[Ca^{2+}]_i$ in normal cardiomyocytes.

Discussion

The main findings of the present study are: 1) scaffolding protein SAP97 might mediate the effect of zacopride on I_{K1} by facilitating the forward trafficking of Kir2.1; 2) enhancing I_{K1}/Kir2.1 improves LV remodeling via negative regulation of PI3K/Akt/m-TOR signaling; 3) enhancing I_{K1} improves intracellular calcium homeostasis and autophagy in a LTCC-independent manner.

Enhancing I_{K1} facilitates intracellular calcium homeostasis and attenuates cell apoptosis

Levo (L-) thyroxine sodium is a synthetic tetraiodothyronine (T4) and is mostly converted to triiodothyronine (T3) in the body. Thyrotoxicosis induced remarkable structural remodeling in the present study, including cardiac hypertrophy and apoptosis, interstitial fibrosis, and electrical remodeling, which manifested as reduced I_{K1} (Kir2.1) and increased $[Ca^{2+}]_i$. The present study provides the first observations on I_{K1}/Kir2.1 in the ventricular myocardium after a relatively long-term exposure to L-thyroxine.

Ventricular hypertrophy is the predominant form of ventricular remodeling and an independent risk factor for cardiac death. It is subdivided into two fundamental types, physiological and pathological [24-27]. Physiological hypertrophy occurs in very limited circumstances, and normal or even improved cardiac function remains. Pathological hypertrophy results in ventricular systolic and/or diastolic dysfunction, i.e., HF, with progression. Ca^{2+} plays a piv-

otal role in myocardial excitation-contraction coupling, substance metabolism, cell cycle regulation, cell-cell communication, and gene expression [28]. Intracellular Ca^{2+} overload is a central event in the pathogenesis of cardiac hypertrophy and HF [29, 30]. Abnormalities in calcium-handling proteins, including LTCC, sodium-calcium exchanger (NCX), CaMKII, SERCA2, and ryanodine receptor (RyR), contribute to the disorder of calcium homeostasis.

Membrane LTCC-mediated Ca^{2+} influx is the initial step of cardiac excitation-contraction coupling. Over-activation of LTCC is generally the first consideration in accounting for an elevation of intracellular Ca^{2+} [29, 31-33]. LTCC antagonists should slow Ca^{2+} influx and prevent the development of cardiac hypertrophy [30]. In the present study, we also confirmed that blocking LTCC alleviated intracellular calcium overload. However, LTCCs play critical roles in initiating and maintaining contraction. Therefore, these blockers may impact the Ca^{2+}-pumping function, which limits their clinical application. Zacopride is a specific I_{K1} agonist with no effect on LTCC in normal cardiomyocytes [19]. It's effect on intracellular calcium homeostasis is likely secondary and mediated by a LTCC-independent manner. I_{K1} might be a new target for intracellular calcium handling. By enhancing I_{K1}, zacopride hyperpolarizes RP and moderately shortens action potential duration (APD), which decreases the opening of voltage-dependent LTCCs and consequently inhibits cardiac Ca^{2+} overload. The most convincing data came from the co-application of I_{K1} blocker. Chloroquine is an important agent for the prevention and treatment of malaria. Renewed interest in chloroquine emerged from an observation that low-dose chloroquine (0.3 μmol/L in feline ventricular myocytes) was relatively a specific I_{K1} channel antagonist [34]. Many laboratories use chloroquine as a pharmacological tool for I_{K1}/Kir2.1 blockade [35-37]. The present study used 7.5 μg/kg/d chloroquine *in vivo*, which is distributed in the plasma at approximately 0.3 μmol/L. Chloroquine blunted the cardioprotection of zacopride on electrical and structural remodeling. In cultured H9C2 (2-1) cells, in parallel use of another I_{K1} blocker, $BaCl_2$, we further clarified that the effect of zacopride on cardiomyocyte autophagy and calcium overload is mediated by I_{K1} agonism.

CaMKII is a Ca^{2+}-activated calmodulin (CaM)-dependent protein kinase. When the mem-

brane potential is depolarized, Ca^{2+} influxes through LTCC and induces calcium release from the SR via RyR-regulated Ca^{2+} channels. Elevated Ca^{2+} and CaM form the Ca^{2+}-calmodulin complex, which binds to the regulatory domain of CaMKII [38]. Phosphorylated CaMKII stimulates intracellular calcium cycling and affects multiple ion channels, including sodium channels, potassium channels, chloride channels, and RyR-regulated calcium channels on the SR. Phosphorylation of RyRs prolongs the opening of Ca^{2+} channels and facilitates Ca^{2+} release from the SR, which leads to the accumulation of Ca^{2+} in the cytoplasm [39]. CaMKII also has a slower response to certain stress-related cardiomyocyte growth and hypertrophic gene expression [40-44]. CaMKII was hyperactivated in L-thyrotoxic rat hearts in the present study and inhibited by zacopride treatment. I_{K1} mediated the regulation of Ca^{2+} dyshomeostasis and Ca^{2+}-activated CaMKII signaling.

The effect of TH on I_{K1} is rarely reported. Sakaguchi et al. showed that T3 acutely enhanced I_{K1} *in vitro* [45]. The present study showed that Kir2.1, the dominant subunit of I_{K1} in ventricle, was inhibited after long-term thyroxine overload or T3 infusion. In the *in vivo* study, it may be the result of neurohumoral regulation, e.g., adrenergic stimulation and/or angiotensin II stress. The sympathetic nervous system (SNS) and renin-angiotensin system (RAS) are involved in myocardial hypertrophy, interstitial fibrosis, and electrical remodeling, including I_{K1} inhibition and calcium overload [46-48]. Accumulated data indicate that the impact of TH on the heart is secondary to these two neurohumoral systems [49, 50]. Excess TH and overactivation of SNS and RAS may overlap in hyperthyroidism. In the cultured H9C2 (2-1) cells, Kir2.1 was downregulated by T3 infusion. It might be the straightforward effect of thyroid hormone or the change secondary to SAP97 downregulation or even calcium overload. Downregulation of SAP97 decreases the membrane expression of I_{K1} by hindering forward trafficking. In HF, elevated diastolic Ca^{2+} reduced I_{K1} [51]. Downregulation of I_{K1} and Ca^{2+} overload might be reciprocal causation. Pharmacological upregulation of I_{K1}/Kir2.1 is a compensation for the pathological reduction of I_{K1}.

Calcium ions are major intracellular second messengers and play an important role in

apoptosis. The disruption of Ca^{2+} homeostasis and dramatic Ca^{2+} elevation in the cytoplasm trigger apoptosis [52]. Reducing Ca^{2+} overload and maintaining intracellular Ca^{2+} homeostasis by enhancing I_{K1} may be an important strategy for preventing apoptosis, as demonstrated in the present study.

Enhancing I_{K1} improves LV remodeling via PI3K/Akt/m-TOR-regulated autophagy

Autophagy is an evolutionarily conserved self-digestion pathway which regulates the intracellular homeostatic mechanism that mediates protein and organelle degradation [53, 54]. Some key signaling mechanisms, such as the MAPK/GSK-3β, PI3K/AKT/mTOR, and ROS signaling pathways, regulate autophagy [55]. Evidences showed that through cytosol-localized TRα1, PI3K/Akt/mTOR signaling pathway underlays one of the mechanisms regulating TH- induced hypertrophy [56]. Therefore, the present study focused on the contribution of the PI3K/Akt/m-TOR/autophagy pathway in the cardioprotective effects of an I_{K1} agonist. PI3K-Akt-mTOR is a critical signaling cascade in cellular metabolism, proliferation, and survival, and it plays pivotal roles in cardiac hypertrophy [57]. The class 1A subgroup of PI3K in the heart is a complex of catalytic subunits (p110α, β and δ) and the regulatory subunit p85. The primary consequence of PI3K activation is the generation of phosphatidylinositol 3, 4, 5-triphosphate (PIP3), which functions as a second messenger and recruits Akt to the cell membrane. mTOR is activated in response to hypertrophic stimuli, such as pressure overload, β-adrenergic stimulation, angiotensin II and L-thyroxine, and controls the development of pathological hypertrophy. L-thyroxine induced the hyperphosphorylation of mTOR in the present study, as expected. mTOR signaling generally plays a negative regulatory role in autophagy. Under physiological conditions, the basal level of autophagy is a protective mechanism that facilitates the recycling of defective organelles and protein accumulation to maintain cellular homeostasis. Insufficient autophagy may lead to the accumulation of defective organelles and protein stress, which trigger cell apoptosis and organ dysfunction [58]. Zacopride treatment inhibited the activation of PI3K/Akt/mTOR signaling and consequently promoted autophagy, which is consistent with the improved cardiac remodeling, pump function and decreased apoptosis. Although the exact mechanism connecting I_{K1} and PI3K/Akt/mTOR signaling is not known, the I_{K1} channel is upstream of PI3K/Akt/mTOR signaling. All these data support that I_{K1} activation counteracted TH-induced cardiac hypertrophy and dysfunction via the PI3K/Akt/mTOR/autophagy pathway.

Enhancing I_{K1} prevents LV fibrosis via inhibition of integrin β3

TH exerts effects on cardiomyocytes and non-myocyte cells, such as fibroblasts, endothelial cells, and vascular smooth muscle cells. Integrins are a family of transmembrane glycoproteins that mediate cell-cell and cell-extracellular matrix (ECM) interactions. Integrins are necessary for the maintenance and remodeling of the ECM, and the transmission of signals from the ECM to cardiomyocytes. Mammalian integrins are heterodimers comprising an α and a β chain from 18 α and 8 β subunits [59, 60].

The β3 subunit is most commonly associated with αV (integrin αVβ3), and it is a cell surface receptor for thyroid hormone (T4) [61]. β3 integrins are expressed in cardiac myocytes and fibroblasts [62]. Cardiac fibroblasts in β3 integrin null (β3-/-) mice exhibited a significant reduction in ECM accumulation and fibrosis following pressure overload hypertrophy [63]. These observations establish the importance of β3 integrin signaling in cardiac fibrosis. The present study observed an attenuation of fibrosis following zacopride administration in hyperthyroid rats. The I_{K1} (Kir2.1) channel is present on ventricular fibroblasts, and it is a primary determinant of RP [64]. Zacopride may affect the proliferation and/or apoptosis of ventricular fibroblasts via modulation of I_{K1} and RP. I_{K1} channels on fibroblasts may be a potential antifibrotic target, but the critical mechanism must be further clarified in future studies.

Disclosure of conflict of interest

None.

Address correspondence to: Ji-Min Cao, Key Laboratory of Cellular Physiology (Shanxi Medical University), Ministry of Education, Department of Physiology, Shanxi Medical University, 56 Xinjian South Road, Taiyuan 030001, Shanxi, China. E-mail: caojimin@126.com

References

[1] Klein I and Ojamaa K. Thyroid hormone and the cardiovascular system. N Engl J Med 2001; 344: 501-509.

[2] Dillmann W. Cardiac hypertrophy and thyroid hormone signaling. Heart Fail Rev 2010; 15: 125-132.

[3] Biondi B. Mechanisms in endocrinology: heart failure and thyroid dysfunction. Eur J Endocrinol 2012; 167: 609-618.

[4] Ojaimi C, Qanud K, Hintze TH and Recchia FA. Altered expression of a limited number of genes contributes to cardiac decompensation during chronic ventricular tachypacing in dogs. Physiol Genomics 2007; 29: 76-83.

[5] Ismail HM. Reversible pulmonary hypertension and isolated right-sided heart failure associated with hyperthyroidism. J Gen Intern Med 2007; 22: 148-150.

[6] Mitchell JE, Hellkamp AS, Mark DB, Anderson J, Johnson GW, Poole JE, Lee KL and Bardy GH. Thyroid function in heart failure and impact on mortality. JACC Heart Fail 2013; 1: 48-55.

[7] Lopatin AN and Anumonwo JM. Structural and Molecular Bases of Cardiac Inward Rectifier Potassium Channel Function. In: Zipes DP and Jalife J, editors. Cardiac electrophysiology: from cell to bedside. 6th edition. Philadelphia: WB Saunders Co; 2014. pp. 33-41.

[8] Lopatin AN and Nichols CG. Inward rectifiers in the heart: an update on I(K1). J Mol Cell Cardiol 2001; 33: 625-628.

[9] Janse MJ. Electrophysiological changes in heart failure and their relationship to arrhythmogenesis. Cardiovasc Res 2004; 61: 208-217.

[10] Kuzman JA, Vogelsang KA, Thomas TA and Gerdes AM. L-thyroxine activates Akt signaling in the heart. J Mol Cell Cardiol 2005; 39: 251-258.

[11] Kenessey A and Ojamaa K. Thyroid hormone stimulates protein synthesis in the cardiomyocyte by activating the Akt-mTOR and p70S6K pathways. J Bio Chem 2006; 28: 20666-20672.

[12] Liu QH, Qiao X, Zhang LJ, Wang J, Zhang L, Zhai XW, Ren XZ, Li Y, Cao XN, Feng QL, Cao JM and Wu BW. I$_{K1}$ channel agonist zacopride alleviates cardiac hypertrophy and failure via alterations in calcium dyshomeostasis and electrical remodeling in rats. Front Pharmacol 2019; 10: 929.

[13] Liu CF, Liu EL, Luo TE, Zhang WF and He RL. Opening of the inward rectifier potassium channel alleviates maladaptive tissue repair following myocardial infarction. Acta Biochim Biophys Sin 2016; 48: 687-695.

[14] Godreau D, Vranckx R, Maguy A, Rücker-Martin C, Goyenvalle C, Abdelshafy S, Tessier S, Couétil JP and Hatem SN. Expression, regulation and role of the MAGUK protein SAP-97 in human atrial myocardium. Cardiovasc Res 2002; 56: 433-442.

[15] Leonoudakis D, Conti LR, Radeke CM, McGuire LM and Vandenberg CA. A multiprotein trafficking complex composed of SAP97, CASK, Veli, and Mint1 is associated with inward rectifier Kir2 potassium channels. J Biol Chem 2004; 279: 19051-19063.

[16] Matamoros M, Pérez-Hernández M, Guerrero-Serna G, Amorós I, Barana A, Núñez M, Ponce-Balbuena D, Sacristán S, Gómez R, Tamargo J, Caballero R, Jalife J and Delpón E. Nav1.5 N-terminal domain binding to α1-syntrophin increases membrane density of human Kir2.1, Kir2.2 and Nav1.5 channels. Cardiovasc Res 2016; 110: 279-290.

[17] Milstein ML, Musa H, Balbuena DP, Anumonwo JM, Auerbach DS, Furspan PB, Hou L, Hu B, Schumacher SM, Vaidyanathan R, Martens JR and Jalife J. Dynamic reciprocity of sodium and potassium channel expression in a macromolecular complex controls cardiac excitability and arrhythmia. Proc Natl Acad Sci U S A 2012; 109: E2134-2143.

[18] Vaidyanathan R, Taffet SM, Vikstrom KL and Anumonwo JM. Regulation of cardiac inward rectifier potassium current (I(K1)) by synapse-associated protein-97. J Biol Chem 2010; 285: 28000-28009.

[19] Liu QH, Li XL, Xu YW, Lin YY, Cao JM and Wu BW. A novel discovery of I$_{K1}$ channel agonist: zacopride selectively enhances I$_{K1}$ current and suppresses triggered arrhythmias in the rat. J Cardiovasc Pharmacol 2012; 59: 37-48.

[20] Rodríguez-Menchaca AA, Navarro-Polanco RA, Ferrer-Villada T, Rupp J, Sachse FB, Tristani-Firouzi M and Sánchez-Chapula JA. The molecular basis of chloroquine block of the inward rectifier Kir2.1 channel. Proc Natl Acad Sci U S A 2008; 105: 1364-1368.

[21] Benjamin IJ, Jalil JE, Tan LB, Cho K, Weber KT and Clark WA. Isoproterenol induced-myocardial fibrosis in relation to myocyte necrosis. Circ Res 1989; 65: 657-670.

[22] Pasquier B. Autophagy inhibitors. Cell Mol Life Sci 2016; 73: 985-1001.

[23] Zhang L, Liu Q, Liu C, Zhai X, Feng Q, Xu R, Cui X, Zhao Z, Cao J and Wu B. Zacopride selectively activates the Kir2.1 channel via a PKA signaling pathway in rat cardiomyocyte. Sci China Life Sci 2013; 56: 788-796.

[24] Fazio S, Palmieri EA, Lombardi G and Biondi B. Effects of thyroid hormone on the cardiovascular system. Recent Prog Horm Res 2004; 59: 31-50.

[25] Galli E, Pingitore A and Iervasi G. The role of thyroid hormone in the pathophysiology of heart failure: clinical evidence. Heart Fail Rev 2010; 15: 155-169.

[26] Siu CW, Yeung CY, Lau CP, Kung AW and Tse HF. Incidence, clinical characteristics and outcome of congestive heart failure as the initial presentation in patients with primary hyperthyroidism. Heart 2007; 93: 483-487.

[27] Dorn GW 2nd. The fuzzy logic of physiological cardiac hypertrophy. Hypertension 2007; 49: 962-970.

[28] Aiba T and Tomaselli GF. Electrical remodeling in the failing heart. Curr Opin Cardiol 2010; 25: 29-36.

[29] Muth JN, Bodi I, Lewis W, Varadi G and Schwartz A. A Ca (2+)-dependent transgenic model of cardiac hypertrophy: a role for protein kinase Calpha. Circulation 2001; 103: 140-147.

[30] Sugiura R, Sio SO, Shuntoh H and Kuno T. Molecular genetic analysis of the calcineurin signaling pathways. Cell Mol Life Sci 2001: 58: 278-288.

[31] Bodi I, Muth JN, Hahn HS, Petrashevskaya NN, Rubio M, Koch SE, Varadi G and Schwartz A. Electrical remodeling in hearts from a calcium-dependent mouse model of hypertrophy and failure: complex nature of K$^+$ current changes and action potential duration. J Am Coll Cardiol 2003; 41: 1611-1622.

[32] Song LS, Guia A, Muth JN, Rubio M, Wang SQ, Xiao RP, Josephson IR, Lakatta EG, Schwartz A and Cheng H. Ca(2+) signaling in cardiac myocytes overexpressing the alpha(1) subunit of L-type Ca(2+) channel. Circ Res 2002; 90: 174-181.

[33] Viola HM, Macdonald WA, Tang H and Hool LC. The L-type Ca(2+) channel as a therapeutic target in heart disease. Curr Med Chem 2009; 16: 3341-3358.

[34] Sánchez-Chapula JA, Salinas-Stefanon E, Torres-Jácome J, Benavides-Haro DE and Navarro-Polanco RA. Blockade of currents by the antimalarial drug chloroquine in feline ventricular myocytes. J Pharmacol Exp Ther 2001; 297: 437-445.

[35] El Harchi A, McPate MJ, Zhang Yh, Zhang H and Hancox JC. Action potential clamp and chloroquine sensitivity of mutant Kir2.1 channels responsible for variant 3 short QT syndrome. J Mol Cell Cardiol 2009; 47: 743-747.

[36] Sanson C, Schombert B, Filoche-Rommé B, Partiseti M and Bohme GA. Electrophysiological and pharmacological characterization of human inwardly rectifying K$_{ir}$2.1 channels on an automated patch-clamp platform. Assay Drug Dev Technol 2019; 17: 89-99.

[37] Luo C, Wang K and Zhang H. Modelling the effects of chloroquine on *KCNJ2*-linked short QT syndrome. Oncotarget 2017; 8: 106511-106526.

[38] Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR and Olson EN. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 1998; 93: 215-228.

[39] Grueter CE, Colbran RJ and Anderson ME. CaMKII, an emerging molecular driver for calcium homeostasis, arrhythmias, and cardiac dysfunction. J Mol Med (Berl) 2007; 85: 5-14.

[40] Backs J, Song K, Bezprozvannaya S, Chang S and Olson EN. CaM kinase II selectively signals to histone deacetylase 4 during cardiomyocyte hypertrophy. J Clin Invest 2006; 116: 1853-1864.

[41] Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA and Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 2002; 110: 479-488.

[42] Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR and Olson EN. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. J Clin Invest 2000; 105: 1395-1406.

[43] MacDonnell SM, Weisser-Thomas J, Kubo H, Hanscome M, Liu Q, Jaleel N, Berretta R, Chen X, Brown JH, Sabri AK, Molkentin JD and Houser SR. CaMKII negatively regulates calcineurin-NFAT signaling in cardiac myocytes. Circ Res 2009; 105: 316-325.

[44] Mattiazzi A, Bassani RA, Escobar AL, Palomeque J, Valverde CA, Vila Petroff M and Bers DM. Chasing cardiac physiology and pathology down the CaMKII cascade. Am J Physiol Heart Circ Physiol 2015; 308: H1177-1191.

[45] Sakaguchi Y, Cui G and Sen L. Acute effects of thyroid hormone on inward rectifier potassium channel currents in guinea pig ventricular myocytes. Endocrinology 1996; 137: 4744-4751.

[46] Samson R, Baydoun H, Jaiswal A and Le Jemtel TH. Cardiac adrenergic nervous system and

left ventricular remodeling. Am J Med Sci 2015; 350: 321-326.

[47] Xie M, Burchfield JS and Hill JA. Pathological ventricular remodeling: therapies: part 2 of 2. Circulation 2013; 128: 1021-1030.

[48] Sun Y. Intracardiac renin-angiotensin system and myocardial repair/remodeling following infarction. J Mol Cell Cardiol 2010; 48: 483-489.

[49] Hu LW, Benvenuti LA, Liberti EA, Carneiro-Ramos MS and Barreto-Chaves ML. Thyroxine-induced cardiac hypertrophy: influence of adrenergic nervous system versus renin- angiotensin system on myocyte remodeling. Am J Physiol Regul Integr Comp Physiol 2003; 285: R1473-1480.

[50] Barreto-Chaves ML, Carrillo-Sepúlveda MA, Carneiro-Ramos MS, Gomes DA and Diniz GP. The crosstalk between thyroid hormones and the renin-Angiotensin system. Vascul Pharmacol 2010; 52: 166-170.

[51] Fauconnier J, Lacampagne A, Rauzier JM, Vassort G and Richard S. Ca^{2+}-dependent reduction of I_{K1} in rat ventricular cells: a novel paradigm for arrhythmia in heart failure? Cardiovasc Res 2005; 68: 204-212.

[52] Kim JY, Yu SJ, Oh HJ, Lee JY, Kim Y and Sohn J. Panaxydol induces apoptosis through an increased intracellular calcium level, activation of JNK and p38 MAPK and NADPH oxidase-dependent generation of reactive oxygen species. Apoptosis 2011; 16: 347-358.

[53] Klionsky DJ and Emr SD. Autophagy as a regulated pathway of cellular degradation. Science 2000; 290: 1717-1721.

[54] Durlacher CT, Chow K, Chen XW, He ZX, Zhang X, Yang T and Zhou SF. Targeting Na^+/K^+-translocating adenosine triphosphatase in cancer treatment. Clin Exp Pharmacol Physiol 2015; 42: 427-443.

[55] Sciarretta S, Maejima Y, Zablocki D and Sadoshima J. Annu Rev Physiol 2018; 80: 1-26.

[56] Kenessey A and Ojamaa K. Thyroid hormone stimulates protein synthesis in the cardiomyocyte by activating the Akt-mTOR and p70S6K pathways. J Biol Chem 2006; 281: 20666-20672.

[57] Ikeda M, Ide T, Fujino T, Matsuo Y, Arai S, Saku K, Kakino T, Oga Y, Nishizaki A and Sunagawa K. The Akt-mTOR axis is a pivotal regulator of eccentric hypertrophy during volume overload. Sci Rep 2015; 5: 15881.

[58] Levine B and Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132: 27-42.

[59] Humphries JD, Byron A and Humphries MJ. Integrin ligands at a glance. J Cell Sci 2006; 119: 3901-3903.

[60] Johnston RK, Balasubramanian S, Kasiganesan H, Baicu CF, Zile MR and Kuppuswamy D. Beta3 integrin-mediated ubiquitination activates survival signaling during myocardial hypertrophy. FASEB J 2009; 23: 2759-2771.

[61] Bergh JJ, Lin HY, Lansing L, Mohamed SN, Davis FB, Mousa S and Davis PJ. Integrin alphaVbeta3 contains a cell surface receptor site for thyroid hormone that is linked to activation of mitogen-activated protein kinase and induction of angiogenesis. Endocrinology 2005; 146: 2864-2871.

[62] Chen C, Li R, Ross RS and Manso AM. Integrins and integrin-related protein in cardiac fibrosis. J Mol Cell Cardiol 2016; 93: 162-174.

[63] Balasubramanian S, Quinones L, Kasiganesan H, Zhang Y, Pleasant DL, Sundararaj KP, Zile MR, Bradshaw AD and Kuppuswamy D. Beta3 integrin in cardiac fibroblast is critical for extracellular matrix accumulation during pressure overload hypertrophy in mouse. PLoS One 2012; 7: e45076.

[64] Chilton L, Ohya S, Freed D, George E, Drobic V, Shibukawa Y, Maccannell KA, Imaizumi Y, Clark RB, Dixon IM and Giles WR. K^+ currents regulate the resting membrane potential, proliferation, and contractile responses in ventricular fibroblasts and myofibroblasts. Am J Physiol Heart Circ Physiol 2005; 288: H2931-H2939.