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

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Abstract: Downregulation of inward rectifier potassium (I\textsubscript{K1}) channel is a hallmark in cardiac hypertrophy and failure. The cardioprotection of zacopride (a selective I\textsubscript{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\textsubscript{K1} antagonist) groups. Echocardiography, histopathology, TUNEL assay, western blotting and confocal imaging for intracellular Ca\textsuperscript{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\textsuperscript{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\textsubscript{K1}, SAP97, and [Ca\textsuperscript{2+}]i homeostasis in cardiomyocytes. I\textsubscript{K1} antagonist chloroquine or BaCl\textsubscript{2} reversed these effects. Nifedipine could attenuate intracellular Ca\textsuperscript{2+} overload with no significant effects on I\textsubscript{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\textsubscript{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 prevention of ventricular remodeling and HF are important issues to improve the outcomes and all-cause mortality.

Cardiac I\textsubscript{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\textsubscript{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\textsubscript{K1} attenuated isoproterenol- and myocardial infarction (MI)-induced ventricular remodeling depending on the strength of compensation for I\textsubscript{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\textsubscript{K1} activation. Therefore, the present study aimed to confirm the effects of the I\textsubscript{K1} agonism on cal-
Iₖ₁ agonist attenuates ventricular remodeling

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

Synapse-associated protein-97 (SAP97) is a membrane-associated guanylate kinase (MA-GUK) 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 Iₖ₁, probably by decreasing the number of functional Iₖ₁ 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 Iₖ₁ channel as a target for cardiac remodeling: zacopride, a moderate Iₖ₁ channel agonist/opener [19]; chloroquine and BaCl₂, nonspecific Iₖ₁ 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) and L-thyroxine, a synthetic form of thyroxine (T4).

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...
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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 BaCl2, 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+BaCl2 (1 μmol/L), T3+Zac+CQ (0.3 μmol/L), T3+nifedipine (Nife, 10 μmol/L), T3+Nife+Zac, Zac, BaCl2, 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 CaCl2). 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)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-thy</th>
<th>L-thy+Zac</th>
<th>L-thy+Zac+CQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>BW (g)</td>
<td>240.5 ± 7.2</td>
<td>243.2 ± 5.0</td>
<td>249.0 ± 3.1</td>
<td>231.5 ± 10.5</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>2.70 ± 0.05</td>
<td>4.77 ± 0.11**</td>
<td>3.93 ± 0.09**</td>
<td>4.72 ± 0.12**</td>
</tr>
<tr>
<td>LVW/BW (mg/g)</td>
<td>1.49 ± 0.11</td>
<td>3.26 ± 0.14**</td>
<td>2.68 ± 0.08**</td>
<td>3.10 ± 0.10**</td>
</tr>
</tbody>
</table>

L-thy, L-thyroxine; Zac, zacopride; CQ, chloroquine. **P < 0.01 vs. control, ***P < 0.01 vs. L-thy. ΔP < 0.05, ΔΔP < 0.01 vs. L-thy+Zac 15 µg/kg.

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

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>L-thy</th>
<th>L-thy+Zac</th>
<th>L-thy+Zac+CQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDd (mm/m²)</td>
<td>4.27 ± 0.22</td>
<td>6.57 ± 0.56**</td>
<td>4.77 ± 0.31</td>
<td>5.93 ± 0.60*</td>
</tr>
<tr>
<td>LVIDs (mm/m²)</td>
<td>2.61 ± 0.10</td>
<td>4.76 ± 0.55***</td>
<td>2.94 ± 0.22</td>
<td>4.35 ± 0.42**</td>
</tr>
<tr>
<td>IVSd (mm/m²)</td>
<td>1.60 ± 0.06</td>
<td>1.69 ± 0.13</td>
<td>1.56 ± 0.10</td>
<td>1.77 ± 0.12</td>
</tr>
<tr>
<td>IVSs (mm/m²)</td>
<td>2.09 ± 0.11</td>
<td>2.46 ± 0.25</td>
<td>2.13 ± 0.12</td>
<td>2.14 ± 0.11</td>
</tr>
<tr>
<td>LVPWd (mm/m²)</td>
<td>1.81 ± 0.22</td>
<td>1.75 ± 0.31</td>
<td>1.95 ± 0.13</td>
<td>1.67 ± 0.09</td>
</tr>
<tr>
<td>LVPWs (mm/m²)</td>
<td>2.32 ± 0.31</td>
<td>2.29 ± 0.33</td>
<td>2.45 ± 0.13</td>
<td>2.38 ± 0.05</td>
</tr>
<tr>
<td>EF (%)</td>
<td>75.6 ± 1.4</td>
<td>59.0 ± 3.5***</td>
<td>75.2 ± 1.9</td>
<td>61.4 ± 2.3**</td>
</tr>
<tr>
<td>FS (%)</td>
<td>38.8 ± 1.3</td>
<td>27.4 ± 1.9***</td>
<td>38.2 ± 1.6</td>
<td>28.8 ± 1.4***</td>
</tr>
</tbody>
</table>

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.

The dose of chloroquine (7.5 µg/kg/d) reversed the anti-remodeling effects of 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 µm²) was approximately 4 times larger than the control group (179.2 ± 8.0 µm²) (Figure 1C, P < 0.01). Zacopride treatment significantly decreased the CSA (187.1 ± 10.0 µm²) (P < 0.01) and attenuated the tissue injury. Co-administration of chloroquine (669.7 ± 28.6 µm²) 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).
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 ratio 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 $P < 0.05$).
I\textsubscript{k1} agonist attenuates ventricular remodeling

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\textsubscript{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 \beta3, was up-regulated in L-thyroxine-infused hearts (\(P < 0.01\)). Zacopride decreased the expression of integrin \beta3 (\(P < 0.01\)), and chloroquine reversed this effect (\(P < 0.01\)).

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

Figure 6 shows that the L-thyroxine-induced cardiac hypertrophy was characterized by increased [Ca\textsuperscript{2+}], 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\textsubscript{2} or chloroquine (\(P < 0.01\)). Nifedipine had no significant effects on SAP97 or Kir2.1. BaCl\textsubscript{2} (1 mmol/L) could downregulate the expression of SAP97 and

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 \(\mu\)m. 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.

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.
**I$_{K1}$** agonist attenuates ventricular remodeling

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
**I<sub>K1</sub>** agonist attenuates ventricular remodeling

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<sup>2+</sup> 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<sup>2+</sup> 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<sub>2</sub>, 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.
I_{K1} agonist attenuates ventricular remodeling

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, BaCl2. 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 BaCl2 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. BaCl2 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+}]. 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-
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otah 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 membrane 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
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Apoptosis. The disruption of Ca\textsuperscript{2+} homeostasis and dramatic Ca\textsuperscript{2+} elevation in the cytoplasm trigger apoptosis [52]. Reducing Ca\textsuperscript{2+} overload and maintaining intracellular Ca\textsuperscript{2+} homeostasis by enhancing I\textsubscript{k1} may be an important strategy for preventing apoptosis, as demonstrated in the present study.

Enhancing I\textsubscript{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 Trc1, 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\textsubscript{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\textsubscript{k1} and PI3K/Akt/mTOR signaling is not known, the I\textsubscript{k1} channel is upstream of PI3K/Akt/mTOR signaling. All these data support that I\textsubscript{k1} activation counteracted TH-induced cardiac hypertrophy and dysfunction via the PI3K/Akt/mTOR/autophagy pathway.

Enhancing I\textsubscript{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\textsubscript{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\textsubscript{k1} and RP. I\textsubscript{k1} channels on fibroblasts may be a potential anti-fibrotic target, but the critical mechanism must be further clarified in future studies.

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Disclosure of conflict of interest

None.

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References

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


[61] Bergh JJ, Lin HY, Lansig 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.

