

Intermedin protects thapsigargin-induced endoplasmic reticulum stress in cardiomyocytes by modulating protein kinase A and sarco/endoplasmic reticulum Ca^{2+} -ATPase

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Received April 4, 2020; Accepted September 30, 2020

DOI: 10.3892/mmr.2020.11746

Abstract. Intermedin (IMD) is a calcitonin/calcitonin-related peptide that elicits cardioprotective effects in a variety of heart diseases, such as cardiac hypertrophy and heart failure. However, the molecular mechanism of IMD remains unclear. The present study investigated the effects of IMD on neonatal rat ventricular myocytes treated with thapsigargin. The results of the present study demonstrated that thapsigargin induced apoptosis in cardiomyocytes in a dose- and time-dependent manner. Thapsigargin induced endoplasmic reticulum stress, as determined by increased expression levels of 78-kDa glucose-regulated protein, C/EBP-homologous protein and caspase-12, which were dose-dependently attenuated by pretreatment with IMD. In addition, IMD treatment counteracted the thapsigargin-induced suppression of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) activity and protein expression levels, and cytoplasmic Ca^{2+} overload. IMD treatment also augmented the phosphorylation of phospholamban, which is a crucial regulator of SERCA. Additionally, treatment with the protein kinase A antagonist H-89 inhibited the IMD-mediated cardioprotective effects, including SERCA activity restoration, anti- Ca^{2+} overload, endoplasmic reticulum stress inhibition and antiapoptosis effects. In conclusion, the results of the present study suggested that IMD may protect

cardiomyocytes against thapsigargin-induced endoplasmic reticulum stress and the associated apoptosis at least partly by activating the protein kinase A/SERCA pathway.

Introduction

Intermedin (IMD), also termed adrenomedullin-2, is a member of the calcitonin/calcitonin gene-related peptide family and is widely expressed in the heart, blood vessels, brain, hypothalamus, kidney, lung, spleen, thymus, ovary and adipose tissue (1). The plasma levels of IMD are relatively low, between 100 and 200 pg/ml (2). Following proteolytic cleavage, pre-proIMD generates IMD_{1-53} , IMD_{1-40} and IMD_{1-47} , the major active fragments that act through the calcitonin-like receptor/receptor activity-modifying protein complexes to induce multiple biological effects, such as increased prolactin release, antidiuretic and natriuretic effects, and reduced food intake (3). Accumulating evidence has indicated extensive functions of IMD in maintaining cardiovascular homeostasis, such as accelerating angiogenesis, anti-apoptotic and fibroblast activity, and increasing cardiac contractility and perfusion (4-6). The plasma levels of IMD and the endogenous IMD in cardiomyocytes have been reported to be significantly increased in response to heart failure and acute cardiac infarction stimuli, and IMD supplementation inhibits cardiomyocyte injury induced by heart failure and cardiac infarction (4,7,8). These results also suggested that IMD may be a potential endogenous protector of the heart. Although these studies have confirmed the cardioprotective effects of IMD, the underlying protective mechanisms are still unclear. Teng *et al* (3) and Zhang *et al* (9) have reported that IMD attenuates tunicamycin and dithiothreitol-induced myocardial injury in rats by inhibiting endoplasmic reticulum (ER) stress. Tunicamycin and dithiothreitol induce ER stress via inhibition of protein glycosylation and disulfide bond formation, respectively. Similar to tunicamycin or dithiothreitol, thapsigargin is also an inducer of ER stress. Thapsigargin, originally isolated from the plant *Thapsia garganica*, is a specific and potent inhibitor for sarco/endoplasmic reticulum calcium ATPase (SERCA) (10). By inhibiting SERCAs, thapsigargin interferes with the ER lumen Ca^{2+} flux that subsequently leads to ER stress (10,11).

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Abbreviations: IMD, intermedin; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; CHOP, C/EBP-homologous protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SERCA, sarco/endoplasmic reticulum calcium ATPase; PKA, cAMP-dependent protein kinase A; PLB, phospholamban

Key words: intermedin, thapsigargin, endoplasmic reticulum stress, sarcoplasmic reticulum Ca^{2+} -ATPase, phospholamban, protein kinase A

SERCA has been reported to exert important effects in the heart, and a number of heart diseases are associated with SERCA dysfunction, such as cardiac hypertrophy and heart failure (12,13). Thapsigargin induces ER stress-related apoptosis by inhibiting SERCA activity to partially simulate the pathophysiological processes of various cardiovascular diseases (14).

The SERCA that is present in all organisms is a 110 kDa transmembrane protein encoded by three homologous genes (SERCA1, SERCA2 and SERCA3), with a dominant expression of SERCA2a in cardiomyocytes (15). SERCA2a is crucial for regulating Ca^{2+} homeostasis by transporting cytosolic Ca^{2+} into the sarcoplasmic reticulum (16,17). Phospholamban (PLB) located in the cardiac sarcoplasmic reticulum and, as an endogenous SERCA inhibitor, compromises SERCA affinity for Ca^{2+} (18). PLB phosphorylation is the primary regulator of SERCA activity in cardiomyocytes (19,20).

To date, there have been no published reports on the effects of IMD on cardiomyocyte injury induced by thapsigargin to the best of our knowledge. Therefore, the preset study focused on the role of IMD in thapsigargin-induced cardiomyocyte apoptosis and examined SERCA activity during thapsigargin treatment to explore whether IMD restored ER stress in cardiomyocytes via a SERCA-dependent mechanism. Protein kinase A (PKA) has also been reported to increase SERCA activity (21); to further explore the underlying mechanisms through which IMD regulates SERCA activity and ER stress, the present study examined the involvement of the PKA pathway in the IMD-mediated protective effects in cardiomyocytes.

Materials and methods

Cell culture and treatment. The experimental protocols were approved by the Ethical Committee of Shanxi Medical University (Taiyuan, China) and complied with internationally accepted principles of laboratory animal care and use (22). Animals were housed at 23°C with 50% humidity, 12-h light/dark cycles, and free access to food and water. Briefly, 16 neonatal SD rats (male and female; age, 1-3 days; Laboratory Animal Center of Shanxi Medical University) were anesthetized by pentobarbital sodium (100 mg/kg) and decapitated for cardiac tissue harvesting. Left ventricular tissues were digested with collagenase II (Sigma-Aldrich; Merck KGaA) in Hank's balanced salt solution (Ca^{2+} - and Mg^{2+} -free) for 1 h at 37°C, as described previously (23). After centrifugation at 500 x g for 10 min at 4°C, the supernatants were discarded, and the cells were resuspended; ~90 min later, non-myocytes were attached to the dishes. The viable non-attached cells were collected and plated on 60-mm culture dishes at a density of 4×10^6 cells per dish and cultured in DMEM supplemented with 1% penicillin-streptomycin and 20% FBS (all purchased from Sigma-Aldrich; Merck KGaA) supplemented with 0.1 mM bromodeoxyuridine (Sigma-Aldrich; Merck KGaA). Using this strategy, a myocyte population was obtained, and all experiments were performed within 3 days after incubation. To evaluate the effects of IMD on thapsigargin-induced ER stress, cardiomyocytes were divided into 6 groups: i) Control; ii) IMD, cells incubated with 100 nM IMD for 24 h at 37°C (100 nM; Phoenix Pharmaceuticals, Inc.) under normoxic

conditions (5% CO_2 ; iii) thapsigargin, cells incubated with 3 μM thapsigargin (Sigma-Aldrich; Merck KGaA), a classic ER stress inducer, for 24 h; iv-vi) thapsigargin +I, 10 and 100 nM IMD, cells incubated with the indicated dose of IMD (thapsigargin was administered 30 min after the addition of IMD). To determine the role of PKA in IMD-mediated cardioprotection, cardiomyocytes were pretreated with 10 μM protein kinase A antagonist H-89 (Sigma-Aldrich; Merck KGaA) for 24 h at 37°C.

Western blot analysis. Cardiomyocytes were lysed in the presence of 10 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich; Merck KGaA), and the protein concentration of the lysates was determined using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Proteins from each sample (~30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride membranes. Following incubation with 5% bovine serum albumin for 2 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies against 78 kDa glucose-regulated protein (GRP78; 1:200; cat. no. sc-13539), C/EBP-homologous protein (CHOP; 1:200; cat. no. sc-7351), caspase-12 (1:200; cat. no. sc-21747), GAPDH (1:500; cat. no. sc-32233; Santa Cruz Biotechnology, Inc.), SERCA2 (1:500; cat. no. S1439), phosphorylated (p-)PLB (1:200; cat. no. SAB1305590), total (t-)PLB (1:200; cat. no. HPA026900; Sigma-Aldrich; Merck KGaA). Subsequently, the blots were incubated with the appropriate secondary antibodies (cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 3 h at 37°C. The membranes were developed with an enhanced chemiluminescence detection system (Amersham; Cytiva). Signal intensities were analyzed using a Gel Imaging System (Bio-Rad Laboratories, Inc.). All assays were performed in triplicate. The relative protein expression levels were determined by normalization to GAPDH.

Measurement of intracellular Ca^{2+} concentration. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured using the Ca^{2+} -specific fluorescent probe fluo-3/AM (cat. no. 50013; Biotium, Inc.). Cardiomyocytes were loaded with 5 μM fluo-3/AM for 30 min at 37°C. The mean fluorescence intensity of the cells was monitored using an Olympus FV1000 laser scanning confocal microscope (Olympus Corporation). Fluo-3/AM was excited at 488 nm, and the emission intensity was measured at 528 nm. All experiments were repeated at least four times.

Measurement of SERCA activity. The sarcoplasmic reticulum of cardiomyocytes was obtained using an ER isolation kit (cat. no. ER0100; Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. SERCA activity was measured using a Ca^{2+} -ATPase Assay kit (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions, normalized to protein concentration and expressed as nanomoles of phosphorus ions per milligram of protein per minute (nmol Pi/mg prot/min).

Flow cytometry analysis. Apoptotic cells were detected using an Annexin-V-FITC kit (BD Biosciences) with propidium iodide (PI) staining and flow cytometric analysis according to the manufacturer's instructions. The double-negative

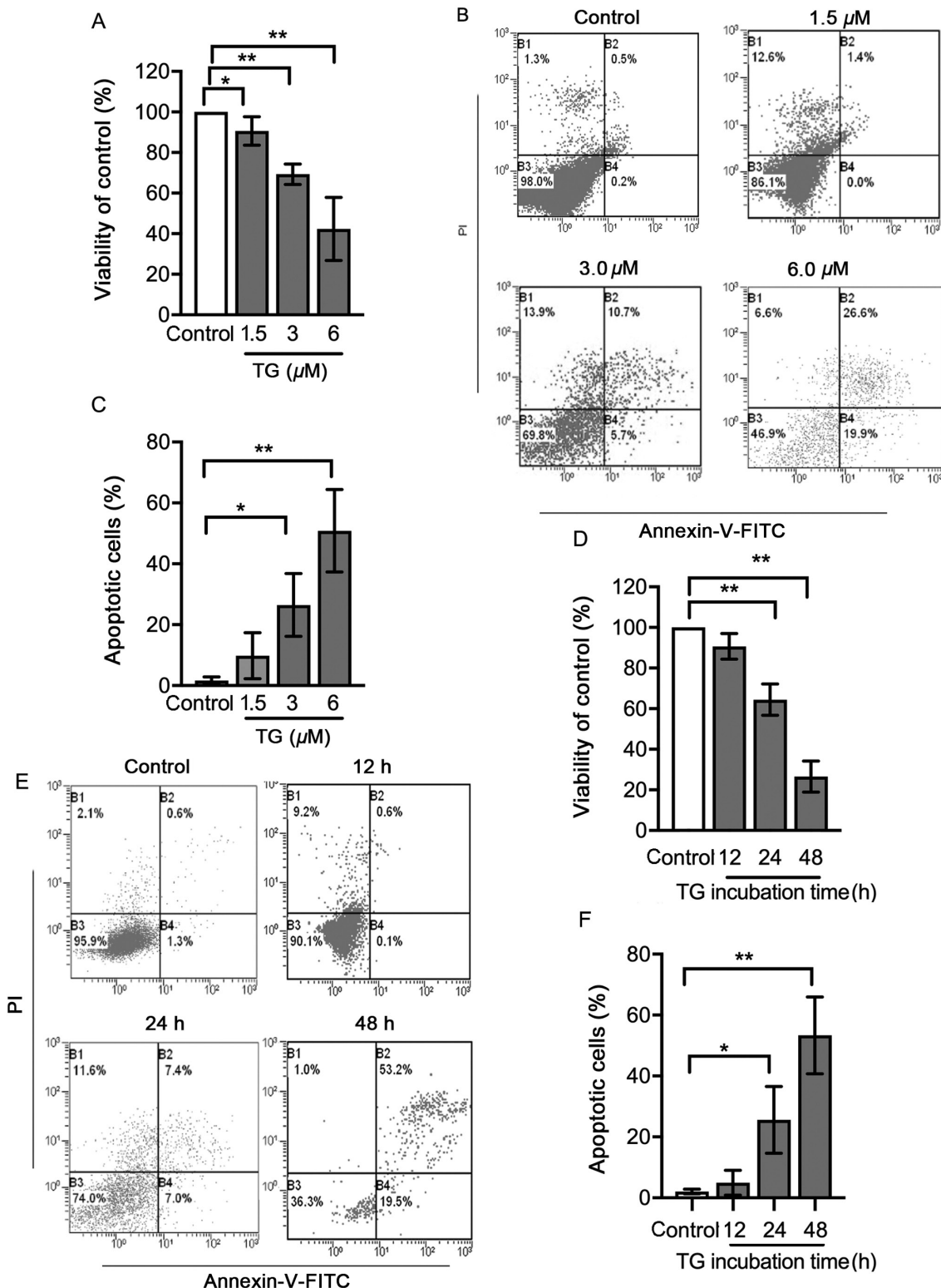


Figure 1. Viability and apoptosis of cells treated with TG. Cultured neonatal rat cardiomyocytes were left untreated or treated with 1.5, 3 or 6 μ M TG for 24 h. Following this, (A) cell viability was determined using an MTT assay, (B) apoptosis was determined using flow cytometry and (C) the rates of apoptotic cells are presented in bar graphs. Cultured neonatal rat cardiomyocytes were left untreated or treated with 3 μ M TG for 12, 24 or 48 h. Following this, (D) cell viability was determined using an MTT assay. (E) Apoptosis was assessed using flow cytometry analysis and (F) the rates of apoptotic cells are presented in bar graphs. In the flow cytometry plots, the horizontal axis represents the Annexin V intensity, and the vertical axis represents PI staining. Lower left quadrant, living cells; upper left quadrant, necrotic cells; right quadrants, apoptotic cells. All data on dose response and time series were analyzed with a one-way ANOVA followed by a Tukey's test. Data are presented as the mean \pm SD. n=3. *P<0.05 and **P<0.01. TG, thapsigargin; PI, propidium iodide.

(Annexin V-negative/PI-negative) cells were defined as viable, the single-positive populations were considered to be early apoptotic (Annexin V-positive/PI-negative) or necrotic

(Annexin V-negative/PI-positive) cells, and double-positive (Annexin V-positive/PI-positive) cells were considered to be in a late stage of apoptosis. Apoptotic cells were analyzed using

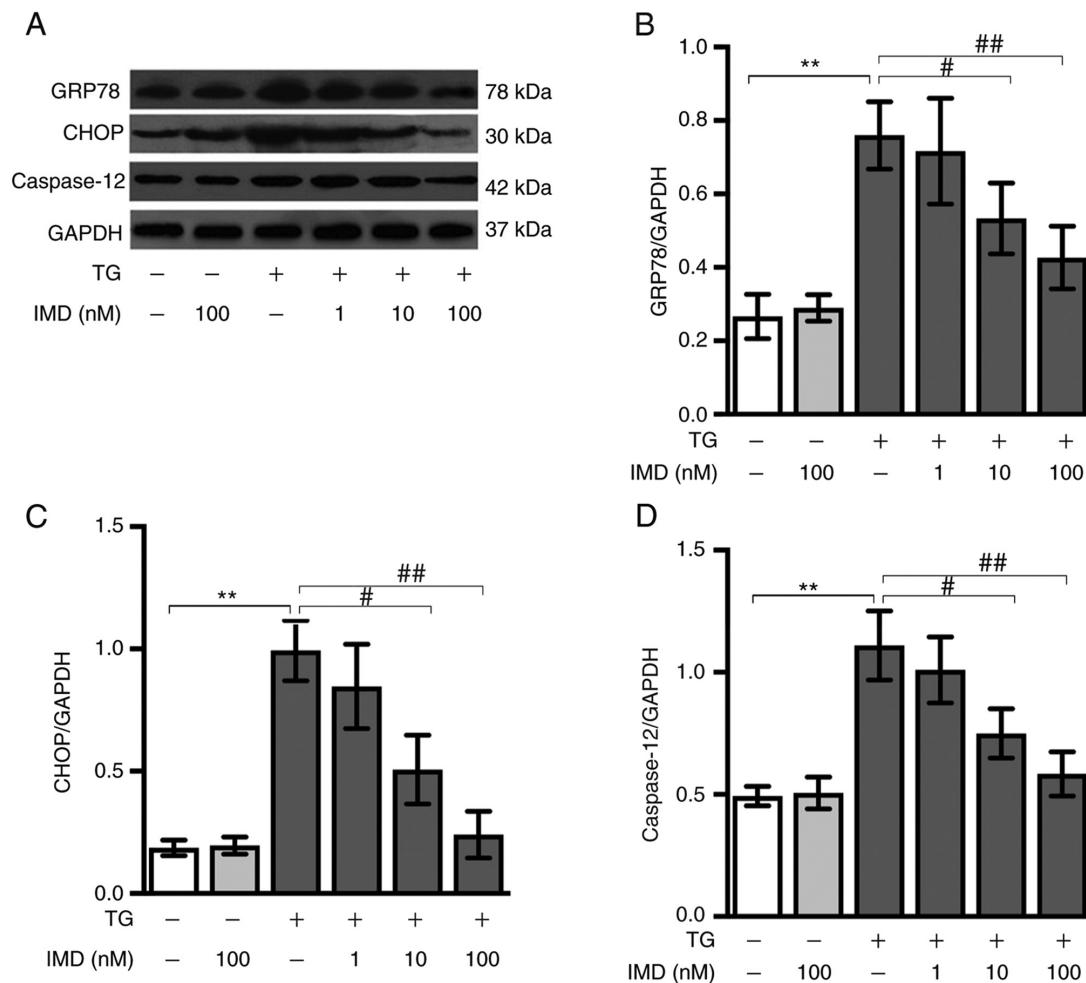


Figure 2. IMD suppresses TG-induced ER stress in cardiomyocytes. Neonatal rat cardiomyocytes were treated with 3 μ M TG and the indicated dose of IMD for 24 h. The expression levels of ER stress markers (GRP78, CHOP and caspase-12) were detected by western blotting. (A) Representative western blots for GRP78, CHOP and caspase-12. Semi-quantification of the protein levels of (B) GRP78, (C) CHOP and (D) caspase-12. Data on dose response were analyzed with a one-way ANOVA followed by the Tukey test. Data are presented as the mean \pm SD. $n=3$. ** $P<0.01$; # $P<0.05$; ## $P<0.01$. IMD, intermedin; ER, endoplasmic reticulum; TG, thapsigargin; GRP78, 78 kDa glucose-regulated protein; CHOP, C/EBP-homologous protein.

a FACScan flow cytometer (BD Biosciences) and CellQuest Pro software (version 5.1; BD Biosciences). Apoptosis was calculated as the total of early and late apoptotic cells.

Statistical analysis. Data are expressed as the mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). All data on dose response and time series were analyzed with a one-way ANOVA followed by a Tukey's post hoc test. Additional data were analyzed with student's t-test for two group comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Thapsigargin induces cardiomyocyte apoptosis in a dose- and time-dependent manner. Neonatal rat cardiomyocytes were incubated with 1.5, 3 or 6 μ M thapsigargin for 24 h to determine the working concentration of thapsigargin. Cell viability was determined using an MTT assay. As demonstrated in Fig. 1A, treatment with increasing concentrations of thapsigargin led to a dose-dependent loss of cardiomyocyte viability compared with the control group. Following exposure to 3 μ M

thapsigargin, 69.33 \pm 4.63% of the cardiomyocytes were viable relative to the control group ($P<0.01$). However, with 6 μ M thapsigargin, the viability of cells was significantly reduced to 44.52 \pm 6.88% (Fig. 1A). Apoptosis was analyzed by flow cytometry using the Annexin V/PI double staining assay. The percentages of apoptotic cells were 9.8 \pm 7.55, 26.47 \pm 10.31 and 50.87 \pm 13.51% in the 1.5, 3 and 6 μ M thapsigargin groups, respectively (Fig. 1B and C).

In order to further study the effects of thapsigargin, cardiomyocytes were treated with 3 μ M thapsigargin for 12, 24 or 48 h. After exposure to 3 μ M thapsigargin for the indicated times, cell viability was significantly decreased in the 24 and 48 h groups compared with that of the control group (Fig. 1D). The apoptotic rates in the 24 and 48 h groups, but not the 12 h group, were significantly higher compared with those in the control group (Fig. 1E and F). Thapsigargin induced apoptosis in cardiomyocytes in a dose- and time-dependent manner; thus, 3 μ M thapsigargin treatment for 24 h was used in subsequent experiments.

IMD inhibits thapsigargin-induced ER stress in cardiomyocytes. Cardiomyocytes were incubated with 3 μ M

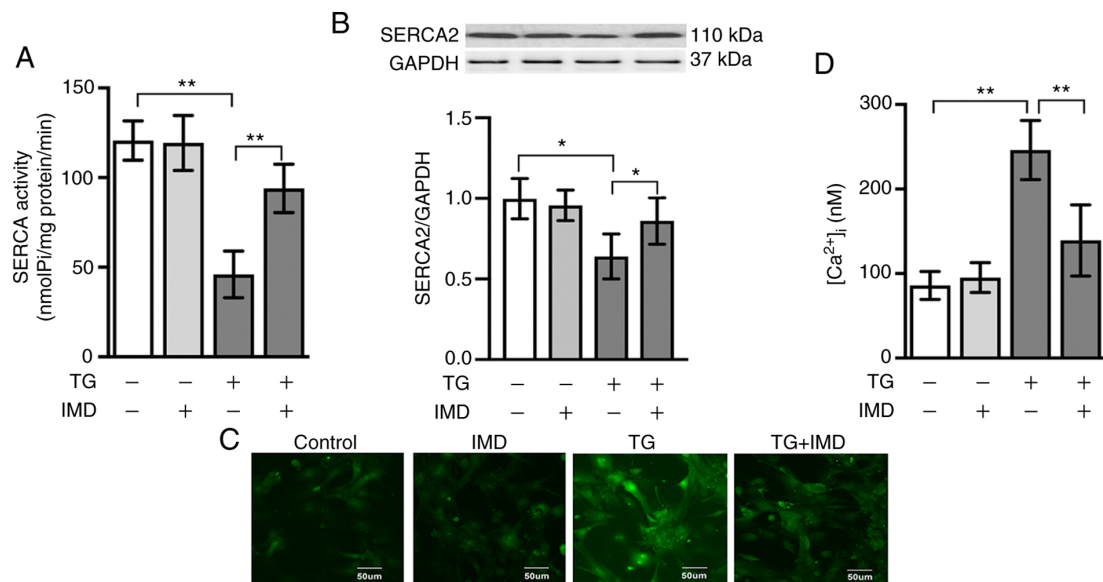


Figure 3. IMD inhibits TG-induced endoplasmic reticulum stress by regulating SERCA activity and $[Ca^{2+}]_i$ homeostasis. Cardiomyocytes were treated with $3 \mu M$ TG in the presence or absence of 100 nM IMD for 24 h. (A) SERCA activity. (B) Representative western blots and semi-quantification of SERCA2 protein levels. (C and D) $[Ca^{2+}]_i$ in cardiomyocytes. The intensity of $[Ca^{2+}]_i$ fluorescence was measured using fluo-3AM with a laser scanning confocal microscope. Data were analyzed with student's t-test for two group comparison. Data are presented as the mean \pm SD. $n=3$. * $P<0.05$ and ** $P<0.01$. IMD, intermedin; SERCA, sarco/endoplasmic reticulum calcium ATPase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; TG, thapsigargin; nmol Pi/mg prot/min, nanomoles of phosphorus ions per milligram of protein per minute.

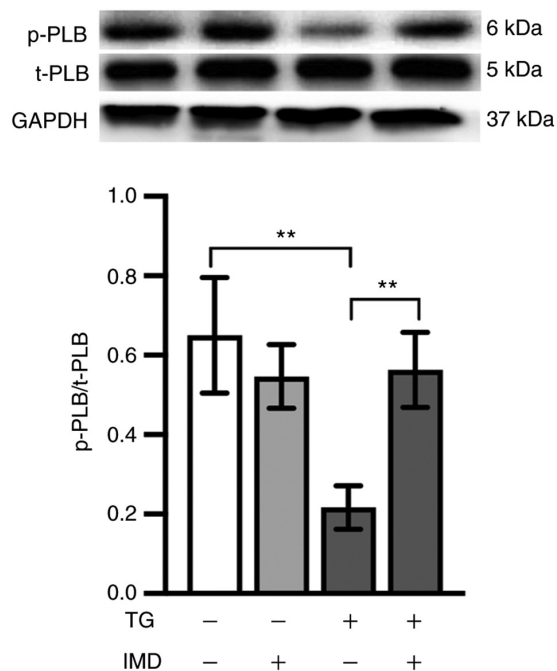


Figure 4. IMD improves SERCA activity by regulating PLB phosphorylation. Cardiomyocytes were treated with $3 \mu M$ TG in the presence or absence of 100 nM IMD for 24 h. p-PLB and t-PLB protein expression levels were assayed by western blot analysis. Representative western blots and semi-quantification of p-PLB and t-PLB. Data were analyzed with a student's t-test for two group comparison. Data are presented as the mean \pm SD. $n=3$. ** $P<0.01$. IMD, intermedin; SERCA, sarco/endoplasmic reticulum calcium ATPase; PLB, phospholamban; p-, phosphorylated; t-, total; TG, thapsigargin.

the thapsigargin group exhibited significantly upregulated protein expression levels of GRP78, CHOP and caspase-12, indicating that thapsigargin treatment induced ER stress. IMD (10 and 100 nM) reduced the thapsigargin-mediated upregulation of ER stress in a dose-dependent manner. Pretreatment with 100 nM IMD resulted in a strong protective effect against thapsigargin-induced injury (Fig. 2A-D). Collectively, these results demonstrated that IMD pretreatment inhibited the thapsigargin-induced ER stress in a dose-dependent manner.

IMD attenuates SERCA suppression and $[Ca^{2+}]_i$ overload induced by thapsigargin in cardiomyocytes. Thapsigargin is a highly selective inhibitor of SERCA. Therefore, the present study examined SERCA activity in cardiomyocytes treated with thapsigargin in the presence or absence of IMD (100 nM) to further investigate the possible relationship between IMD and SERCA. As demonstrated in Fig. 3A and B, thapsigargin treatment significantly reduced the activity and protein expression levels of SERCA2, which were reversed by IMD treatment.

Since SERCA serves a crucial role in Ca^{2+} homeostasis, subsequent experiments were performed to determine whether IMD decreased cytosolic Ca^{2+} overload in cardiomyocytes using the fluorescent indicator fluo-3AM. As presented in Fig. 3C and D, the intensity of $[Ca^{2+}]_i$ fluorescence was significantly elevated in the thapsigargin group compared with that in the vehicle group. This increase in $[Ca^{2+}]_i$ fluorescence was significantly reduced by IMD pretreatment (Fig. 3C and D).

IMD improves SERCA function by regulating PLB phosphorylation. PLB is a key regulatory protein of SERCA (25). Unphosphorylated PLB binds SERCA and inhibits its activity, which is abolished upon PLB phosphorylation (25).

thapsigargin in the presence or absence of IMD for 24 h. CHOP, GRP78 and caspase-12 are molecular markers specific for ER stress (24). Compared with the control group,

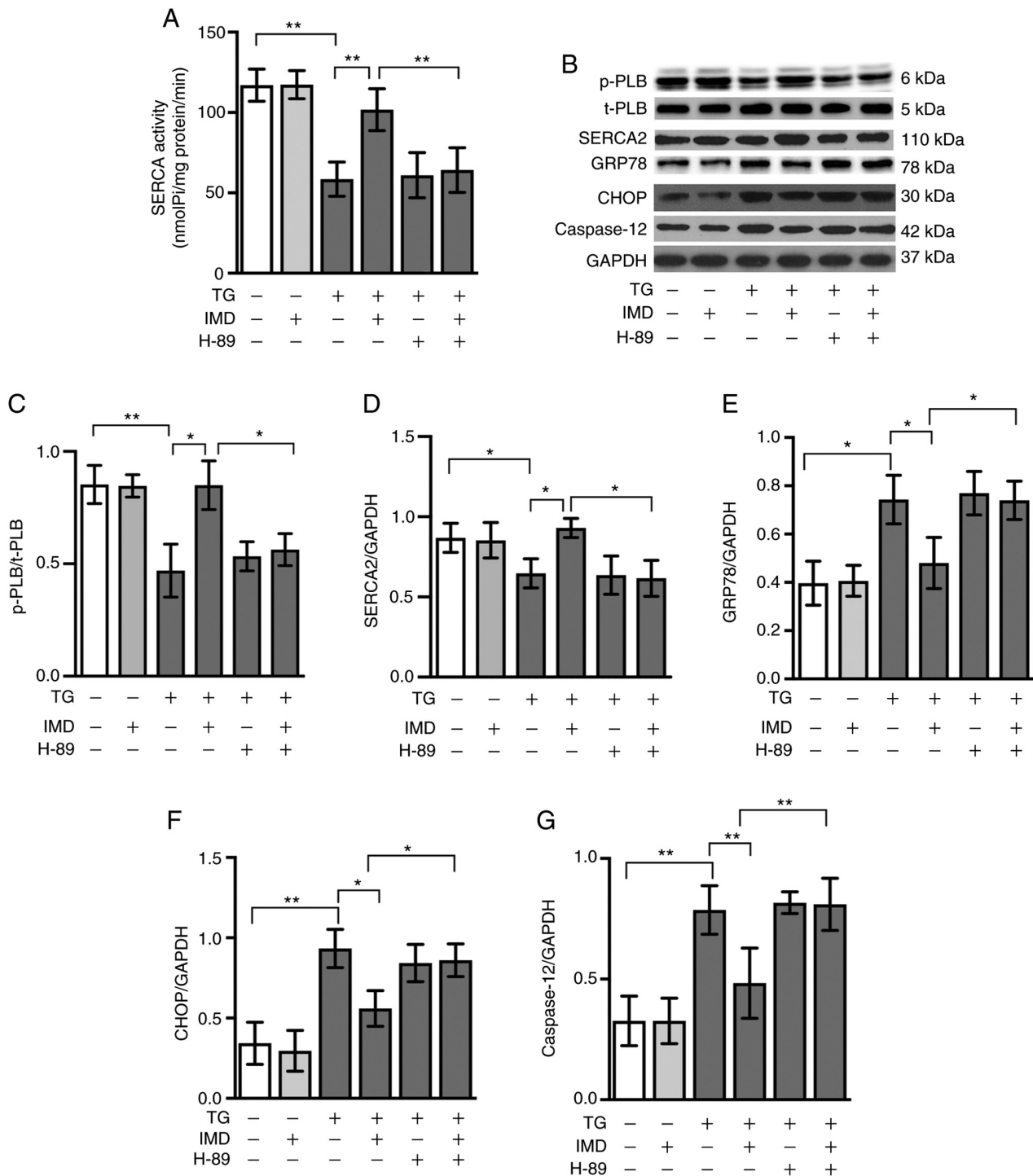


Figure 5. PKA signaling pathway is involved in the effects of IMD on SERCA activity and endoplasmic reticulum stress. Cardiomyocytes were treated with TG in the presence or absence of 100 nM IMD or 10 μ M PKA inhibitor H-89 for 24 h. (A) SERCA activity. (B) Representative western blot images of p-PLB, t-PLB, SERCA2, GRP78, CHOP and caspase-12. Semi-quantification of (C) p-PLB/t-PLB, (D) SERCA2, (E) GRP78, (F) CHOP and (G) caspase-12 protein levels. Data were analyzed with a student's t-test for two group comparison. Data are presented as the mean \pm SD. n=3. *P<0.05 and **P<0.01. IMD, intermedin; SERCA, sarco/endoplasmic reticulum calcium ATPase; PLB, phospholamban; p-, phosphorylated; t-, total; GRP78, 78 kDa glucose-regulated protein; CHOP, C/EBP-homologous protein; PKA, protein kinase A; TG, thapsigargin; nmol Pi/mg prot/min, nanomoles of phosphorus ions per milligram of protein per minute.

Therefore, elucidating the changes in the SERCA regulatory protein PLB may help understand the protective mechanisms of IMD. Thus, t-PLB and p-PLB protein levels were analyzed in the present study by western blotting. As demonstrated in Fig. 4, the levels of p-PLB, which is the active form of the SERCA regulatory protein, were reduced in the thapsigargin

group compared with those in the vehicle group. This reduction was reversed by IMD pretreatment (Fig. 4).

PKA contributes to SERCA function and ER stress regulated by IMD. As PKA regulates PLB phosphorylation and SERCA activity (21,26), the present study further examined whether

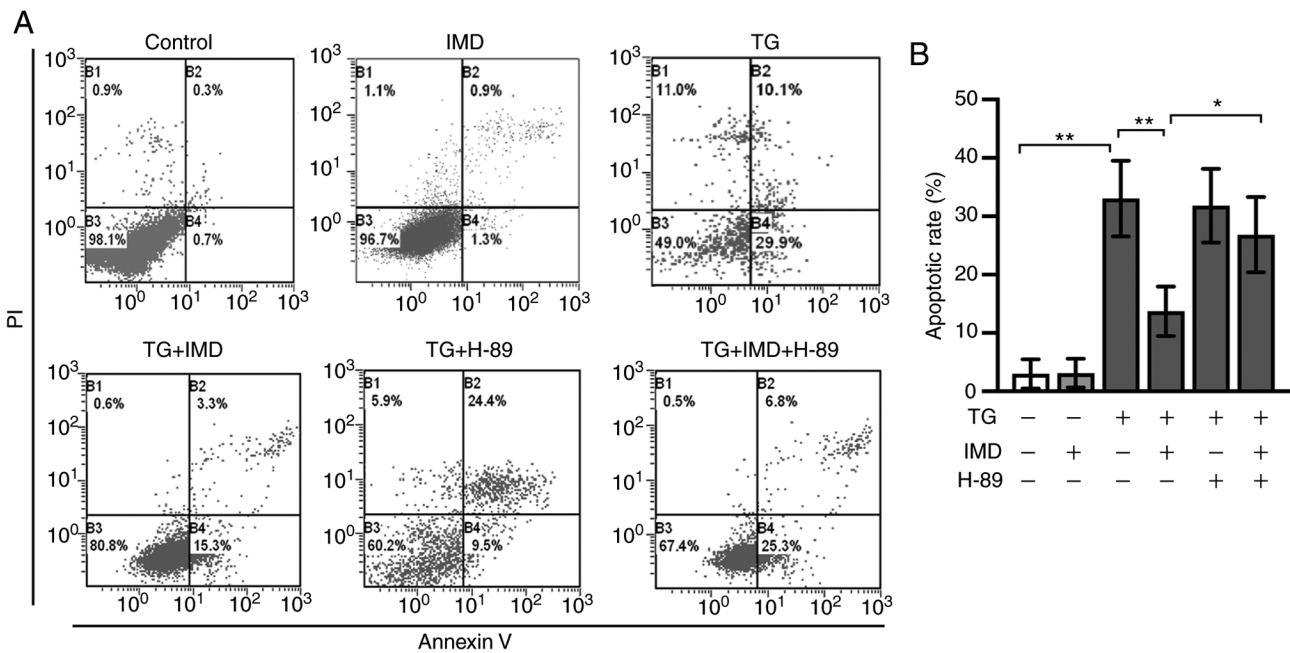


Figure 6. IMD reduces endoplasmic reticulum stress-mediated apoptosis in cardiomyocytes. Neonatal rat cardiomyocytes were treated with TG in the presence or absence of IMD or H-89 for 24 h. Apoptotic rates were assessed using flow cytometry analysis. (A) Representative flow cytometric dot plots for each treatment group. Horizontal and vertical axis represent Annexin V and PI staining. Lower left quadrant, living cells; upper left quadrant, necrotic cells; and right quadrants, apoptotic cells. (B) Quantitative analysis of apoptotic cells. Data were analyzed with a student's t-test for two group comparison. Data are presented as the mean \pm SD. n=3. *P<0.05 and **P<0.01. IMD, intermedin; PI, propidium iodide; TG, thapsigargin.

the PKA signaling pathway was involved in IMD-mediated cardioprotection, PLB phosphorylation and SERCA function. As presented in Fig. 5A-D, co-treatment with the PKA inhibitor H-89 (10 μ M) inhibited the effects of IMD on PLB phosphorylation and SERCA activity in cardiomyocytes treated with thapsigargin. These results suggested that IMD-induced PLB phosphorylation and restoration of SERCA activity were mediated in part by the PKA pathway.

PKA inhibitor H-89 treatment also abolished the protective effects of IMD on ER stress and ER stress-related cardiomyocyte apoptosis induced by thapsigargin, as demonstrated by western blotting and Annexin V/PI double staining assay (Figs. 5B, 5E-G, 6A and 6B). These results suggested that IMD may ameliorate ER stress and ER stress-related cardiomyocyte apoptosis induced by thapsigargin at least in part via the PKA/SERCA pathway.

Discussion

The results of the present study demonstrated that IMD decreased the thapsigargin-induced upregulation of GRP78, CHOP and caspase-12, the specific markers of ER stress. IMD alleviated thapsigargin-induced ER stress by restoring SERCA activity and Ca^{2+} overload in cultured cardiomyocytes. PLB phosphorylation may also contribute to IMD-enhanced SERCA activity. Co-treatment with the PAK inhibitor H89 appeared to counteract the protective effects of IMD on PLB phosphorylation and restoration of SERCA activity, suggesting the involvement of the PKA signaling pathway in the effects of IMD.

IMD belongs to a multifunctional calcitonin/calcitonin gene-related peptide superfamily and shares common

receptors with calcitonin gene-related peptide, adrenomedullin and amylin, with unique and important cardioprotective functions, including improving cardiac function, pro-angiogenesis, anti-oxidation and anti-ER stress (5). ER stress is increasingly recognized as an important contributor to myocardial injury (27). ER, as the primary site of proper folding and sorting of proteins, is vulnerable to ischemia, hypoxia and oxidative stress. Various pathophysiological conditions disturb the ER function and initiate the unfolded protein response to promote cell survival (24). However, persistent and excessive ER stress triggers apoptosis and aggravates cardiovascular diseases, including heart failure, cardiac hypertrophy and ischemic heart disease. ER stress and its induced apoptosis have been highlighted as important mechanisms underlying myocardial injury (28,29). Alleviating ER stress is accepted as a promising therapeutic approach for the treatment of cardiovascular diseases (30).

In previous studies, IMD has been demonstrated to exert a cardioprotective effect against ER stress induced by tunicamycin and dithiothreitol (6,7). The pathways by which IMD inhibits ER stress and protects myocardial injury are not well understood. Thapsigargin is also a classic ER stress inducer. Tunicamycin and dithiothreitol induce ER stress via inhibition of protein glycosylation or disulfide bond formation, respectively (31), whereas thapsigargin possesses a unique mechanism to induce ER stress. To date, there have been no published reports on the effects of IMD on thapsigargin-induced cardiomyocyte injury. The present study assessed the protein expression levels of ER stress-related markers GRP78, CHOP and caspase-12 in cardiomyocytes by western blotting; thapsigargin treatment significantly upregulated the expression levels of GRP78, CHOP and

caspase-12, whereas IMD pretreatment significantly ameliorated these changes in a dose-dependent manner, suggesting that IMD inhibited thapsigargin-induced ER stress. To the best of our knowledge, the present study demonstrated for the first time that IMD may rescue cardiomyocytes from thapsigargin-triggered ER stress.

Experimental and clinical studies have indicated that IMD suppresses cardiac hypertrophy and heart failure, a major health issue that is a leading cause of death worldwide (32-34). During this process, cytosolic Ca^{2+} overload serves a crucial role in the development of pathological cardiac hypertrophy and heart failure. Cytosolic Ca^{2+} homeostasis is tightly controlled by Ca^{2+} -handling enzymes, proteins, channels and transporters in the plasma membrane and Ca^{2+} storage organelles (35). The ER is the major Ca^{2+} storage organelle that releases Ca^{2+} predominately via the Inositol 1,4,5-trisphosphate and the ryanodine receptor, and uptakes Ca^{2+} via SERCA, which is the only active Ca^{2+} transporter from the cytosol to the ER in the heart (36). Suppression of SERCA activity and subsequent alteration of cytosolic Ca^{2+} signaling can severely impair the systolic and diastolic function of the heart, which are major etiologies of cardiac hypertrophy and heart failure (37-39). As the primary function of SERCA is to replenish the sarcoplasmic reticulum Ca^{2+} load during the contraction-relaxation cycle of the heart, resulting in cytosolic Ca^{2+} overload, which is associated with heart failure and cardiac hypertrophy, it was hypothesized in the present study that restoration of SERCA activity may mediate the cardioprotective effects of IMD on ER stress and ER stress-related apoptosis. Thapsigargin is a highly selective inhibitor of SERCAs (40). By inhibiting SERCAs, thapsigargin disrupts Ca^{2+} transport into the ER lumen, leading to an increase in cytoplasmic Ca^{2+} concentration, and subsequently activates ER stress (41). To further investigate this signaling pathway, thapsigargin was used in the present study, as it is a well-established model to study ER stress and SERCA activity (42). The present results demonstrated that thapsigargin attenuated the protein expression and activity of SERCA2a in cardiomyocytes, and IMD pretreatment reversed this change. Furthermore, $[\text{Ca}^{2+}]_i$ in thapsigargin-treated cardiomyocytes was significantly increased, whereas IMD pretreatment inhibited this increase, suggesting that modulating SERCA function and Ca^{2+} homeostasis may contribute to IMD-mediated cardioprotection. Thus, the results of the present study indicated that the regulation of SERCA activity may serve an important role in the IMD-mediated protection of cardiomyocytes against ER stress, which in part explains the underlying mechanism of IMD improving cardiac hypertrophy and heart failure.

PLB inhibits SERCA activity by reducing its affinity for Ca^{2+} (42). Unphosphorylated PLB binds SERCA to inhibit SERCA activity; this inhibition is abolished upon PLB phosphorylation (25). PLB is a key regulator of SERCA (42). Considering the relationship between PLB and SERCA, the present study assessed the protein levels of p-PLB and t-PLB in cardiomyocytes treated with thapsigargin in the presence or absence of IMD to further investigate the underlying mechanisms of IMD. The results demonstrated that the expression of p-PLB was decreased in the thapsigargin group compared with those in the vehicle group, whereas IMD reversed this change, suggesting that PLB phosphorylation may be associated with

the protective effects of IMD on SERCA activity and cytosolic Ca^{2+} influx.

IMD stimulates cardiomyocyte PKA activity (43), and SERCA activity can be increased by PKA (20), which provides a potential mechanism by which IMD stimulates SERCA function and subsequently attenuates ER stress-related apoptosis in thapsigargin-treated cardiomyocytes. To investigate this in the present study, cardiomyocytes were co-pretreated with the PKA inhibitor H-89 and/or IMD, and incubated with thapsigargin. The results demonstrated that the PKA inhibitor H-89 inhibited the protective effect of IMD on ER stress and SERCA function, as indicated by the changes in the expression levels of GRP78, CHOP and caspase-12, and SERCA activity. H-89 pretreatment also abrogated the antiapoptotic effects of IMD. These results suggested that IMD may exert antiapoptotic effects at least partly via the regulation of the PKA signaling pathway in cardiomyocytes treated with thapsigargin.

H-89 is a highly selective, but not exclusive inhibitor of PKA, and reportedly also binds to other protein kinases, including ribosomal protein S6 kinase $\beta 1$, ribosomal protein S6 kinase $\alpha 5$, rho-associated protein kinase 2, RAC- α serine/threonine-protein kinase and ribosomal protein S6 kinase $\alpha 1$, with a low binding affinity (44,45). Thus, the possibility that these protein kinases may in part contribute to the thapsigargin-induced cardiomyocyte apoptosis cannot be eliminated. In addition, the present study did not investigate whether other Ca^{2+} -handling enzymes, proteins, channels and transporters, such as inositol 1,4,5-trisphosphate receptor type, ryanodine receptor 1 and stromal interaction molecule 1, are involved in the protection of IMD on thapsigargin-induced cardiomyocyte apoptosis. Accumulating evidence has suggested that thapsigargin-induced apoptosis is mediated by autophagy in cardiomyocytes (46,47). The present results indicated that IMD attenuated thapsigargin-induced cardiomyocyte apoptosis by inhibiting ER stress with the possible involvement of the PKA/SERCA signaling pathway. The present study focused on the mechanisms upstream of ER stress, but did not explore the involvement of autophagy, which is downstream of ER stress. This is a limitation of this study, and future studies should evaluate whether autophagy contributes to the effects of IMD on ER stress-related apoptosis. In addition, PLB has been demonstrated to be a major regulator of SERCA activity. It is the only SERCA-associated protein directly involved in the development of cardiac disease, including heart failure (18). Thus, the present study focused on the PLB/SERCA signaling pathway. Accumulating evidence has indicated that sarcolipin also inhibits the affinity of SERCA for Ca^{2+} (48,49). In future studies, we will explore whether the effect of IMD on cardiomyocyte apoptosis is associated with sarcolipin and other SERCA regulators.

In conclusion, the results of the present study identified an additional signaling pathway through which IMD responds to ER stress. The protective role of IMD in attenuating thapsigargin-induced cardiomyocyte apoptosis may be mediated by inhibiting ER stress with the possible involvement of the PKA/SERCA signaling pathway. These findings provided a novel insight into the mechanisms underlying the cardioprotective effects of IMD.

Acknowledgements

Not applicable.

Funding

The present work was supported by the National Natural Science Foundation of China (grant no. 81600256), the Natural Science Foundation of Shanxi Province (grant no. 2014021038-2) and the Postdoctoral Research Startup Fund of the First Hospital of Shanxi Medical University.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MZ and ZL designed the experiments. ZL and JG performed the experiments. YB and MZ analyzed the data. ZL and JG wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experimental protocols were approved by the Ethical Committee of Shanxi Medical University (approval no. SYXk:2015-0507) and complied with the internationally accepted principles of laboratory animal care and use.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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