Intermedin1–53 Inhibits NLRP3 Inflammasome Activation by Targeting IRE1α in Cardiac Fibrosis

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Abstract—Intermedin (IMD), a paracrine/autocrine peptide, protects against cardiac fibrosis. However, the underlying mechanism remains poorly understood. Previous studies have shown that activation of the nucleotide-binding oligomerization domain (NOD)–like receptor family pyrin domain containing 3 (NLRP3) inflammasome contributes to cardiac fibrosis. In this study, we aimed to investigate whether IMD mitigated cardiac fibrosis by inhibiting NLRP3. Cardiac fibrosis was induced by angiotensin II (Ang II) infusion for 2 weeks in rats. Western blot, real-time PCR, histological staining, immunofluorescence assay, RNA sequencing, echocardiography, and hemodynamics were used to detect the role and the mechanism of IMD in cardiac fibrosis. Ang II infusion resulted in rat cardiac fibrosis, as shown by over-deposition of myocardial interstitial collagen and cardiac dysfunction. Importantly, NLRP3 activation and endoplasmic reticulum stress (ERS) were found in Ang II–treated rat myocardium. Ang II infusion decreased the expression of IMD and increased the expression of the receptor system of IMD in the fibrotic rat myocardium. IMD treatment attenuated the cardiac fibrosis and improved cardiac function.

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IMD inhibited the upregulation of NLRP3 markers and ERS markers induced by Ang II. In vitro, IMD knockdown by small interfering RNA significantly promoted the Ang II–induced cardiac fibroblast and NLRP3 activation. Moreover, silencing of inositol requiring enzyme 1 α (IRE1α) blocked the effects of IMD inhibiting fibroblast and NLRP3 activation. Pre-incubation with PKA pathway inhibitor H89 blocked the effects of IMD on the anti-ERS, anti-NLRP3, and anti-fibrotic response. In conclusion, IMD alleviated cardiac fibrosis by inhibiting NLRP3 inflammasome activation through suppressing IRE1α via the cAMP/PKA pathway.

KEY WORDS: Intermedin; cardiac fibrosis; NLRP3 inflammasome; ER stress; cAMP/PKA

INTRODUCTION

Cardiac fibrosis is a common response to many clinical disorders such as hypertension, vascular diseases, and cardiomyopathy [1, 2]. Renin–angiotensin–aldosterone system (RAAS) dysregulation, inflammation, oxidative stress, endoplasmic reticulum stress (ERS), and abnormal growth factors participate in the pathogenesis of myocardial fibrosis [3–7]. Angiotensin II (Ang II), an important member of RAAS, plays key roles in the process of cardiac fibrosis [7]. Ang II activates cardiac fibroblasts (CFs), promotes CFs transforming into myofibroblasts, increases collagen synthesis, and reduces collagen degradation [8]. Therefore, inhibiting the roles of Ang II has important clinical significance for cardiac fibrosis in patients with hypertension.

As is well known, inflammation is responsible for cardiac fibrosis and cardiac hypertrophy [5, 13–16]. The NOD–like receptor (NLR) family members are central players in controlling the inflammatory response. The NLR protein family participates in multiprotein complexes, termed inflammasomes [17]. NOD–like receptor family pyrin domain containing 3 (NLRP3) inflammasome, the most fully characterized inflammasome, mediates caspase-1-dependent maturation of interleukin-1β (IL-1β) and IL-18 [17–20]. NLRP3 inflammasome activation contributes to CFs transforming into myofibroblasts and collagen deposition [19, 20]. Ang II, transforming growth factor-β1 (TGF-β1) and lipopolysaccharide (LPS) can activate NLRP3 inflammasome in CFs, leading to extracellular matrix deposition [20–22]. Therefore, inhibition of NLRP3 may be a therapeutic target for alleviating myocardial fibrosis.

Many studies indicate that the crosstalk between inflammation and endoplasmic reticulum stress (ERS) plays an important role in the regulation of inflammation in cardiac fibrosis. ERS inhibition may attenuate cardiac inflammation in cardiac remodeling [23, 24]. Several ER transmembrane sensors mediate the effects of ERS, including protein kinase–like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1α (IRE1α). In particular, the activation of IRE1α initiates inflammatory responses to promote proinflammatory cytokine signaling [45–48]. Furthermore, ERS especially IRE1α activation can initiate NLRP3 inflammasome, which provides insights into the link between ERS and chronic inflammatory diseases [52]. ERS may act as the trigger and activator of the NLRP3 inflammasome in cardiovascular diseases [17, 43, 51]. However, it is unclear whether IRE1α promotes NLRP3 activation in cardiac fibrosis.

In our previous study, ERS is inhibited by intermedin (IMD, or adrenomedullin 2), which is a paracrine/autocrine bio-active polypeptide belonging to the calcitonin/calcitonin gene-related peptide (CGRP) family. Prepro-IMD is composed of 148 amino acids, which yield IMD1–47, IMD8–47, and IMD1–53 by proteolytic cleavage and amidation. IMD has extensive cardiovascular protective effects via its calcitonin receptor–like receptor (CRLR)/receptor activity modifying protein (RAMP) complexes [25, 26]. Our previous research shows that endogenous IMD is significantly down-regulated in response to Ang II treatment in CFs, and administration of IMD1–53 suppresses Ang II–induced activation of CFs and cardiomyocyte hypertrophy [27, 28]. Moreover, IMD prevents myocardial ischemia injury and vascular smooth muscle cell calcification by inhibiting ERS [29, 30]. IMD inhibits inflammation in hyperlipidemic, diabetic, and salt-sensitive hypertensive rats [31–33]. However, whether IMD inhibits NLRP3 inflammasome activation in cardiac fibrosis is unknown.

In the present study, we investigated whether IMD inhibited Ang II–activated NLRP3 inflammasome by suppressing ERS in cardiac fibrosis.
MATERIALS AND METHODS

Animals and Materials

All animal care and experimental protocols complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011) and were approved by the Animal Care Committee of Peking University Health Science Center. Male Sprague–Dawley (SD) rats were obtained from the Animal Center, Peking University Health Science Center (Beijing). Synthetic human IMD1–53 and human Ang II were from Phoenix Pharmaceuticals (Belmont, CA, USA). Antibodies for collagen I and III, glucose-regulated protein 78 (GRP78), GRP94, phosphorylated IRE1α (p-IRE1α), and IRE1α, ATF6, ATF4, and spliced-X-box binding protein 1 (s-XBP-1) were from Abcam PLC (Cambridge, UK). Antibodies for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-smooth muscle actin (α-SMA), NLRP3, IL-1β, IL-18, CRLR, RAMP1, RAMP2, RAMP3, and all secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for phosphorylated PERK (p-PERK) and PERK and phosphorylated eukaryotic translation initiation factor 2α (p-eIF2α) and eIF2α were from Cell Signaling Technology (Danvers, MA, USA). Apoptosis-associated speck-like protein containing CARD (ASC) antibody, caspase-1 antibody, phenyl butyric acid (PBA), taurine (TAU), LPS, H89, LY294002, PD98059, and Hoechst 33,342 were from Sigma (St. Louis, MO, USA). Other chemicals and reagents were of analytical grade.

Cardiac Fibrosis Model in Rats

The rat cardiac fibrosis model was prepared according to the methods [34–36] with minor modification. Eight-week-old male SD rats (250 ± 10 g) were randomly divided into 3 groups: (1) control (Con); only sham surgery; ended the experiment 2 weeks later; (2) Ang II: Ang II (555 ng/kg/min, dissolved in sterile saline) was infused subcutaneously via Alzet mini-osmotic pumps for 2 weeks; (3) Ang II plus IMD: IMD1–53 (100 ng/kg/h dissolved in sterile saline) was infused subcutaneously via Alzet mini-osmotic pumps for 2 weeks at the same time as the Ang II treatment described above. All of the rats were given normal drinking water and normal diet. After hemodynamic measurements, all of the animals were killed by exsanguination and their hearts were quickly collected for further analysis.

Echocardiography

At the end of the experiment, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). The rats underwent ultrasonography to visualize the left ventricle (LV) with a Vevo 770TM Imaging System (Visual Sonics, Toronto, Canada). The probe, which was 17.5 MHz, was placed at the sternal of the rats. A 2-D directional M-mode image of the LV short axis was used to analyze the LV internal diameter in systole (LVID; s), LV posterior wall thickness in systole (LPW; s), LV anterior wall thickness in systole (LVAW; s), LV volume in systole (LVVol; s), LV internal diameter in diastole (LVID; d), LV posterior wall thickness in diastole (LVPW; d), LV anterior wall thickness in diastole (LVAW; d), and LV volume in diastole (LVVol; d). Ejection fraction (EF) and fractional shortening (FS) were quantified by the above data. All echocardiographic parameter measurements represent an average of at least 5 consecutive cardiac cycles.

Hemodynamic Measurements

After echocardiography, a 2.0-F micromanometer conductance catheter was placed into the right carotid artery of the rats for hemodynamic measurements. Then, the catheter was advanced into the LV to measure cardiac function. Data were obtained by PowerLab with the BL-420F instrument (TaiMeng, Chengdu, China).

Non-invasive Tail Arterial Blood Pressure Measurement

Arterial blood pressure was measured by the BP98A Softron tail-cuff system; the measurement was repeated at 0, 4, 7, 11, and 14 days. The mean of three blood values was analyzed. The time of pressure measurement was kept consistent, and the temperature of the rat tails was maintained at 30–33 °C.

Preparation of Primary Neonatal CFs and Ang II Treatment

Neonatal rat CFs were isolated from 1- to 2-day-old SD rats. Briefly, after being washed in Hank’s balanced salt solution (HBSS), the rat myocardium was cut into pieces and digested in HBSS including trypsin (0.05%) and collagenase (0.055%). The supernatant was collected and added to high-glucose Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum.
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(FBS). After centrifugation for 10 min at 1000 rpm, the cell suspension was filtered with a sterile stainless-steel cell crible and plated at 37 °C for 90 min to allow CFs to attach to culture dishes. Then, the medium, which mostly contained cardiomyocytes, was decanted, and purified CFs were cultured in fresh DMEM containing 10% FBS. The shape of the fibroblasts was fusiform or stellate under the microscope (Fig. S3a). The purity of the fibroblasts was determined by Vimentin staining (Fig. S3b). The activation of the fibroblasts was observed by immunofluorescence assay of myofibroblasts differentiated from fibroblasts stained with α-SMA antibody (Fig. S3c). Before reagent treatment, the cells were starved with serum-free medium for 24 h. After incubation with IMD1–53 (1 × 10⁻⁷ mol/L) for 30 min, the CFs (1 × 10⁵/mL) were stimulated with Ang II (1 × 10⁻⁷ mol/L) for 24 h as described [41, 42]. The CFs were stimulated with LPS (100 ng/mL) for 24 h as the positive control.

Western Blot Analysis

Heart tissue and rat CFs were homogenized in lysis buffer. Equal amounts of protein samples were loaded and separated on 10% SDS-PAGE, then transferred to nitrocellulose membranes for 3 h at 4 °C and 200 mA. After incubation in 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies overnight at 4 °C. After 3 washes for 5 min each in TBST (20 mmol/L Tris–HCl (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween 20), the membranes were incubated with secondary antibodies (horseradish peroxidase–conjugated anti-mouse, anti-goat, or anti-rabbit IgG) for 1 h at room temperature (RT). The reaction was visualized by enhanced chemiluminescence. Protein levels were analyzed by use of NIH Image and normalized to that of GAPDH. All experiments were repeated at least 3 times.

Quantitative Real-Time PCR Analysis

Trizol reagent was used to extract total RNA from heart tissue. An amount of 2.0 µg RNA was reverse-transcribed into cDNA. Real-time PCR (7500 Fast Real-Time PCR System, Applied Biosystems, America) was used to amplify cDNA. The amount of PCR product formed in each cycle was evaluated by Eva Green fluorescence. Relative quantification involved the 2⁻ΔΔCt method, with GAPDH as a reference. The primers for real-time PCR are in Supplement Table 3.

Enzyme-Linked Immunosorbent Assay (ELISA) for IMD

Cultured CFs were stimulated with Ang II (1 × 10⁻⁷ mol/L) for 24 h as described above. Next, the supernatant and cell lysate of the CFs were collected as samples for the test. The levels of IMD were measured in the samples prepared from the cultured CFs as per the manufacturer’s instructions provided in the ELISA kit (Jiangsu Meimian Industrial Co. Ltd., China). The concentration of IMD in the samples is determined by comparing the O.D. of the samples to the standard curve.

Immunofluorescence Assay of CFs

After a rinse with phosphate-buffered saline (PBS) 3 times, CFs were fixed with 4% paraformaldehyde at RT for 15 min, permeabilized with 0.1% TritonX-100 at RT for 10 min, sealed with 3% bovine serum albumin (BSA)/PBS at RT for 10 min, and incubated with the antibody for α-SMA (host is rabbit) at 37 °C for 1 h. Negative control was incubated with PBS at 37 °C for 1 h, then anti-rabbit IgG at 37 °C for 1 h in the dark, and then Hoechst 33,342 (10 µg/mL, diluted in 0.5% BSA/PBS) at RT for 5 min in the dark; mounted with 50% glycerin; and observed under an immunofluorescence microscope.

Hematoxylin and Eosin (H&E) Staining

Hearts were excised and fixed in 4% paraformaldehyde; embedded in paraffin; cut into three 5-µm-thick sections, one of which was used as negative control; and stained with hematoxylin for 15 min and with eosin for 3 min; underwent ethanol dehydration, xylene transparency, and neutral gum mounting; and were observed under a microscope. Five visual fields were randomly selected, and standardized light conditions were always used to take photos. Fifty cells were randomly selected from each visual field, Image J was used to measure the cross-sectional area of the cardiomyocyte, and the average value was taken.

Picrosirius Red Staining

Hearts were excised and fixed in 4% paraformaldehyde; embedded in paraffin; cut into three 5-µm-thick sections, one of which was used as negative control; and stained with hematoxylin for 15 min and with eosin for 3 min; underwent ethanol dehydration, xylene transparency, and neutral gum
mounting; and were observed under a microscope. Image J was used to quantify the percentage of fibrotic areas in 5 randomly selected visual fields, and the average value was taken.

**siRNA Transfection and Identification**

Small interfering RNAs (siRNAs) were designed to target IMD and IRE1α in rat CFs. A scramble siRNA was used as negative control. After seeding into 6-well plates for 24 h, the CFs were cultured in serum-free medium for an additional 24 h, then transfected with IMD, IRE1α, or scramble siRNA by using transfection reagent (Lipofectamine RNAiMAX, Invitrogen).

**RNA Sequencing and Analysis of RNA-Seq Data**

As described previously [37], cDNA was synthesized and purified by mRNA, which was enriched and fragmented into short fragments. Subsequently, samples were amplified by PCR and sequenced on the Illumina HiSeq 2000. Because of the existence of poor samples, we detected the expression of gold standard marker genes of cardiac remodeling, followed by sample clustering and filtering. Then, differentially expressed genes were identified by 1.5-fold change in expression and false discovery rate (FDR) < 0.1 between 2 conditions (control vs. Ang II and Ang II vs. IMD). Gene ontology (GO) analysis was used to classify genes most likely to be associated with the development of Ang II–induced cardiac fibrosis [38, 39].

**Statistical Analysis**

Graphpad software (GraphPad Software Inc., San Diego, CA, USA) was used for analyzing data, which were expressed as mean ± SD. Comparisons of 2 groups were analyzed by Student’s t test. Comparisons of more than 2 groups were analyzed by one-way ANOVA followed by Student–Newman–Keuls test. P < 0.05 was considered significant.

**RESULTS**

**IMD1–53 Inhibited NLRP3 Inflammasome Activation Induced by Ang II in Cardiac Fibrosis**

Firstly, we examined the level of IMD and found that the expression of IMD was decreased and its receptor system was increased in the Ang II–treated myocardium of rats (Fig. 1(a, b)). In addition, we found the protein content of IMD was decreased in cultured fibroblast and its supernatants stimulated by Ang II (Fig. 1(c)). Next, we determined the effects of IMD1–53 on cardiac fibrosis induced by Ang II administration. It was found that IMD1–53 administration decreased collagen deposition in rat myocardial interstitial tissue, and reduced mRNA and protein levels of collagen I and III (Fig. 1(d–f)) induced by Ang II compared with control. And the anti-fibrotic effects of IMD were further confirmed in CFs in vitro. IMD1–53 pre-administration decreased the levels of collagen I and III and α-SMA (Fig. 1(g–h)) in the Ang II–treated group. Then, we investigated whether IMD had protective effects against Ang II–treated myocardial hypertrophy and hypertension. IMD1–53 infusion attenuated the cross-sectional area of cardiomyocytes (Fig. 2(a)) and decreased the markers of hypertrophy (ANP, BNP, and HW/BW) (Fig. 2(b)) treated with Ang II. Echocardiography revealed that IMD significantly improved cardiac function markers LVPWd, LVPWs, LVAWd, LVAWs, FS, LVIdD, LVIdS, and E/A with Ang II + IMD1–53 versus Ang II alone (Fig. 2(c) and Supplement Table S1). Dynamic changes in SBP, DBP, MBP, and HR in rats were monitored on days 0, 4, 7, 10, and 14 non-invasively via the tail artery. Ang II treatment increased the functional parameters of SBP, DBP, and MBP time-dependently, which was reversed by IMD1–53 administration (Fig. 2(d–f)). However, neither Ang II nor IMD1–53 treatment affected HR (Fig. 2(g)). On day 14, hemodynamics were measured by carotid intubation invasively. SBP, DBP, MBP, LVSP, LVEDP, LV + dp/dt max, and LV − dp/dt max were reduced in the Ang II + IMD group as compared with the Ang II–alone group (Supplement Table S2). These data indicated that IMD attenuated cardiac fibrosis, hypertrophy, and cardiac dysfunction and limited hypertension induced by Ang II.

To explore the mechanism of IMD protecting against myocardial fibrosis, RNA-sequencing (RNA-seq) was used to profile the changes in gene expression in rat hearts with Ang II and IMD treatment. Overall, 1017 genes were Ang II–upregulated and IMD–downregulated. In contrast, 429 genes were downregulated by Ang II and upregulated by IMD (Fig. 3(a)). On gene ontology (GO) analysis, the most statistically significantly representative term was inflammatory response, which was involved in the biological process in the development of myocardial fibrosis (Fig. 3(b)). Moreover, differentially expressed genes with Ang II upregulated and IMD downregulated
mainly involved 3 ERS–related genes and more than 100 genes of inflammatory response and immune regulation, such as Il18, Il1r2, Il1rm, Tlr2, Tlr8, C3, C4a, and C6 (Fig. 3(c)). These results suggested that IMD attenuated the Ang II–induced myocardial fibrosis was mainly related to inflammation and ERS.

To confirm whether the anti-fibrotic effect of IMD is involved in its inhibition of inflammation, we evaluated the inflammasome generation in vivo and in vitro. In vivo, IMD1–53 treatment decreased the Ang II–induced protein levels of NLRP3, ASC, IL-1β, IL-18, and caspase-1 (Fig. 3(d, e)) in rat hearts. In vitro, IMD1–53
**Fig. 1** IMD$_{1–53}$ inhibited myocardial fibrosis and hypertrophy. (a) Quantitative real-time PCR analysis of mRNA levels of IMD, calciotinin receptor-like receptor (CRLR), receptor activity modifying protein (RAMP) 1, RAMP2, and RAMP3 in rat myocardium. Results are relative to GAPDH. Data are mean±SD (n=4 in each group). *P<0.05, **P<0.01. (b) Western blot analysis of protein levels of IMD, CRLR, RAMP1, RAMP2, and RAMP3 in rat myocardium. GAPDH or β-actin is a control for protein loading. Data are mean±SD (n=3 in each group), *P<0.05, **P<0.01. (c) The protein content of IMD in cultured fibroblasts (left panel) and its supernatants (right panel) analyzed by ELISA. Data are mean±SD (n=6 in each group), *P<0.05, **P<0.01. (d) Picosirius red staining of myocardial interstitial collagen deposition in rat. The bar represents 50 µm. Data are mean±SD (n=4 in each group), *P<0.05, **P<0.01. (e) Quantitative real-time PCR analysis of mRNA levels of collagen I and III in rat myocardium. Results are relative to GAPDH. Data are mean±SD (n=4 in each group), *P<0.05, **P<0.01. (f) Western blot analysis of protein levels of collagen I and III in rat myocardium. (g) Western blot analysis of protein levels of collagen I and III, α-smooth muscle actin (α-SMA) in rat cardiac fibroblasts (CFs). GAPDH was a control for protein loading. Data are mean±SD (n=3 in each group), *P<0.05, **P<0.01. (h) Immuno-fluorescence assay of myofibroblasts differentiated from CFs stained with α-SMA antibody and Hoechst 33,342 and treated with Con, Ang II, and Ang II+IMD. The bar represents 100 µm.

attenuated NLRP3, ASC, caspase-1, IL-1β, and IL-18 protein expression that was increased by Ang II, which has a similar effect induced by LPS in CFs (Fig. 3(f, g)). Thus, inhibition of NLRP3 inflammasome activation may play an important role in IMD alleviating cardiac fibrosis.

**IMD$_{1–53}$ Inhibited NLRP3 Inflammasome Via ERS Mediated by the IRE1α Pathway in Vitro**

We further explored the potential mechanism of IMD inhibiting NLRP3. One mechanism contributing to cardiac disease is by enhancing ERS, a key event in fibrosis [12, 53]. In this study, we found that IMD$_{1–53}$ treatment decreased the protein expression of ERS markers (GRP78, GRP94, ATF6, ATF4, p-PERK, p-eIF2α, IRE1α, and s-XBP-1) in rat hearts (Fig. S1a, b). These effects of IMD on ERS were confirmed in Ang II–treated CFs (Fig. S2a, b). It has been reported that ERS can initiate NLRP3 activation [51]. In particular, the IRE1α pathway is activated in response to ERS, which can induce transcription of multiple cytokines and inflammatory molecules [45–48]. Therefore, we hypothesized IMD$_{1–53}$ inhibited NLRP3 inflammasome via the IRE1α pathway. We knocked down IRE1α and IMD by siRNA in CFs (Fig. 4(a, b)). IMD silencing further increased the Ang II–increased protein expression of NLRP3, ASC, caspase-1, IL-1β, and IL-18, which was reversed by IRE1α silencing (Fig. 4(c, d)). Moreover, we further confirmed that IMD silencing aggravated the Ang II–evoked fibrotic response, which was blocked by IRE1α silencing (Fig. 4(e, f)). These data suggested that the IMD$_{1–53}$ inhibited NLRP3 inflammasome via the IRE1α pathway.

**IMD$_{1–53}$ Inhibited ERS, NLRP3 Inflammasome Activation, and Cardiac Fibrosis Via the cAMP/PKA Pathway**

The post-receptor signal pathways of IMD, such as the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway, PI3K/Akt pathway, and extracellular signal–regulated kinase 1/2 (ERK1/2) pathway, are involved in its bioactive effects [27, 29, 30, 49, 50]. To determine the post-receptor pathway of IMD in Ang II–induced cardiac fibrosis, H89, LY294002, and PD98059 were used to block the cAMP/PKA, PI3K/Akt, and ERK1/2 pathways in CFs, respectively. Preincubation with H89 reversed the effect of IMD$_{1–53}$ inhibiting Ang II–induced ERS, but LY294002 or PD98059 had no effect (Fig. 5(a, b)). Next, we further explored whether IMD inhibited NLRP3 inflammasome and cardiac fibrosis through the cAMP/PKA pathway. We found H89 blocked the effect of IMD$_{1–53}$ in anti-inflammation and anti-cardiac fibrosis, but LY294002 or PD98059 could not (Fig. 5(c–f)). These results suggested that IMD played its biological role mainly through cAMP/PKA signaling in cardiac fibrosis.

**DISCUSSION**

The major finding of this study is that IMD can protect against myocardial fibrosis induced by Ang II and the mechanisms may be involved in attenuating NLRP3 inflammasome activation by inhibiting the IRE1α pathway via cAMP/PKA signaling (Fig. 6).

Cardiac fibrosis is a common response to many clinical disorders such as vascular diseases, hypertension, and cardiomyopathy [1, 2]. RAAS activation plays a key role in myocardial fibrosis, and Ang II is an important member of the RAAS [8]. According to previous literatures [34–36], we established an Ang II–induced cardiac fibrosis model in rats with pressure overload, which had a similar pathogenesis to that of cardiac fibrosis occurring in humans with hypertension. Here, Ang II–treated rats showed significantly increased collagen deposition
**Fig. 2** IMD$_{1-53}$ inhibited myocardial hypertrophy and hypertension induced by Ang II in vivo. (a) Hematoxylin–eosin staining of cardiomyocyte cross-sectional area in rats. The bar represents 50 µm. Data are mean±SD (n=6 in each group), *P<0.05, **P<0.01. (b) Quantitative real-time PCR analysis of mRNA levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in rat myocardium. Results are relative to GAPDH. Data are mean±SD (n=4 in each group), *P<0.05, **P<0.01. Ratio of heart weight to body weight (HW/BW) in rats. Data are mean±SD (n=6 in each group), *P<0.05, **P<0.01. (c) Echocardiographic parameters of rats. (d) Systolic blood pressure (SBP), (e) diastolic blood pressure (DBP), (f) mean blood pressure (MBP), and (g) heart rate (HR). Data are mean±SD (n=6 in each group), *P<0.05, **P<0.01.
Fig. 3  IMD1–53 attenuated NLRP3 inflammasome activation induced by Ang II in vivo and in vitro. (a) Left, differentially expressed genes upregulated by Ang II and downregulated by IMD. Right, differentially expressed genes downregulated by Ang II and upregulated by IMD. The left circle indicates Ang II vs control; the right circle indicates Ang II vs IMD. Gene ontology analysis of genes involved in biological processes with (b) Ang II upregulation and IMD downregulation and (c) AngII downregulation and IMD upregulation. Western blot analysis of protein levels of (d, e) NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), caspase-1, interleukin-1β (IL-1β), and IL-18 in rat myocardium in vivo and (f, g) NLRP3, ASC, caspase-1, IL-1β, and IL-18 in CFs in vitro. GAPDH was a control for protein loading. Data are mean ± SD (n=3 in each group). *P < 0.05, **P < 0.01.
Fig. 4 IMD inhibited Ang II–activated NLRP3 inflammasome and fibrotic response via IRE1α pathway in vitro. (a) Quantitative real-time PCR analysis of mRNA levels of IMD and IRE1α in CFs. (b–f) Western blot analysis of protein levels of IRE1α, NLRP3, ASC, caspase-1, IL-1β, IL-18, collagen I, collagen III, and α-SMA in CFs. GAPDH was a control for protein loading. Results are relative to GAPDH. Data are mean ± SD (n = 3 in each group). *P < 0.05, **P < 0.01.
Fig. 5 IMD1–53 inhibited NLRP3 inflammasome via the cAMP/PKA pathway. (a–f) Western blot analysis of protein levels of collagen I, collagen III, α-SMA, GRP78, GRP94, ATF6, ATF4, p-eIF2α, s-XBP-1, NLRP3, ASC, caspase-1, IL-1β, and IL-18 in CFs treated with H89, LY294002, or PD98059 to block the cAMP/PKA, PI3K/Akt, and ERK1/2 pathways. GAPDH was a control for protein loading. Data are mean ± SD (n = 3 in each group), *P < 0.05, **P < 0.01.
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and expression of fibrotic biomarkers such as collagen I and III in myocardium, which indicated a fibrotic process. These were in accordance with other reports [7, 8]. Then, we found a marked decrease in IMD mRNA and protein levels and increase in CRLR/RAMP mRNA and protein levels in the Ang II–treated rat heart. To further determine whether IMD in heart tissue was partly derived from fibroblasts, we measured the protein level of IMD in cultured fibroblasts and their supernatants. We found the level of IMD was decreased after Ang II stimulation both in supernatant and cells. The changes in IMD and its receptors in the systemic imbalance and local damage–induced cardiac fibrosis suggested that endogenous IMD played an important role in cardiac fibrosis.

Then, we investigated whether IMD had a protective effect on Ang II–treated myocardial fibrosis in vivo.

Prepro-IMD is composed of 148 amino acids, which yield IMD1–47, IMD8–47, and IMD1–53 by proteolytic cleavage and amidation. It is reported that IMD1–53 is the major bioactive fragment of prepro-IMD [64]. Meanwhile, IMD1–53 has stronger biological effects than IMD1–47 and IMD8–47, especially in hemodynamics [65] and cardiovascular protection [25]. And a large number of studies have proved that IMD1–53 has strong protective effects in cardiovascular disease, such as cardiac hypertrophy [28], fibrosis [62], atherosclerosis [66], abdominal aortic aneurysm [40], and vascular calcification [37]. Therefore, we chose IMD1–53 instead of IMD1–47 or IMD8–47 to detect the roles of IMD in cardiac fibrosis. In the present study, IMD1–53 inhibited the expression of myocardial fibrotic biomarkers such as collagen I and III and reduced myocardial interstitial collagen deposition in Ang II–treated rats. IMD1–53 also

Fig. 6 A working model of mechanism through which IMD attenuates cardiac fibrosis induced by Ang II. IMD inhibits IRE1α expression via combining the CRLR/RAMP receptor complex and activating cAMP/PKA signaling. Inhibition of IRE1α by IMD inhibits NLRP3 activation, thus preventing the development of cardiac fibrosis.
attenuated cardiomyocyte cross-sectional area, HW/BW, and mRNA levels of ANP and BNP in the myocardium of rats treated with Ang II. Moreover, IMD relieved hypertension and reduced fibrosis. We tested whether IMD acted directly on the heart. We found that IMD1–53 directly attenuated collagen I and III synthesis in CFs and inhibited CFs transforming into myofibroblasts in vitro. Hence, IMD1–53 conferred significant cardio-protection against Ang II–induced myocardial fibrosis and hypertrophy. Thus, IMD acted as an autocrine/paracrine modulator of cardiac fibrosis and had a protective role in cardiac fibrosis. These were in accordance with our previous studies [27, 53, 54]. However, it is unclear whether the anti-hypertrophic effect of IMD contributes to reducing fibrosis. The crosstalk between cardiac fibroblasts and cardiomyocytes involves paracrine signaling [56], mechanical stimuli [57], direct cell-to-cell interactions [58], and ECM–mediated signaling [59]. Especially, Ang II and TGF-β are important paracrine effectors in the crosstalk between myocardial hypertrophy and fibrosis [60]. The fibroblast can be activated by Ang II or TGF-β, which is secreted from injured cardiomyocytes [61]. It is found that the TGF-β signaling pathway is inhibited by IMD in cardiac fibrosis in a previous study [62]. In addition, many studies indicate that cardiac hypertrophy and fibrosis induced by Ang II are significantly inhibited by IMD [27, 63]. Therefore, we speculate that the protection effect of IMD on cardiac fibrosis may be involved in alleviating cardiac hypertrophy. However, as an auto/paracrine factor, whether IMD mediates the crosstalk between cardiomyocytes and fibroblasts will be further explored in future studies.

Next, we investigated the protective mechanisms of IMD against myocardial fibrosis. IMD infusion significantly attenuated inflammation in the rat cardiac fibrosis, which was consistent with previous studies [53, 55]. Inflammation has been identified as a critical player contributing to cardiac fibrosis and cardiac hypertrophy [5, 13–16]. NLRP3 inflammasome, a significant inflammation regulator, participates in CFs transforming into myofibroblasts and collagen deposition [17, 19, 20, 44]. Other factors such as Ang II, TGF-β, and LPS can also activate NLRP3 inflammasome in CFs, leading to extracellular matrix deposition [20–22]. In the present study, IMD1–53 inhibited NLRP3-inflammasome biomarkers such as NLRP3, ASC, caspase-1, IL-18, and IL-1β induced by Ang II in vivo, which was also confirmed in CFs in vitro. These results suggested that the anti-fibrotic response of IMD1–53 was potentially involved in its anti-NLRP3 inflammasome activation.

We further investigated the mechanism of IMD inhibiting inflammation. Recent data have indicated that ERS can initiate NLRP3 inflammasome activation [17]. In this study, we found that NLRP3 inflammasome activation was mediated by inhibiting ERS at least in part. ERS, a cascade of pathways, named the adaptive unfolded protein response (UPR), is initiated to recover and maintain homeostasis. However, in the event of prolonged and excessive ERS, terminal UPR is initiated and results in apoptosis, inflammation, fibroblast activation, and ultimately fibrosis development [11, 12]. ERS is found to aggravate cardiac hypertrophy and fibrosis in recent studies [9, 10]. Our previous studies show that IMD1–53 reduces myocardial ischemia injury and vascular calcification by inhibiting ERS [29, 30]. In the present study, we found that IMD1–53 inhibited myocardial ERS, as shown by increased levels of ERS markers in Ang II–treated rats and cultured CFs. Then, we further investigated which specific ERS pathways mediated IMD inhibiting Ang II–activated NLRP3 inflammasome and the fibrotic response in vitro. The IRE1α pathway is activated in response to ERS, which induces transcription of multiple cytokines and inflammatory molecules [45–48]. So, we used two kinds of siRNAs to knock down IMD and IRE1α mRNA expression in CFs. IRE1α silencing significantly decreased Ang II–activated NLRP3–inflammasome and the fibrotic response in vitro, so Ang II activated these effects via the IRE1α pathway. In addition, IMD silencing significantly increased Ang II–activated NLRP3–inflammasome and fibrotic response, which suggested that endogenous IMD had protective effects on CF inflammation and fibrosis. Moreover, IRE1α silencing significantly reversed these effects of IMD silencing on Ang II–treated CFs, which indicated that the IRE1α pathway potentially mediated these anti-NLRP3–inflammasome and anti-fibrosis effects of IMD.

Next, we revealed the signaling pathway which was involved in the role of IMD inhibiting NLRP3 and ERS. IMD exerts its biological effects by nonselective interaction with the CRLR/RAMP complex. Several signaling pathways are downstream of CRLR/RAMPs, such as cAMP/PKA, PI3K/Akt, and mitogen-activated protein kinase. However, in different cells, the post receptor signaling pathway activated by IMD is different [40]. In this study, we found that H89 but not LY294002 and PD98059 could block the effects of IMD on inflammation and ERS. The actions of IMD are associated with the activation of cAMP, which is the main pathway for IMD exerting its effect. The protective effects of IMD on vascular calcification, cardiomyocyte hypertrophy, apoptosis,
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and myocardial infarction [49, 50] are mediated by the activation of cAMP/PKA signaling, which supports our finding that cAMP/PKA activation may mediate roles of IMD downregulating NLRP3 and IRE1α expression. In previous studies, it was found that IMD1–53 inhibits the expression of TGF-β and the phosphorylation of Smad3 in cardiac fibrosis induced by aldosterone [67] or Ang II [62]. In addition, NLRP3–deficient cardiac fibroblasts display impaired differentiation and Smad activation in response to TGF-β or Ang II [68]. Meanwhile, IL-1β, which is produced by NLRP3, promotes TGF-β expression [69]. Therefore, we speculate that cAMP/PKA/IRE1α/NLRP3 may be the upstream pathway of TGF-β pathway by reducing NLRP3 activation. However, the exact role and mechanism between NLRP3 and TGF-β in cardiac fibrosis needs to be further clarified.

In summary, we provided experimental evidence that the endogenous cardiovascular-protective peptide IMD could be a paracrine/autocrine factor that prevents cardiac fibrosis by inhibiting NLRP3 via reducing the cAMP/PKA pathway. Thus, IMD may be an effective therapeutic target for cardiac fibrosis.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

Yong-Fen Qi, Chao-Shu Tang, and Jin-Sheng Zhang designed the study. Lin-Shuang Zhang, Jin-Sheng Zhang, Yue-Long Hou, Wei-Wei Lu, Xian-Qiang Ni, Fan Lin, and Xiu-Ying Liu performed all the experiments. Lin-Shuang Zhang and Jin-Sheng Zhang also performed the data analysis and drafted the manuscript. Yong-Fen Qi, Xiu-Jie Wang, Yan-Rong Yu, Mo-Zhi Jia, Ling Han, and San-Bao Chai critically revised the manuscript. All the authors reviewed the final manuscript.

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AVAILABILITY OF DATA AND MATERIAL

The data and material are available from the corresponding author on reasonable request.

DECLARATIONS

Competing Interests The authors declare no competing interests.

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