Effects of intermedin1-53 on myocardial fibrosis

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Introduction

Myocardial fibrosis is one of the important pathological features during cardiac remodeling. Regression of myocardial fibrosis may alleviate cardiac ventricle stiffness and reduce mortality of cardiovascular incident [1]. Therefore, to seek for new substances or drugs that suppress and relieve myocardial fibrosis is of great value.

As a member of the calcitonin/calcitonin gene-related peptide (CGRP) family, intermedin (IMD) is closely related to adrenomedullin (ADM) and is therefore called ADM2 [2,3]. IMD shares about 30% amino acid sequence homology with ADM. Similar to ADM, IMD is found in many tissues, including gastrointestinal tract, pancreas, and lung [4,5]. Moreover, both ADM and IMD are localized in tissues of cardiovascular systems [6,7]. It has been demonstrated that ADM and IMD seem to display their biological actions, mainly through stimulation of calcitonin receptor-like receptor/receptor activity-modifying protein complexes (CRLR/RAMP) [3]. ADM and IMD may act in an autocrine or paracrine manner to regulate cardiac function [8,9]. Additionally, IMD has similar or more potent vasodilatory and hypotensive actions compared with ADM and CGRP [7]. Our previous study discovered that expression of IMD was increased in myocardial tissue in a sub-acute myocardial ischemic injury and a hypertrophy model induced by chronic administration of isoprenaline in rat [9]. IMD gene transfer prevented endothelial cell loss, kidney damage, inflammation, and fibrosis in salt-induced hypertensive rats [4,10]. Furthermore, administration of IMD exerted a positive inotropic effect, attenuated cardiomyocyte hypertrophy and protected against deleterious effects of oxidative stress associated with ischemia–reperfusion injury [11–13]. Thus, IMD may be a potent endogenous cardio-protective substance.

Human IMD gene is considered to encode a prepropeptide of 148 amino acids [14]. Amino acid sequence analysis shows that cleavage sites are located between two basic...
Amino acids at Arg93-Arg94 resulting in the production of IMD95-147, namely IMD1-53 [3]. Our previous study showed that IMD1-53 antagonized the hypertensive effects after intravenous IMD administration [15]. However, other effects of IMD1-53 in cardiac system, for example, myocardial fibrosis, are still not clear. The aim of this study is to observe effects of IMD1-53 on myocardial fibrosis.

Materials and Methods

Preparation of animal model

The experimental procedure used in this study was approved by Ningxia Medical University Animal Care Committee (Yinchuan, China). Briefly, 35 male Sprague–Dawley (SD) rats were randomly divided into operated group (myocardial infarction model, n = 20) and sham-operated group (n = 15). Male SD rats were anesthetized by chloral hydrate (0.3 ml/100 g, intraperitoneal injection). The trachea was intubated for artificial ventilation with room air. Thoracotomy was performed at the fourth intercostals. The pericardium was opened and the heart was exposed. A 6-0 silk suture was passed around the left anterior descending coronary artery, which was then occluded by pulling on the suture tightly. Sham-operated animals underwent the same surgical procedures, but the suture around the left anterior descending coronary artery was not fastened. Afterward, penicillin was injected for anti-infection. Four weeks later, right carotid artery was separated and catheter was guided into left ventricle to determine heart function including left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), left ventricular diastolic pressure (LVEDP), left ventricular dp/dt (LV ± dp/dt) by a BL-420F Biological function measuring instrument (Taimeng, Chengdu, China). Left ventricle apical was fixed with 4% paraformaldehyde for detection of mesenchymal collagen. Other ventricle muscles were collected at −80°C for further use.

Western blot analysis

Myocardium tissue was homogenized in a lysis buffer. Then 15 μg of protein extracts were resuspended in sample buffer after protein concentration was determined. Protein samples were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred onto a polyvinylidene fluoride membrane for 3 h at 4°C and 200 mA. Nonspecific proteins were blocked by incubating the membrane with 5% defatted milk powder in Tris-buffered saline with Tween-20 (TBS-T) for 1 h at room temperature with agitation. Then the polyvinylidene fluoride membranes were incubated with the rabbit monoclonal primary antibodies (1:1000 dilution in TBS-T; Abcam, Austin, USA) overnight at 4°C. Secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG (dilution 1:5000) was added and incubated for 1 h at room temperature. The reaction was visualized by enhanced chemiluminescene (ECL; Beyotime, Haimen, China) and exposed to X-ray film. The films were scanned and analyzed by NIH image software (National Institutes of Health, Wisconsin, USA). Density ratios of different protein levels were normalized to β-actin.

Reverse transcriptase polymerase chain reaction determination

Left ventricles were separated. Then total RNA was isolated by extraction with Trizol (Invitrogen, Carlsbad, USA). Full-length cDNA was synthesized from RNA templates by using a first-strand cDNA synthesis kit (Fermentas, Ontario, Canada). Primer pairs and amplification reaction parameters for semi-quantitative polymerase chain reaction were shown in Table 1. Amplification products were detected by 2% agarose electrophoresis.

Rat cardiac fibroblast isolation

Adult male SD rats (1–3-day old; a total of four rats were used for this study) were killed and hearts were rapidly removed under sterile conditions and cut into 0.5–1.0 mm³ scrap. Then, the scrap was digested with 0.1% trypsin (Sigma, St Louis, USA) and incubated at 37°C with 5% CO2 in a humidified incubator (SHELLAB, Cornelius, USA) for 1 h. Cardiac fibroblast were obtained by differential adherence. Isolated CFbs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Gaithersburg, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Sigma), 100 μg/ml of streptomycin and 100 U/ml of penicillin. Cells were adjusted to 5×10⁴ cells/well for further use. The 2nd–4th generation cells were used in this study.

3-[4,5-dimehyl-2-thiazoly]-2,5-diphenyl-2H-tetrazolium bromide assay

Cell growth and proliferation were accessed by 3-[4,5-dimehyl-2-thiazoly]-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) assay. In brief, 200 μl aliquots of the resulting CFbs suspensions were dispensed into 96-well plates in DMEM at 37°C with 5% CO2 for 24 h. Medium were replaced with DMEM with 1% FBS and the cells continued to be incubated for 24 h. Then cells were divided into five groups: (i) 1% FBS control group: cells were cultured in DMEM with 1% FBS; (ii) 10% FBS group: cells were cultured in DMEM with 10% FBS; and (iii–v) Aldosterone (ALD; Sigma) groups: cells were exposed to different concentrations of ALD (10⁻⁸, 10⁻⁷ and 10⁻⁶ M) containing 1% FBS. Twenty hours later, supernatant were removed and MTT solutions (20 μl) were added. After being incubated at 37°C for 4 h, the mixture was removed. Then, the precipitates were dissolved in 150 μl aliquots of dimethyl sulfoxide. Absorbance was measured using a microplate reader (Bio-Rad, Hercules, USA) at a wavelength of 570 nm.
To investigate the effect of the IMD1-53 on ALD-induced CFbs proliferation, CFbs were incubated with both ALD and IMD1-53. The procedures were performed as described above.

Determination of hydroxyproline contents in supernatants

Cells were divided into six groups: (i) 1% FBS control group; (ii) ALD group: CFbs were incubated with ALD (10^{-6} M); (iii) IMD1-53 group: CFbs were cultured with 10^{-7} M IMD1-53; and (iv–vi) IMD1-53 treated-groups: medium contains 10^{-6} M ALD and different concentrations of IMD1-53 (10^{-8}, 10^{-7} and 10^{-6} M). Then, hydroxyproline contents in supernatant were detected as described previously [16].

To investigate the effect of the IMD1-53 receptor antagonist, CGRP8-37 (10^{-6} M) and ADM22-52 (10^{-6} M), the agents were administered 30 min before the addition of IMD1-53 and the other procedures were performed as described above.

Statistical analysis

GraphPad Prism 4 (La Jolla, USA) was used to analyze the results. Data were expressed as the mean ± SD. One-way analysis of variance was used to compare more than two groups, and Student’s t-test was used for two groups’ comparison. P < 0.05 was considered as statistically significant.

Results

Heart function decreased in operated animals

To confirm whether heart function was decreased in the operated group, we determined the hemodynamic changes of the cardiac. It is clear that levels of systolic blood pressure (SBP), diastolic blood pressure (DBP), and LVSP in the operated group were lower than those in the sham-operated group (Table 2). The ± LVdp/dt max in the operated group was also decreased while LVEDP was increased as compared with those in the sham-operated group (Table 2).

Collagen expression increased in operated animal

To find out whether peri-infarcted zone got fibrosis, collagen amount in cardiac tissue were determined by Sirius-red stain and western blot analysis. After Sirius-red stain,

<table>
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<th>Sham-operated group</th>
<th>Operated group</th>
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<tr>
<td>SBP (mmHg)</td>
<td>124.30 ± 2.04</td>
<td>108.60 ± 3.34**</td>
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<tr>
<td>DBP (mmHg)</td>
<td>95.01 ± 3.99</td>
<td>78.01 ± 2.74**</td>
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<tr>
<td>LVSP (mmHg)</td>
<td>124.30 ± 2.77</td>
<td>110.20 ± 3.73*</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>1.49 ± 0.57</td>
<td>15.54 ± 1.32**</td>
</tr>
<tr>
<td>+LVdp/dt max (mmHg/s)</td>
<td>3946.00 ± 92.33</td>
<td>3041.00 ± 142.40**</td>
</tr>
<tr>
<td>−LVdp/dt max (mmHg/s)</td>
<td>−3523.00 ± 108.70</td>
<td>−2624.00 ± 84.89**</td>
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<tr>
<td>HR (bpm)</td>
<td>409.00 ± 18.00</td>
<td>421.00 ± 25.00</td>
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SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LV ± dp/dt, left ventricular dp/dt.

*P < 0.05, **P < 0.01 vs sham-operated group.
normal myocardium was stained yellow while mesenchyme collagen was dyed red. In the operated group, mesenchyme collagen was significantly increased in both infarcted zone and non-infarcted zone (region remote to the infarct) compared with those in the sham-operated group (Fig. 1). Consistently, I and III type collagen expression in the non-infarcted zone was also significantly increased in the operated group (Fig. 1).

Gene and protein expression levels of IMD, CRLR/RAMP complex, and ADM receptor boosted in cardiac fibrosis
To make clear whether CGRP and ADM receptor were involved in cardiac fibrosis, we examined the mRNA and protein levels of IMD, CRLR/RAMP complex, and ADM receptor in the non-infarcted zone. Protein expression levels of IMD, CRLR, RAMP1, RAMP2, and RAMP3 in the operated group were increased about 146%, 289%, 45%, 36%, and 24% compared with the sham-operated group [P < 0.05 or P < 0.01; Fig. 2(A,B)]. Consistent with protein expression, mRNA expression of IMD, CRLR, RAMP1, RAMP2, and RAMP3 were found to be 81%, 44%, 65%, 61%, and 72% in the operated group more than those in the sham-operated group [P < 0.05 or P < 0.01; Fig. 2(C,D)].

ALD precursor content increased in the peri-infarcted zone
Because of the crucial effect of ALD in cardiac fibrosis, we determined the mRNA level of ALD precursor, CYP11B2, in the peri-infarcted zone. As shown in Fig. 3, after the left anterior descending coronary artery was occluded, compared with the sham-operated group, mRNA level of CYP11B2 in the peri-infarcted zone was up-regulated by 39% (P < 0.01).

IMD1-53 decreased ALD-induced CFbs proliferation
Cell proliferative responses were augmented when the cells were cultured with the high concentration of FBS. Results from the in vitro experiment showed when CFbs were treated with 10% FBS, cell numbers increased significantly compared with 1% FBS [P < 0.01; Fig. 4(A)]. CFbs proliferation was significantly promoted with ALD treatment of different concentrations (10^-8, 10^-7 and 10^-6 M) in a dose-dependent manner compared with 1% FBS group [P < 0.05 or P < 0.01; Fig. 4(A)]. However, there were no significant differences between 10% FBS group and 10^-6 M ALD group (P > 0.05). Consequently, 10^-6 M ALD was applied in subsequent experiments.

To determine whether IMD1-53 had a direct inhibitory effect on CFbs proliferation, CFbs were incubated with IMD1-53 for 24 h. Results showed that IMD1-53 treatment failed to affect proliferation and activity of CFbs (P > 0.05). After IMD1-53 treatment, ALD-induced CFbs proliferation were decreased approximate 14%, 22%, and 33% [P < 0.05 or P < 0.01; Fig. 4(B)]. These results indicated that IMD1-53 inhibited ALD-induced CFbs proliferation in a dose-dependent manner.

IMD1-53 reduced hydroxyproline amount after ALD-stimulation
CFbs incubated with 10^-6 M ALD released more hydroxyproline contents than that with 1% FBS control group [P < 0.01; Fig. 4(C)]. Hydroxyproline contents were about 20%, 36%, and 45% decreased in IMD1-53 treatment groups (10^-8, 10^-7, and 10^-6 M) as compared with ALD group (10^-6 M) (P < 0.05 or P < 0.01). However, IMD1-53 alone did not affect hydroxyproline amounts [P > 0.05; Fig. 4(C)]. Incubation of IMD1-53 receptor antagonist (ADM22-52 and CGRP8-37) with IMD1-53 and ALD made hydroxyproline contents increased about 53% and 33% in CFbs supernatant than with IMD1-53 and ALD only [P < 0.05 or P < 0.01; Fig. 4(D)].

Discussion
Previous studies of our laboratory and other groups have proved the presence of IMD mRNA and protein expression in the heart [7]. Our present study indicates that IMD1-53 may alleviate cardiac fibrosis in myocardial infarction rat and ALD-induced CFbs fibrosis.
As an important risk factor, myocardial fibrosis is a common pathophysiological process in the development of many heart diseases. Cardiocytes are easy-to-get fibrosis in various pathological conditions such as myocardial ischemia [17]. Excessive CFbs numbers and abundant collagens deposition are markers of cardiac fibrosis. The present data showed I and III type collagen protein expression in the non-infarcted zone was increased and mesenchyme collagen in both peri-infarcted zone and non-infarcted zones was also augmented, which indicated that myocardial fibrosis model was successfully constructed after the left anterior descending coronary artery was ligated. Moreover, cardiac function in the operated group was also decreased, which was assessed by lower levels of SBP, DBP, LVSP, and $+LVdp/dt_{max}$ and higher level of LVEDP.

ALD is an important hormone in the rennin–angiotensin–aldosterone system (RAAS) and plays a crucial role in the development of hypertension, heart failure, and other cardiovascular diseases [18]. RAAS acts in a paracrine/autocrine manner and is of great significance in maintaining homeostasis in functions and structure of cardiovascular system [19]. As an essential hormone in the RAAS system [18], ALD promotes collagen deposition and fibrosis, thus leading to fibrosis and structural remodeling of the heart [20]. In this study, we found that the mRNA expression of ALD precursor, CYP11B2, in the peri-infarcted zone was increased after the left anterior descending coronary artery was occluded. Moreover, in vitro experiments confirmed ALD promoted CFbs proliferation and hydroxyproline release in a dose-dependent manner. Consistent with other
report [21], these results suggest that ALD is involved in cardiac fibrosis in vivo and in vitro. We therefore chose ALD as a stimulator and used the concentration of $10^{-6}$ M for further in vitro study.

IMD is a vasoactive peptide of the CGRP family [2]. Administration of IMD protects the isolated rat heart against the deleterious effects of oxidative stress associated with ischemia–reperfusion injury [12]. IMD infusion decreased severe cardiac impairment and lowered LDH activity, suggesting IMD might ameliorate target-organ damage [22]. These studies indicated that IMD, very similar to another CGRP member ADM, may play multiple roles in different pathophysiological process [15,23]. This study showed that ADM had a strong attribution in regulating heart function. However, it was confirmed that IMD1-53 could generate higher blood pressure and faster heart rate in a dose-dependent manner than ADM did after being injected with equivalent dose of IMD1-53 and AMD on rats [24]. In cardiomyocytes of adult spontaneously hypertensive rats, IMD (but not ADM) is markedly up-regulated as it is in response to oxidative stress [25]. Hirose et al. [25] confirmed that ADM mRNA in coronary ligation rats was much more abundantly expressed than IMD while the

**Figure 3** mRNA expression of CYP11B2 in rats mRNA levels were determined by semi-quantitative reverse transcriptase polymerase chain reaction (A) and expressed as the ratio of the density of the tested mRNA band over the density of β-actin mRNA band (B). Data were expressed as the mean ± SD. *$P < 0.05$ vs sham-operated group. Lanes 1–3, sham-operated groups; lanes 4–6, operated groups. $n = 6$.

**Figure 4** Effect of IMD1-53 on ALD-induced cell proliferation and collagen synthesis in cultured CFbs (A) Fibroblasts were treated with different concentrations of ALD ($10^{-8}$, $10^{-7}$ and $10^{-6}$ M) for 24 h ($n = 3$/group). Proliferation of CFbs was measured by MTT assay. Different concentrations of FBS (1% or 10%) were used as the control group. (B) Different concentrations of IMD1-53 ($10^{-8}$, $10^{-7}$ and $10^{-6}$ M) were added to culture medium after ALD induction. (C) CFbs were treated with different concentrations of IMD ($10^{-8}$, $10^{-7}$ and $10^{-6}$ M) and ALD for 24 h. Intracellular collagen level was determined by measuring hydroxyproline in the supernatant. (D) Hydroxyproline contents in the supernatant after CFbs were treated with IMD, ALD, or/ and ADM22-52/CGRP8-37 for 24 h. *$P < 0.05$, **$P < 0.01$ vs control group; *$P < 0.05$, **$P < 0.01$ vs ALD ($10^{-6}$ M) group; *$P < 0.05$, **$P < 0.01$ vs IMD group.
increase of the expression of ADM mRNA in coronary ligation rats was not so marked than that of IMD mRNA. Therefore, IMD may be extremely effective to some cardiovascular stress in contrast to ADM, even though it may be expressed in the cardiovascular system of a healthy body at a level lower than ADM. These findings suggest a much more prominent role for IMD than ADM locally in the myocardium as an endogenous regulator of cardiac. In the present study, mRNA and protein expression of IMD1-53 in the non-infarcted zone was found to be up-regulated in operated rats, indicating IMD1-53 might be involved in cardiac fibrosis. IMD has been reported to increase regional blood flows in normal rats [9]. To our surprise, IMD1-53 had no remarkable inhibitory action on basic cultured CFbs. However, IMD1-53 decreased ALD-induced CFbs proliferation and hydroxyproline secretion in a dose-dependent manner. Our results suggested that IMD1-53 might be a strong anti-fibrosis agent on rats.

The biological actions of ADM and IMD are mediated via binding to their receptors [8]. The overlapping biological effects of these two peptides are due to the similar structure and cross-reactivity between homologous receptors [3]. ADM has two receptors formed by respective combination of CRLR and RAMP 2 or 3 [8]. It also has some affinity for CGRP receptors, which are composed of CRLR and RAMP1 [26]. Like ADM, IMD works through CRLR/RAMP receptor complexes [27]. IMD acts non-selectively at all three CRLR/RAMP complexes [3,25]. CRLR receptor blocker or RAMP inhibitors prevented the effects of IMD on cardiac function [26]. Cardiovascular effects of IMD microinjection into the nucleus tractus solitarius were significantly attenuated by an antagonist of ADM [24]. Ren et al. [24] reported that IMD increased heart rate through the CRLR to produce a CRLR/RAMP receptor complex, while CGRP8-37 and ADM22-52, the receptor antagonists of CRLR/RAMP and ADM, inhibited this kind of effect. Therefore, up-regulation of IMD and its relevant receptors might be an adaptive response to myocardial damage. We found that the expression levels of RAMP1/2/3 and CRLR were increased at both transcriptional and translational levels on operated rats, which was consistent with the expression of IMD. Moreover, the presence of the receptor antagonists CGRP8-37 and ADM22-52 remarkably decreased the anti-fibrosis effect of IMD after ALD stimulation by in vitro experiment. These results indicated the important role of IMD in cardiac fibrosis, at least partly, might be closely related to binding of this peptide to CRLR/RAMP complex and ADM receptor.

The present study has revealed some new findings concerning the effect of IMD on cardiac function. Our results showed that cardiac fibrosis is associated with enhanced IMD1-53, CRLR, and RAMP1/2/3 protein levels in the non-infarcted zone of cardiac tissue. In addition, treatment with antagonists of CRLR/RAMP and ADM also accelerates the process of cardiac fibrosis. This study suggested that IMD1-53 is involved in cardiac fibrosis. It alleviates cardiac fibrosis most probably through the combination with CRLR/RAMP complex and ADM receptor. Our further study will focus on the exact molecular mechanism of IMD on cardiac fibrosis.

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