



Alarin alleviated cardiac fibrosis via attenuating oxidative stress in heart failure rats

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Abstract

The present study was to explore whether alarin could alleviate heart failure (HF) and attenuate cardiac fibrosis via inhibiting oxidative stress. The fibrosis of cardiac fibroblasts (CFs) was induced by angiotensin (Ang) II. HF models were induced by ligation of the left anterior descending artery to cause ischemia myocardial infarction (MI) in Sprague–Dawley rats. Alarin (1.0 nM/kg/d) was administrated by intraperitoneal injection for 28 days. The decreases of left ventricular (LV) ejection fraction (EF), fractional shortening (FS), the maximum of the first differentiation of LV pressure ($LV \pm dp/dt_{max}$) and LV systolic pressure (LVSP), and the increases of LV volume in systole (LVVS), LV volume in diastole (LVVD), LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) in MI rats were improved by alarin treatment. The increases in the expression levels of collagen I, collagen III, and transforming growth factor (TGF)- β were inhibited by alarin treatment in CFs and in the hearts of MI rats. The levels of NADPH oxidase (Nox) activity, superoxide anions and malondialdehyde (MDA) levels were increased, and the level of superoxide dismutase (SOD) activity was reduced in Ang II-treated CFs, which were reversed by alarin. Nox1 overexpression reversed the effects of alarin on attenuating the increases of collagen I, collagen III and TGF- β expression levels induced by Ang II in CFs. These results indicated that alarin improved HF and cardiac fibrosis via inhibiting oxidative stress in HF rats. Nox1 played important roles in the regulation of alarin effects on attenuating CFs fibrosis induced by Ang II.

Keywords Alarin · Cardiac fibroblasts · Fibrosis · Oxidative stress · Heart failure

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Introduction

Heart failure, a complex syndrome due to structural or functional cardiac disorders, is characterized by forming interstitial fibrosis of heart, including increases in the expression of collagen I, collagen III and transforming growth factor- β (TGF- β) (Liu et al. 2018; Wang et al. 2019a; Yue et al. 2017). Coronary artery ligation-induced HF rats showed markedly impaired cardiac function and hemodynamics (Gan et al. 2014). Fibrosis in the heart is an important driver of disease progression of chronic heart failure (CHF) (Tarone et al. 2014), and excessive cardiac fibrosis causes large infarct scars, leading to cardiac dysfunction and cardiac dilatation (Kong et al. 2014; Heineke and Molkentin 2006). Cardiac fibroblasts (CFs) are currently considered as the main source of cardiac fibrosis in response to ischemic injury (Tallquist and Molkentin 2017). CFs played important roles in postinfarction remodeling, which can ultimately result in pathological cardiac fibrosis and HF (Philip et al.

2019). Cardiac fibrosis is a hallmark of HF, and there is no effective drug to treat it.

Alarin, a bioactive peptide consisted of 25 amino acids, was identified as the fourth member of the galanin peptide family, just next to galanin, galanin message-associated protein (GMAP) and galanin-like peptide (GALP) (Fang et al. 2020; Miko et al. 2014). Alarin was discovered as an alternate transcript of the GALP gene (Santic et al. 2006, 2007), and can be detected in circulating blood (Hu et al. 2019). It showed that galaninergic system can be considered a promising target to reduce energy dysregulation and attenuate oxidative damage in myocardial injury induced by ischaemia/reperfusion (I/R) of rats (Studneva et al. 2019b). An agonist of galanin receptors GalR1-3 can attenuate doxorubicin-induced cardiotoxicity (Studneva et al. 2019a). Alarin has no detectable affinity toward any of the known three galanin receptor subtypes (Santic et al. 2007; Boughton et al. 2010), therefore its similar effects cannot be expected. Whether alarin could alleviate heart failure and cardiac fibrosis is not well known.

Oxidative stress is often detected and considered to play key roles in pathological cardiac remodeling and the transition to cardiac failure (Sag et al. 2014; Madamanchi and Runge 2013). Angiotensin (Ang) II-induced collagen production is mediated via reactive oxygen species (ROS) generation in adult rat cardiac fibroblasts (CFs). Ang II activates ROS-sensitive kinases that are critical in regulating fibrotic remodeling of the heart (Ohtsu et al. 2005). N-terminal galanin fragment 2–15 (G) decreased H9C2 cells apoptosis and mitochondrial ROS production stimulated by hypoxia and reoxygenation conditions (Pisarenko et al. 2017). However, whether alarin administration attenuated cardiac fibrosis in MI-induced HF rats via inhibiting oxidative stress is not well known. The current study was designed to explore the anti-fibrosis effects of alarin in MI-induced HF rats, and whether oxidative stress was involved in the effects of alarin on alleviating HF.

Materials and methods

Animals

Experiments were carried out using 150–180 g male Sprague–Dawley (SD) rats (Vital River Biological Co., Ltd, Beijing, China). The rats were kept in a temperature-controlled room on a 12 h light–dark cycle with free access to standard chow and tap water. All procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

Myocardial infarction model

The myocardial infarction (MI) rats were induced by coronary artery ligation with sterile techniques as previously reported (Gan et al. 2011). Briefly, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and randomly subjected to the ligation of the left anterior descending coronary artery and sham-operated (Sham) groups. The heart was exposed through a left intercostal thoracotomy, and left coronary artery was looped by a single nylon suture (7-0). Finally, the heart was quickly repositioned into the chest. The Sham rats were treated the same as the coronary ligation rats except that their coronary arteries were not ligated. One day later, the rats were randomly assigned to four groups, including sham + saline group, MI + saline group, sham + Alarin group, and MI + Alarin group. Alarin rat (1.0 nM/kg/d in 300 µl saline, 026–33, Phoenix Pharmaceuticals, CA, USA) was administered by intraperitoneal injection for 28 days, rats in the sham group and the MI group received the same volume of saline. In another experiment, an alarin antagonist alarin 6–25 Cys (Wang et al. 2020; Fraley et al. 2013) (Ala6-25 Cys, 2.0 nM/kg/d in 300 µl saline, GL Biochem, Shanghai, China), or galanin receptor antagonists M35 (2.0 nM/kg/d in 300 µl saline, American Peptide Co., CA, USA) or C7 (2.0 nM/kg/d in 300 µl saline, American Peptide Co.) were administered by intraperitoneal injection following alarin.

Echocardiography

Twenty-eight days after coronary ligation, transthoracic echocardiography was performed using an ultrasound system (VisualSonics, Toronto, Canada) with a 21-MHz probe under isoflurane anesthesia (2.0–3.0%). The left ventricular end-systolic diameter (LVESD), LV end-diastolic diameter (LVEDD), LV volumes in diastole (LVVd) and LV volumes in systole (LVVs) were measured. The LV ejection fraction (EF), and fractional shortening (FS) were calculated. Measurements were averaged over three consecutive cardiac cycles. Two days later, hemodynamic monitoring was determined.

Hemodynamic monitoring

Rats were anesthetized with isoflurane (2.0–3.0%). Conductance micromanometer catheter (1.4F, Millar Instruments, TX, USA) was inserted into the LV chamber via the right carotid artery across the aortic valve. The left ventricle systolic pressure (LVSP), and LV end-diastolic

pressure (LVEDP), maximum of the first differentiation of LV pressure ($LV + dp/dt$) and decline ($LV - dp/dt$), were obtained with a PowerLab data acquisition system (AD Instruments, Sydney, Australia).

Cardiac sample collection

After hemodynamic monitoring determination, the rats were euthanasia by cervical dislocation after anesthetized with 2.5% isoflurane. The heart was removed after phosphate-buffered saline (PBS, Biochannel Biotechnology Co., Ltd, Nanjing, China) perfusion. LV was collected to use in the next experiments. Approximately 50 mg LV sample was used to isolate the RNA or protein.

Sirius red staining

Cardiac sections (5 μ m) were examined by Sirius red staining (Service Biological Technology Co., Ltd, Wuhan, China) according to the manufacture instruments to measure the fibrosis of cardiomyocytes. Three to five random fields (around 30–50 cells per field) were selected from each of three sections from each animal for observation under a light microscope (Carl Zeiss GmbH, Oberkochen, Germany). Images were analyzed using Image-Pro Plus software (Media Cybernetics, Inc., MD, USA).

Culture of cardiac fibroblasts

Rat cardiac fibroblasts (CFs) were isolated from 1 to 3 days old SD rats. Briefly, CFs were separated from cardiomyocytes by gravity separation and grown to confluence on 10 cm cell culture dishes with growth media (DMEM including 10% FBS, 1% penicillin and 1% streptomycin) at 37 °C in humid air with 5% CO₂ and 95% O₂. CFs from the second passage were used for the subsequent experiments. CFs were incubated with angiotensin II human (Ang II, A9525, 10⁻⁶ M, Sigma, MO, USA) for 24 h to induce the fibrotic phenotype. CFs were assigned to four groups, including PBS group, Ang II group, alarin (10 nM) group, Ang II + alarin (10 nM) group. Alarin and Ang II were devolved in PBS and added into the growth media in 6 cm plate (10⁵ cells/plate) at the same time.

Quantitative real time-PCR (qRT-PCR)

The total RNA in samples was extracted with Trizol (Ambion, TX, USA). cDNA was extracted from the RNA with reverse transcription using random primers in a total volume of 10 μ L according to the instructions of the PrimeScript™ RT Master Mix (Takara, China). All cDNA were stored at -70 °C before used. Collagen I, collagen III and transforming growth factor (TGF)- β mRNA levels were determined with SYBR Green I fluorescence (Invitrogen; Thermo Fisher Scientific, Inc). All samples were amplified in triplicates for 45 cycles in a 384-well plate. GAPDH was used as the internal controls for mRNA (Yang et al. 2020). Relative expression levels were quantified using the 2^{- $\Delta\Delta C_q$} method. The primers are shown in Table 1.

Western blotting

The samples were sonicated in RIPA lysis buffer (Bio-Channel Biotechnology Co., Ltd.) and homogenized. The debris was removed by centrifugation at 12,000 \times g for 10 min at 4 °C and the supernatant was collected. Subsequently, about 30–40 μ g protein was separated by gel electrophoresis, transferred to PVDF membrane. The membrane was blocked with 5% skimmed milk powder at room temperature for 1 h and probed with primary antibodies overnight at 4 °C against collagen I, collagen III, TGF- β and Nox1 (Abcam, MA, USA). Then, a secondary antibody (Abcam) was added and incubated at room temperature for 1 h and GAPDH (1 Abcam) was used as an internal control. The bands were visualized via ECL (Beyotime, Shanghai, China). Images were analyzed using Image-Pro Plus software (XRayScan; CAD/CAM Services, Inc.).

NADPH oxidases 1 overexpression

Recombinant adenoviral vectors harboring green fluorescent protein (Ad-GFP) or NADPH oxidases 1 (Ad-Nox1) were obtained from Genechem (Shanghai, China). Adenovirus (10⁸ TU/ml) was added to the growth media.

Table 1 List of utilized primers for qRT-PCR

Gene	Species	Forward primer	Reverse primer
Collagen I	Rat	TCAAGATGGTGGCCGTTAC	CTGCGGATGTCTCAATCTG
Collagen III	Rat	CGAGATTAAAGCAAGAGGAA	GAGGCTTCTTACATACCAC
TGF- β	Rat	CAGGGAGTAAGGGACACGA	ACAGCAGTTAGGAACCCAGAT
GAPDH	Rat	GGCACAGTCAAGGCTGAGAATG	ATGGTGGTGAAGACGCCAGTA

TGF- β transforming growth factor- β , GAPDH glyceraldehyde-3-phosphate dehydrogenase

Measurement of Nox activity

The Nox activity in the CFs was measured by enhanced lucigenin chemiluminescence. Briefly, the CFs were sonicated and homogenized. The debris was removed by centrifugation at $12,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was collected. NADPH ($100\text{ }\mu\text{M}$) was added to the supernatant as a substrate to react with Nox and generate superoxide anions. The light emission produced by the reaction of lucigenin ($5\text{ }\mu\text{M}$) with superoxide anions was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values represented the Nox activity and were expressed as the mean light units (MLU) per minute per milligram of protein.

Measurement of superoxide anions

Superoxide anions level in the CFs was determined by lucigenin-derived chemiluminescence. Briefly, the CFs were sonicated and homogenized. The debris was removed by centrifugation at $12,000\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$ and the supernatant was collected. The reaction with superoxide anions was started by adding dark-adapted lucigenin ($5\text{ }\mu\text{M}$) to each sample to cause photon emission, which was measured with a microplate reader (BioTek, VT, USA) once every minute for 10 min. The values representing the superoxide anions level were expressed as the MLU per minute per milligram of protein.

Malondialdehyde level in the heart

The CFs samples were homogenized in lysis buffer (Thermo Fisher Scientific, MA, USA). The malondialdehyde (MDA) level was determined using an ELISA kit (USCN Business Co., Ltd., Wuhan, China) following the manufacturer's instructions.

Superoxide dismutase (SOD) activity level

The CFs samples were collected and homogenated. Superoxide dismutase (SOD) measurement was performed according to the manufacturer's instructions (Jiancheng Bioengineering Institute, Nanjing, China) using a microplate reader (BioTek, VT, USA).

Statistical analyses

Data were presented as the mean \pm standard error of the mean (SEM) and were analyzed using GraphPad Prism 7.0 (GraphPad software Inc., CA, USA). Statistics were completed using one-way ANOVA, followed by Bonferroni

test for post hoc analysis when multiple comparisons were made. A two-tailed p value < 0.05 was considered statistically significant.

Results

Effects of alarin on cardiac function and fibrosis in HF rats

EF (29.72 ± 2.11 vs. 64.81 ± 3.17) and FS (19.72 ± 1.61 vs. 31.80 ± 1.64) were reduced in MI-induced HF rats, EF and FS were increased by 50.14% and 69.42% after alarin treatment in MI rats. MI induced the increases in LVVS (81.80 ± 8.02 vs. $23.63 \pm 2.99\text{ }\mu\text{l}$), LVVD (106.50 ± 7.60 vs. $52.20 \pm 4.41\text{ }\mu\text{l}$), LVESD (9.43 ± 1.09 vs. $2.01 \pm 0.23\text{ mm}$) and LVEDD (12.71 ± 1.35 vs. $4.43 \pm 0.45\text{ mm}$), which were reversed after alarin. LV + dp/dt_{max} (2351.67 ± 123.97 vs. $3365.53 \pm 135.18\text{ mm Hg/s}$) and LVSP (106.43 ± 2.91 vs. $132.11 \pm 2.19\text{ mm Hg}$) were reduced in MI rats, and these decreases were enhanced 68.96% and 66.15% by alarin treatment, respectively. LVEDP was increased in the HF rats (8.33 ± 0.70 vs. $3.36 \pm 0.44\text{ mm Hg}$), which was reversed 64.99% by alarin (Table 2).

Effects of alarin on cardiac fibrosis in HF rats

The fibrosis of the heart in MI rat was increased, which was attenuated 54.44% by alarin treatment (Fig. 1A). The expression levels of collagen I, collagen III and TGF- β were increased in the heart of MI-induced HF rats, and these increases were alleviated by alarin (Fig. 1B).

Effects of alarin and galanin receptor antagonists

Ala6-25 Cys, an antagonist of alarin, reversed the attenuating effects of alarin on the levels of collagen I, collagen III and TGF- β in the heart of MI-induced HF rats. Ala6-25 Cys didn't further increase the expressions of collagen I, collagen III and TGF- β in the heart of MI rats (Fig. 2A). Galanin receptor antagonist M35 had no effect on the roles of alarin in inhibiting the increases of collagen I, collagen III, and TGF- β in the heart of MI rats (Fig. 2B). Galanin receptor antagonist C7 had no effect on the roles of alarin in inhibiting the increases of collagen I, collagen III, and TGF- β in the heart of MI rats (Fig. 2C). Ala6-25 Cys, M35 and C7 reduced EF and FS of rats compared with the saline group. LVESD, LVEDD, LVVS and LVVD were increased in the rats of treating with Ala6-25 Cys, M35 and C7 (Fig. 2D).

Table 2 Echocardiographic examination of the left ventricular function

Variables	Sham + Saline	MI + Saline	Sham + Alarin	MI + Alarin
EF (%)	64.81 ± 2.11	29.72 ± 3.17*	65.80 ± 2.17	47.36 ± 3.52 [#]
FS (%)	31.80 ± 1.64	19.72 ± 1.61*	33.84 ± 1.68	28.19 ± 1.57 [#]
LVESD (mm)	2.01 ± 0.23	9.43 ± 1.09*	2.16 ± 0.30	4.15 ± 0.58 [#]
LVEDD (mm)	4.43 ± 0.45	12.71 ± 1.35*	4.85 ± 0.47	6.50 ± 0.86 [#]
LVVS (μl)	23.63 ± 2.99	81.80 ± 8.02*	30.00 ± 4.45	44.40 ± 6.31 [#]
LVVD (μl)	52.20 ± 4.41	106.50 ± 7.60*	49.00 ± 3.51	68.50 ± 6.60 [#]
LV + dp/dt _{max} (mm Hg/s)	3365.53 ± 135.18	2351.67 ± 123.97*	3474.96 ± 125.03	3050.83 ± 168.48 [#]
LVSP	132.11 ± 2.19	106.43 ± 2.91*	131.35 ± 2.25	123.47 ± 3.16 [#]
LVEDP	3.36 ± 0.44	8.33 ± 0.70*	3.62 ± 0.44	5.10 ± 0.49 [#]

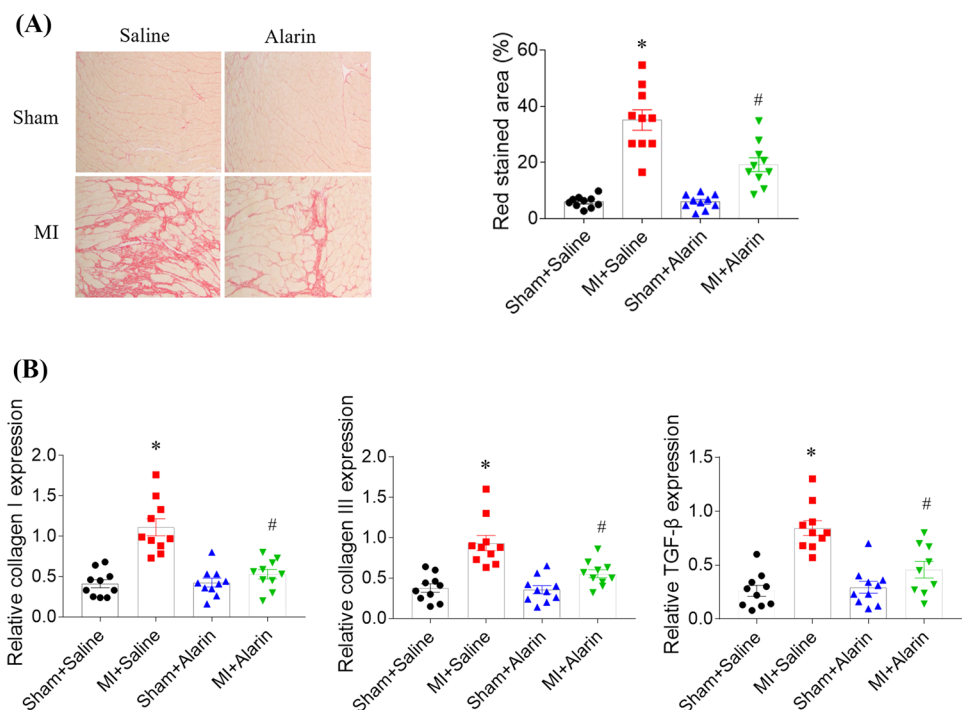
The results are expressed as the mean ± SEM of 10 rats per group

EF ejection fraction, FS fractional shortening, LV left ventricular, LVESD LV end-systolic diameter, LVEDD LV end-diastolic diameter, LVVD LV volume in diastole, LVVS LV volume in systole

* $p < 0.05$ versus the Sham + Saline group

[#] $p < 0.05$ versus the MI + Saline group

Fig. 1 Alarin improved cardiac fibrosis in myocardial infarction (MI) induced heart failure (HF) rats. **A** alarin alleviated cardiac fibrosis in MI-induced HF rats. **B** alarin inhibited the increases of collagen I, collagen III, and transforming growth factor (TGF)- β in the heart of MI rats. The results are expressed as mean ± SEM. $N = 10$ for each group. * $p < 0.05$ versus the Sham + Saline group; [#] $p < 0.05$ versus the MI + Saline group



Effects of alarin on CFs fibrosis induced by Ang II

Treatment with low dose alarin (1.0 nM) had no effect on the increases of collagen I, collagen III and TGF- β induced by Ang II in CFs. Both the middle (10 nM) and high (100 nM) doses of alarin inhibited the increases of collagen I, collagen III and TGF- β induced by Ang II in CFs (Fig. 3A). Middle dose of alarin (10 nM) was selected in the following experiments.

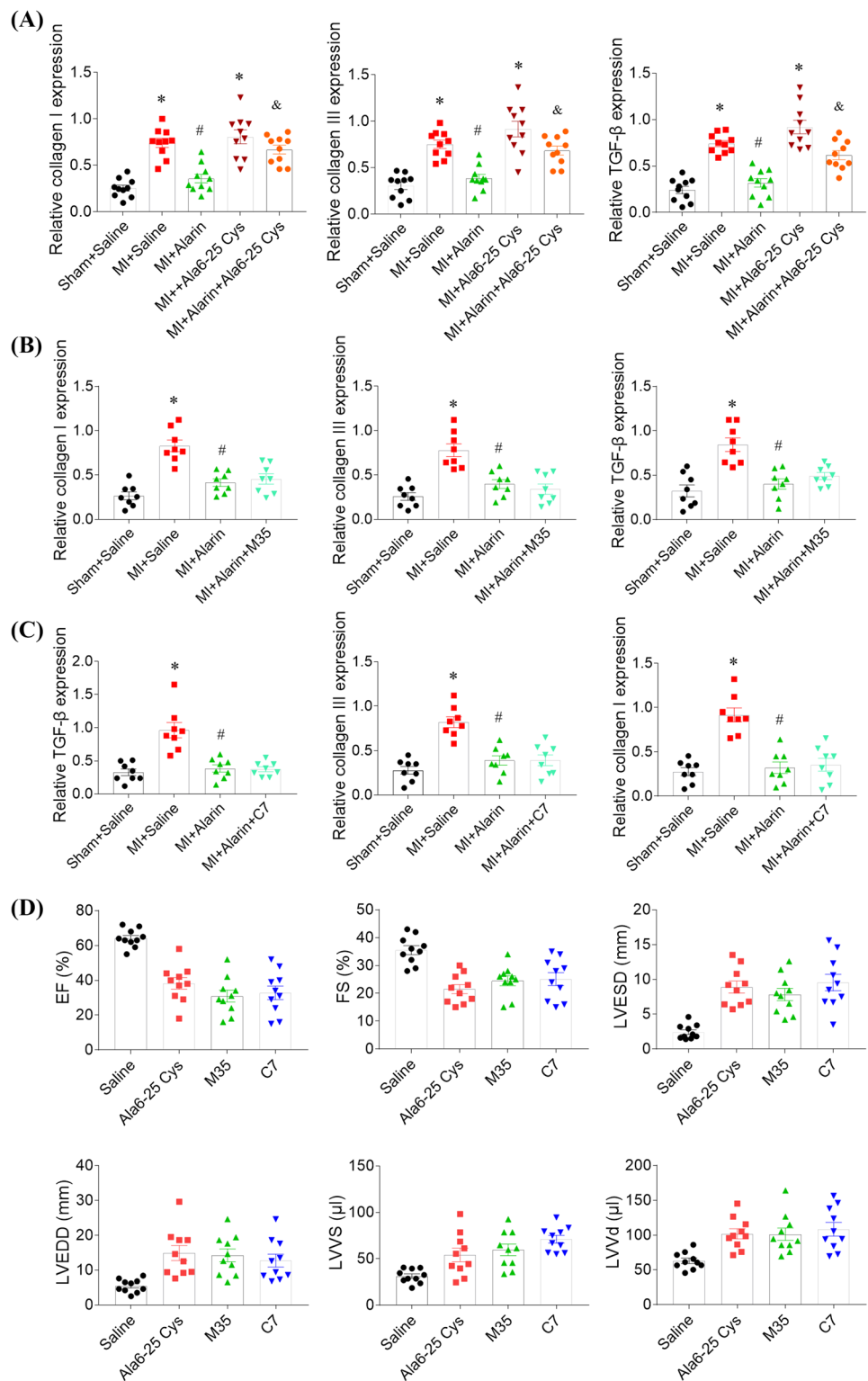
Ala6-25 Cys reversed the attenuating effects of alarin on the increases of collagen I, collagen III and TGF- β induced by Ang II in CFs (Fig. 3B). The protein expression levels of

collagen I, collagen III and TGF- β in CFs were heightened treated with Ang II, which was revised by alarin (Fig. 3C).

Effects of alarin on oxidative stress

Nox activity level was increased in the heart of MI rats (18.11 ± 1.61 vs. 8.21 ± 0.82 MLU/min/mg protein). Alarin alleviated the increase of Nox activity level in the heart of MI rats (10.89 ± 0.95 vs. 18.11 ± 1.61 MLU/min/mg protein). The increase in the level of superoxide anion in the heart of MI rats (17.56 ± 2.18 vs. 7.66 ± 1.01 MLU/

Fig. 2 Effects of alarin and galanin receptor antagonists. **A** Alarin 6-25 Cys (Ala6-25 Cys), an antagonist of alarin, reversed the attenuating effects of alarin on the increases of collagen I, collagen III and transforming growth factor (TGF)- β in the heart of myocardial infarction (MI) rats. **B** Galanin receptor antagonist M35 had no effect on the roles of alarin in inhibiting increases of collagen I, collagen III, and TGF- β in the heart of MI rats. **C** Galanin receptor antagonist C7 had no effect on the roles of alarin in inhibiting increases of collagen I, collagen III, and TGF- β in the heart of MI rats. **D** Alarin and galanin receptor antagonists alleviated cardiac dysfunction in myocardial infarction (MI) induced HF rats. The results are expressed as mean \pm SEM. $N=8$ for each group. * $p < 0.05$ versus the Sham + Saline (**A**, **B** and **C**) or Saline (**D**) group; # $p < 0.05$ versus the MI + Saline group; & $p < 0.05$ versus the MI + Alarin group

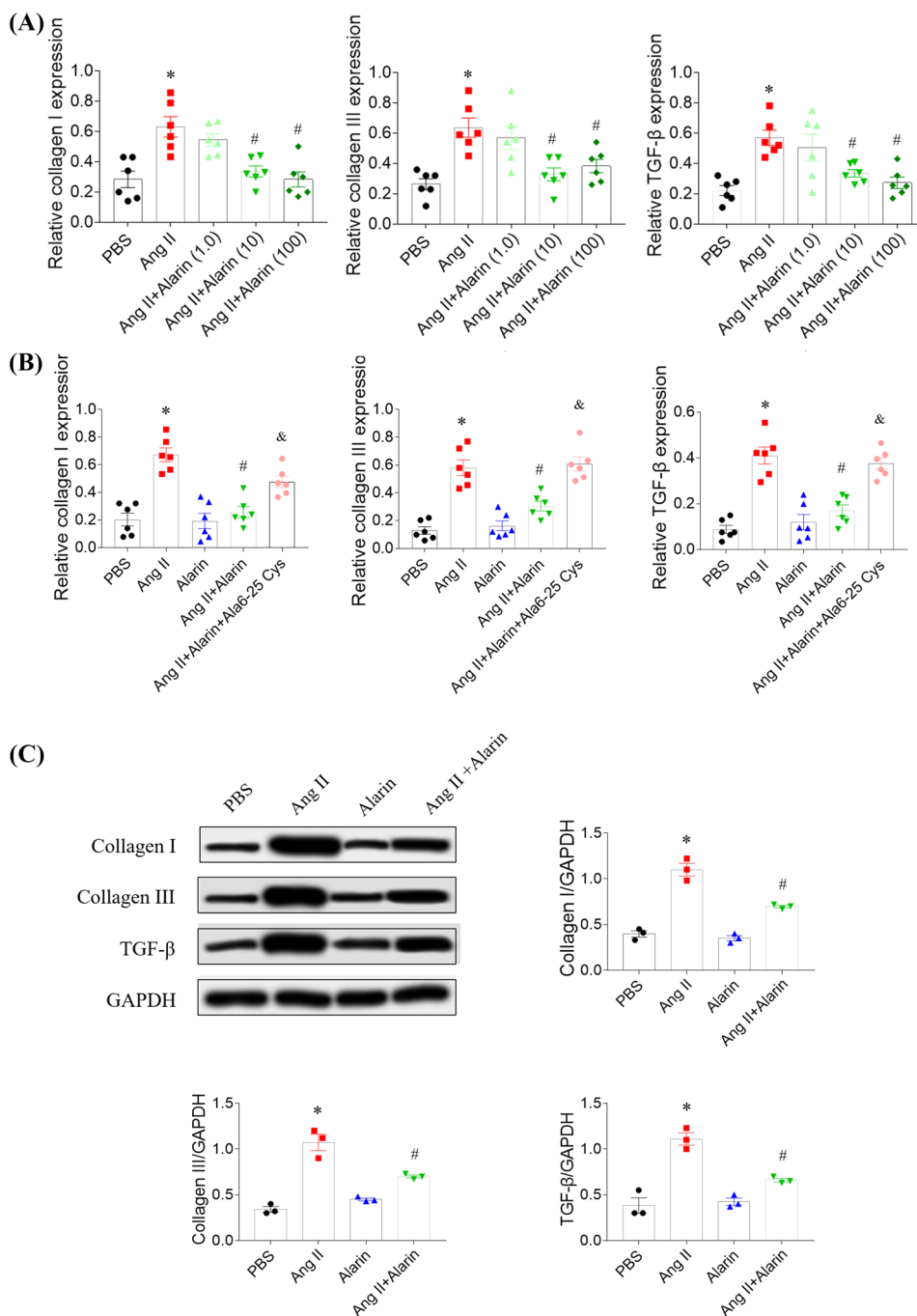


min/mg protein) was attenuated after alarin administration (11.85 ± 0.97 vs. 17.56 ± 2.18 MLU/min/mg protein).

MDA level was increased in the heart of MI rats (201.24 ± 19.30 vs. 67.09 ± 6.53 mmol/mg protein), which was attenuated by alarin treatment (109.17 ± 10.42 vs.

201.24 ± 19.30 mmol/mg protein). SOD activity level was reduced in the heart of MI rats (142.81 ± 12.75 vs. 365.25 ± 25.88 U/mg protein), and alarin administration enhanced the decrease of SOD activity level in the heart of

Fig. 3 Alarin alleviated cardiac fibroblasts (CFs) fibrosis induced by angiotensin (Ang) II. **A** Low dose had no effect, and middle and high doses of alarin inhibited the increases of collagen I, collagen III and transforming growth factor- β (TGF- β) mRNA levels in CFs induced by Ang II. **B** Alarin 6-25 Cys (Ala6-25 Cys), an antagonist of alarin, reversed the attenuating effects of alarin on the increases of collagen I, collagen III and transforming growth factor (TGF)- β induced by Ang II in CFs. **C** alarin attenuated the increases of collagen I, collagen III and TGF- β protein levels in CFs induced by Ang II. The results are expressed as mean \pm SEM. * p < 0.05 versus the PBS group; # p < 0.05 versus the Ang II group



MI rats (237.82 ± 16.52 vs. 142.81 ± 12.75 U/mg protein) (Fig. 4A).

Nox activity level was increased in Ang II-treated CFs (13.57 ± 1.23 vs. 4.43 ± 0.39 MLU/min/mg protein). Alarin attenuated the increase of Nox activity level induced by Ang II in CFs (6.95 ± 0.95 vs. 13.57 ± 1.23 MLU/min/mg protein). The increase in the level of superoxide anion induced by Ang II in CFs (12.98 ± 1.07 vs. 5.98 ± 0.60 MLU/min/mg protein) was inhibited after alarin

treatment (7.18 ± 0.98 vs. 12.98 ± 1.07 MLU/min/mg protein). MDA level was increased in Ang II-treated CFs (248.67 ± 45.21 vs. 99.67 ± 7.58 mmol/mg protein), which was attenuated by alarin treatment (186.17 ± 29.71 vs. 248.67 ± 45.21 mmol/mg protein). Ang II inhibited SOD activity level in CFs (112.17 ± 9.66 vs. 254.83 ± 33.13 U/mg protein), and alarin administration enhanced the decrease of SOD activity level in Ang II-treated CFs (180.17 ± 19.76 vs. 112.17 ± 9.66 U/mg protein) (Fig. 4B).

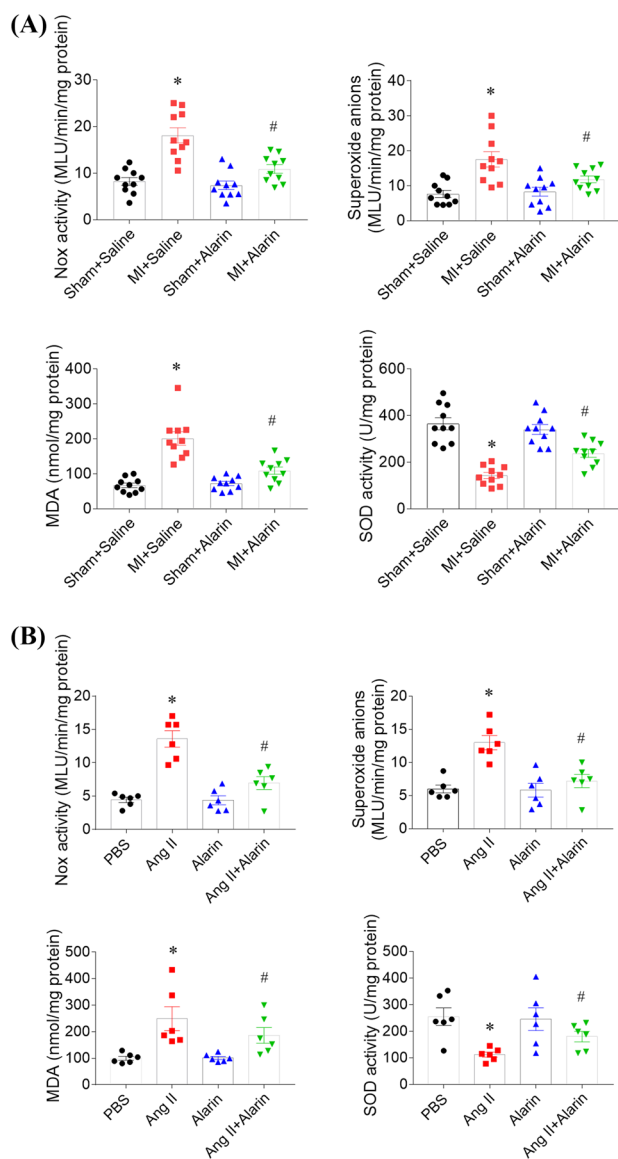


Fig. 4 Alarin alleviated the oxidative stress in the heart of myocardial infarction (MI) rats and in the cardiac fibroblasts (CFs) induced by angiotensin (Ang) II. **A** Alarin reversed the increases of NADPH oxidase (Nox) activity, superoxide anions and malondialdehyde (MDA), and the decrease of superoxide dismutase (SOD) activity levels in the heart of MI rats. **B** Alarin reversed the increases of NADPH (Nox) activity, superoxide anions and malondialdehyde (MDA), and the decrease of superoxide dismutase (SOD) activity levels induced by Ang II in CFs. The results are expressed as mean \pm SEM. * $p < 0.05$ versus the Sham + Saline (**A**) or PBS (**B**) group; # $p < 0.05$ versus the MI + Saline (**A**) or Ang II (**B**) group

Nox1 overexpression reversed the effects of alarin on inhibiting Ang II-induced CFs fibrosis

The expression was increased in the CFs treated with Ang II, and this increase was inhibited by alarin administration (Fig. 5A). Nox1 expression in Ad-Nox1 treated CFs was

2.88 times of Ad-GFP treated CFs (Fig. 5B). Nox1 overexpression reversed the effects of alarin on attenuated the increases of collagen I, collagen III and TGF- β levels the heart of MI rats; while, Nox1 overexpression elevated the expression levels of collagen I, collagen III and TGF- β in the heart of rats (Fig. 5C). Nox1 overexpression reversed the effects of alarin on inhibiting Ang II-induced increases of collagen I, collagen III and TGF- β levels in CFs; while, Nox1 overexpression elevated the expression levels of collagen I, collagen III and TGF- β in CFs (Fig. 5D).

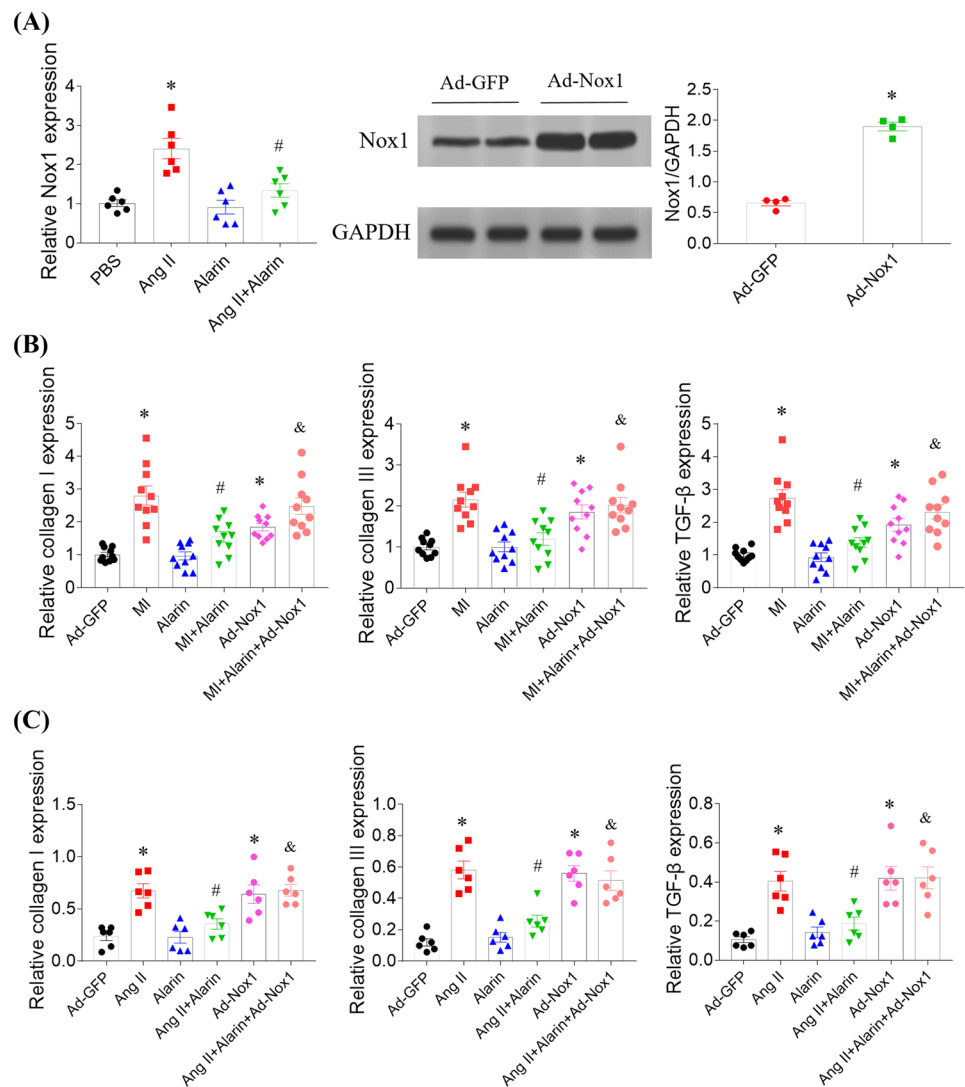
Discussion

Alarin was identified as the fourth member of the galanin peptide family. It found that galanin level was correlated with humoral biomarkers with HF, pro-BNP and copeptin (Ozkaramanli Gur et al. 2017). Treatment with galanin (2–11) enhanced cell viability in a dose-dependent manner, attenuated cell apoptosis and excessive mitochondrial reactive oxygen species (ROS) production in response to oxidative stress in H9C2 cells (Timotin et al. 2017). We present found that alarin treatment improved HF and cardiac fibrosis in the rats, and inhibited Ang II-induced CFs fibrosis via attenuating oxidative stress.

Heart failure is a complex syndrome due to structural or functional cardiac disorders that impairs the ability of the ventricle to fill with or eject blood (Gonzalez et al. 2018; Patel and Deoghare 2015). It is characterized by progressive loss of contractility and ejection fraction, ventricular wall thinning, cardiac dilatation, dysregulated fluid homeostasis, cytokine and neurohumoral activation (Dorn and Molkentin 2004). There have been considerable advances in the pharmacological management of HF over the past 30 years. Despite effective medical interventions, mortality and morbidity remain substantial. In the present study, we found that EF, FS, LV + dp/dt_{max} and LVSP of LV were reduced in MI rats, which were reversed by alarin treatment. In MI rats, LVVVS, LVVD, LVESD and LVEDD were elevated, and alarin administration inhibited these increases. These results indicated that alarin can attenuate MI-induced HF, and improved cardiac dysfunction of HF.

Heart failure is characterized by forming cardiac fibrosis. Myocardial interstitial fibrosis led to left ventricular dysfunction resulting in the development of HF (Gonzalez et al. 2018). Study showed that galanin receptor antagonist M40 effectively improved cardiac function of contraction, and attenuated cardiac fibrosis of MI-HF rats. However, it is not clear whether the relationship between alarin and fibrosis of heart in HF. In our present study, the results showed that the levels of collagen I, collagen III and TGF- β were increased in the heart of MI rats, and alarin treatment relieved these increases. Renin-angiotensin system plays an important role

Fig. 5 NADPH oxidase 1 (Nox1) overexpression reversed the effects of alarin on inhibiting cardiac fibrosis. **A** Alarin inhibited the increase of Nox1 in cardiac fibroblasts (CFs) induced by angiotensin (Ang) II. **B** Nox1 expression was significantly increased by overexpression. **C** Nox1 overexpression reversed the effects of alarin on inhibiting the increases of collagen I, collagen III and transforming growth factor- β (TGF- β) mRNA levels in the heart of myocardial infarction (MI) rats. **D** Nox1 overexpression reversed the effects of alarin on inhibiting Ang II-induced increases of collagen I, collagen III and TGF- β mRNA levels in CFs. The results are expressed as mean \pm SEM. * p < 0.05 versus the PBS (A) or Ad-GFP (B, C and D) group; # p < 0.05 versus the Ang II (A and C) or MI (B) group; & p < 0.05 versus the MI + Alarin (B) or Ang II + Alarin (C) group



in the cardiac remodeling (Guimaraes et al. 2021). Ang II level was increased in MI-induced heart failure model (Peng et al. 2020). Activated myofibroblasts express Ang II, and elevated Ang II consequently regulates the differentiation of myofibroblasts or the expression of extracellular matrix proteins (Cai et al. 2019). Hence, Ang II is commonly used in CFs to simulate cardiac fibrosis of MI (Wang et al. 2019a). We present found that the increases of collagen I, collagen III and TGF- β levels induced by Ang II treatment were attenuated after alarin administration in CFs. These results demonstrated that cardiac fibrosis were elevated in MI rats, and alarin improved fibrosis of the heart in HF rats.

So far, three types of galanin receptors have been described, including galanin receptor 1 (GalR1), GalR2 and GalR3. However, the receptor of alarin is still unknown (Sipkova et al. 2017). Previous study showed that Ala6-25 Cys is an antagonist of alarin (Wang et al. 2020), and C7 and M35 are the antagonists of galanin receptors (Zhang et al. 2019;

Bhandari et al. 2010). We present found that Ala6-25 Cys, but not M35 or C7 reversed the attenuating effects of alarin on MI-induced cardiac fibrosis. In addition, Ala6-25 Cys reversed the effects of alarin on inhibiting the CFs fibrosis induced by Ang II. The results indicated that alarin produce the biological effects via an unknown receptor, but not a galanin receptor. Next, we found that administration of Ala6-25 Cys, M35 or C7 significantly reduced cardiac function of rats, indicating galanin family regulates the cardiac function on physiological state.

Reactive oxygen species (ROS), as important molecules in living organisms, are involved in many signaling pathways. As a key contributor to organ damage, oxidative stress is associated with various of diseases (Honda et al. 2019), including fibrosis of the heart (Kura et al. 2020). The increases of cell apoptosis and mitochondrial ROS production in H9C2 cells treated with hypoxia and reoxygenation were reduced exposure of short N-terminal galanin fragment

2–15 (Pisarenko et al. 2017). Antioxidants were showed to improve cardiac function and produce anti-fibrosis effect (Wang et al. 2019b; Liu et al. 2021). We present found that Nox activity, superoxide anion and MDA levels were increased, and SOD level was reduced in the heart of MI rats and CFs treated with Ang II, and alarin reversed these changes. So far, seven isoforms of Nox (Nox1–Nox5, dual oxidase 1 and dual oxidase 2) have been identified (Zhang et al. 2020). Our current study showed that Nox1 level was increased in Ang II-treated CFs, and this increase was inhibited by alarin administration. Moreover, Nox1 overexpression reversed the effects of alarin on inhibiting the increases of collagen I, collagen III and TGF- β in the heart of MI rats and CFs induced by Ang II. The expression levels of collagen I, collagen III and TGF- β were elevated by Nox1 overexpression in the heart of rats and CFs. These results demonstrated that the oxidants and antioxidants were imbalanced in the heart of HF rats induced by MI, which could be reversed by alarin. Alarin could inhibit fibrosis of heart in HF via attenuating oxidative stress.

In addition to the mechanism of alarin acts directly on CFs, there are other pharmacological/physiological roles of alarin that are diminishing cardiac fibrosis independently of the effects on fibroblasts. Colocalization of galanin with vasoactive intestinal peptide, neuropeptide Y and substance P was observed in many nerve fibers of the heart (Zhu and Dey 1992). Alarin or its receptor may be expressed in the nerve fibers of the heart to regulate cardiac fibrosis via neuromodulation mechanism.

In conclusion, oxidative stress was enhanced in the heart of HF rats, which could be attenuated by alarin. Alarin functioned as an antioxidant to improve cardiac dysfunction, and attenuate cardiac fibrosis in HF rats and fibrosis in CFs treated with Ang II. Nox1 regulated the attenuating effects of alarin in HF and cardiac fibrosis.

Declarations

Conflict of interest All authors declare that no competing financial interests exist.

Ethical approval All procedures were approved by the Experimental Animal Care and Use Committee of Nanjing Medical University and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

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