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Original Article

Relationship between no-reflow phenomenon and local rennin-angiotensin system in myocardium in a rabbit model of acute myocardial infarction and reperfusion

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Abstract: During acute myocardial infarction and reperfusion (AMI/R), no-reflow phenomenon often happens, which mechanisms are not well known. Our purpose was to evaluate the relation between no-reflow phenomenon and variations in angiotensin (Ang) II, angiotensin-converting enzyme (ACE), and interferon gamma (IFN-γ) in myocardium during AMI/R. Twenty-four rabbits were randomized into three groups (n=8 for each): a sham operated group, an AMI 1 h group, and an AMI 4 h group. The animals in the latter two groups were subjected to 1 or 4 h of coronary occlusion followed by 2 h of reperfusion. Areas of no-reflow and necrosis were evaluated pathologically. In the AMI groups, the levels of Ang II and IFN-γ were significantly higher in no-reflow myocardium than in reflow and normal myocardium, while the expression of ACE mRNA was significantly higher in reflow myocardium than in normal and no-reflow myocardium. The areas of no-reflow and necrosis were significantly higher in AMI 4 h group than in AMI 1 h group. In a rabbit model of AMI/R, no-reflow phenomenon is closely related to local RAS and IFN-γ levels in myocardium, and the activation of local RAS may be one of the important mechanisms of no-reflow phenomenon.

Keywords: Interferon gamma, myocardial infarction, no-reflow phenomenon, rennin-angiotensin system, reperfusion

Introduction

Reperfusion therapy is of great importance to acute myocardial infarction (AMI), and its main goal is to restore epicardial and microvascular blood flow to the ischemic myocardium. If the pathophysiological response is severe during AMI and reperfusion (AMI/R), slow flow or no-reflow phenomenon often happens, which restores epicardial flow but provides only poor perfusion to distal tissue [1]. No-reflow results in a bad prognosis for left ventricular remodeling and function, acute and long-term clinical events, and survival [2]. Therefore, it is essential to reduce the extent of no-reflow for reperfusion therapy for AMI [3, 4].

The mechanism of no-reflow phenomenon is complex. Microvascular spasm, distal embolization of atheroma and thrombus, microvascular plugging by platelets and leukocytes, endothelial swelling, tissue edema compressing the microvasculature, oxidative stress, and inflammation may all play a role [5, 6]. However, the mechanism responsible for no-reflow phenomenon has not been completely understood till now.

Renin-angiotensin system (RAS) is a very important endocrine system in the body, with a high biological activity, which exists widely in the circulation and tissues. The notion of a local tissue-specific RAS, in addition to the classic circulating RAS [7, 8], is now widely recognized [9-12]. All RAS components have been identified in the cardiac tissue [9, 13-15]. Most angiotensin (Ang) II found in the heart is synthesized *in situ* by local production, unlike blood-derived Ang I [16-18]. In fact, local Ang II concentrations may exceed plasma levels and play important roles in the control of cardiac function [19] in cardiac pathophysiology, such as hypertrophy

and infarction [20, 21]. It has been confirmed that myocardial ischemia in ischemia/reperfusion can activate cardiac local RAS, which causes coronary constriction, promotes myocardial ischemia or reperfusion arrhythmia, and leads to ventricular hypertrophy or myocardial remodeling, thus resulting in impaired cardiac function [22].

Interferon gamma (IFN-γ) is an inflammatory cytokine secreted by endothelial cells, mononuclear macrophages, and lymphocytes. It participates in immunopathogenesis and destroys the structure and function of blood vessel endothelium [23]. It has also been shown that IFN-γ increases the excretion of C-reactive protein, which accelerates the development of atherosclerosis and thrombosis [24]. The expression of IFN-γ is upregulated in the myocardium of patients with unstable angina pectoris, which reflects the immunological activity of endothelial cells, and the activity of ACE is also enhanced [23].

However, few studies have reported on the changes in the expression levels of local RAS components in the development of no reflow phenomenon, and on the relation between IFN-γ, local myocardial RAS, and no-reflow phenomenon. The present study explored the changes in Ang II and ACE, and the relation between local myocardial RAS, IFN-γ, and no-reflow phenomenon, so as to evaluate the effects of local myocardial RAS on myocardial no-reflow during AMI/R, and to investigate the mechanisms of no-reflow phenomenon.

Materials and methods

Rabbit model of AMI/R [25, 26]

The animals and protocols used in the study were approved by the Institutional Animal Care and Use Committee of Binzhou Medical University (Binzhou, China).

Twenty-four New Zealand white male rabbits (Laboratory Animal Center of Shandong University, Jinan, China), 5-6 months old and weighing 2.5-3.0 kg, were anesthetized intravenously with 30 mg/kg pentobarbital sodium and ventilated with a respirator (SV900; Siemens-Elema, Solna, Sweden) using room air enriched with 1.5 L/min oxygen. A left lateral thoracotomy was performed in the third to fourth intercostal space, and the heart was suspended in a pericardial cradle. The middle portion of a major branch of the left circumflex coronary artery (LCX) was encircled with a suture. The two ends of the suture were threaded through a piece of plastic tubing to form a snare that could be tightened to achieve coronary artery occlusion.

The animals were divided randomly into three groups of eight: a sham-operated group, an AMI 1 h group, and an AMI 4 h group. Animals in the latter two groups were subjected to 1 or 4 h of coronary occlusion followed by 2 h of reperfusion. In the sham-operated animals, the LCX was encircled by a suture, but not occluded.

Assessment of ligation area, area of no reflow, and area of necrosis

After 2 h of reperfusion, the area of no-reflow (ANR) was delineated by intra-atrial injection of 1 mL/kg of the fluorescent dye thioflavin S (Sigma Chemical Co., St. Louis, MO, USA) which was dissolved in 0.9% saline and centrifuged at 1500 rpm for 5 min. The LCX was then re-occluded, and Evans blue dye was injected into the left atrium to determine the ligation area (LA). The animals were then euthanized with an overdose of xylazine (100 mg, intravenous) and 12 mEq KCl (intra-atrial), and the hearts were removed. The left ventricle was cut into five or six slices parallel to the atrioventricular groove. The areas without perfusion of thioflavin S were identified under ultraviolet light in a dark room. The LA was defined as the region unstained by Evans blue dye, and the ANR was defined as the nonfluorescent area within the LA.

The other slices were incubated in a 1% solution of triphenyl tetrazolium chloride for 15 min at 37°C. The regions that failed to demonstrate red staining were considered as areas of necrosis (NA). All slices were photographed. The outlines of the left ventricular wall area, LA, ANR, and NA were analyzed using the Image-Pro Plus software (Media Cybernetics Co., Rockville, MD, USA). LA is reported as a percentage of the left ventricular wall area; ANR and NA are reported as a percentage of the LA. The experimental protocol was performed as previously described [25, 26].

Assay of Ang II in myocardial tissue

Myocardial tissue samples were obtained from the normal, reflow, or no-reflow tissue slices immediately after the animals were euthanized. The samples were washed with 0.9% ice-cold

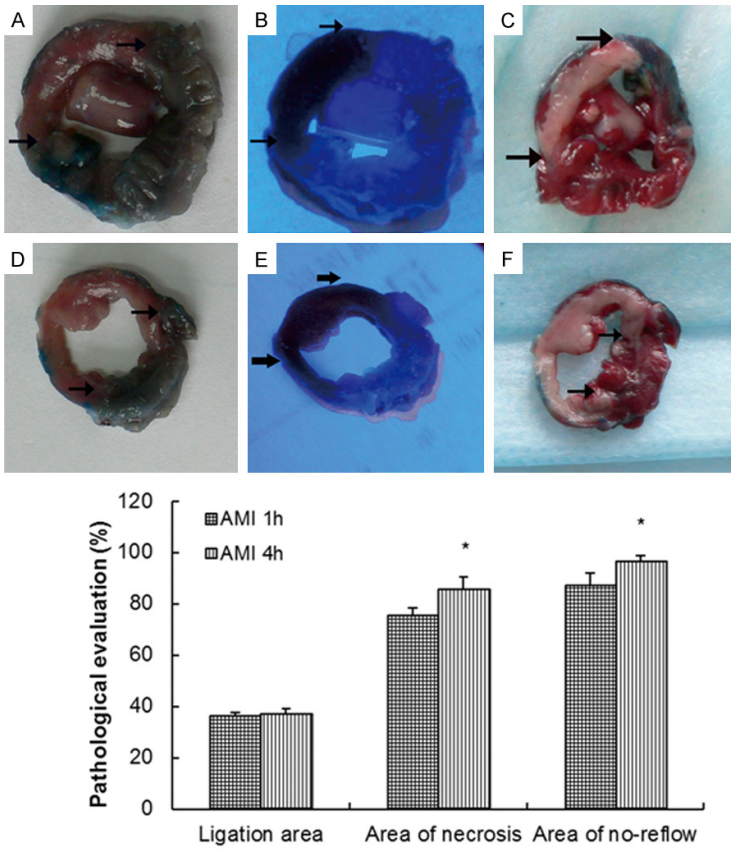

Figure 1. Ligation area (unstained by Evans blue dye), area of no-reflow (unstained by thioflavin S in the ligation area), and area of necrosis (unstained by triphenyl tetrazolium chloride) in the AMI 1 h and AMI 4 h groups. (A-C) pictures belong to the AMI 1 h group; (D-F) pictures belong to the AMI 4 h group. The red areas in (A and D) show the ligation area, black areas in (B and E) show the area of no-reflow, and white areas in (C and F) show necrosis (see black arrows). Comparison of ligation area (ischemic area), area of necrosis, and area of no-reflow evaluated by pathological method between the two AMI groups. $^{*}P < 0.01$, compared with the AMI 1 h group. AMI: acute myocardial infarction.

saline; 2 mL of 0.05 mol/L phosphate buffer and mixed enzyme inhibitor (Kemei Dongya Biotechnology Institute, Beijing, China) were added, and a tissue homogenate was made. The supernatant was separated by centrifugation (4000 rpm, 5 min, 4°C) and stored at -25°C. Ang II in myocardial tissue was determined by radioimmunoassay (Kemei Dongya Biotechnology Institute, Beijing, China).

Assay of IFN-γ in myocardial tissue

Myocardial tissue samples were obtained from the normal, reflow, or no-reflow tissue slices immediately after the experimental procedures. The samples were washed in 0.9% saline, fixed in paraformaldehyde (40 g/L), and embedded in paraffin; the microtome sections (50 μm thick) were obtained. The expression of IFN-γ was assayed using an immunohistochemical technique. The sections were observed under a light microscope (BX-51; Olympus Corporation, Tokyo, Japan) and photographed (200×). Three sections were randomly selected from each group, and three visual fields were randomly selected from each section for semiquantitative analysis using the Image-Pro Plus software (Media Cybernetics Co., Rockville, MD, USA). IFN-γ positivity was reported as the average integral optical density of the nine visual fields evaluated.

Assay of ACE mRNA in myocardial tissue by RT-PCR

Myocardial tissue samples were obtained from the normal, reflow, or no-reflow tissue slices immediately after the experimental procedures, packaged in the foil, labeled, and preserved in the refrigerator at -70°C for determining the expression levels of regional myocardial ACE mRNA by RT-PCR. It involved RNA extraction, reverse transcription, PCR amplification, electrophoresis, and other procedures [27, 28]. The gel analysis system was observed and analyzed. The image was used for densitometric scanning using FR-2000 image analysis system (Furi Science and Technology Ltd., Shanghai, China), to target gene optical density value and samples corresponding to the reference gene optical density value of the ratio as target genes in the sample relative to the transcription level.

Statistical analysis

Statistical analysis was performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, USA). Data were reported as means ± SD. IFN-γ, Ang II, ACE mRNA, LA, ANR, and NA were compared among groups using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons. Two-sided values of $P < 0.05$ were considered to be statistically significant.

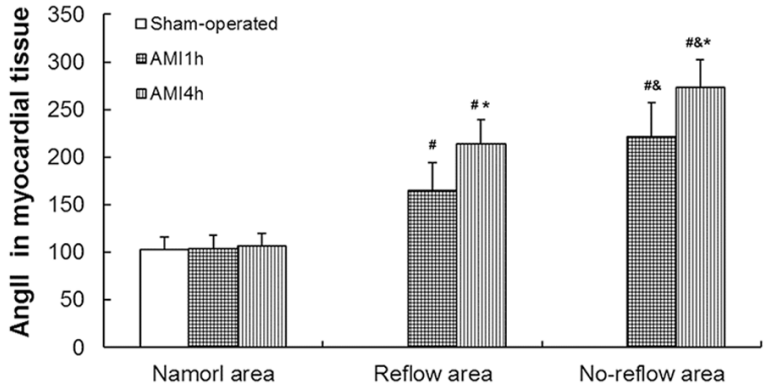

Figure 2. Ang II expression in the myocardium of different regions in the three groups. #$P < 0.01$, compared with the normal region; &$P < 0.01$, compared with the reflow region; *$P < 0.01$, compared with the AMI 1 h group. AMI: acute myocardial infarction.

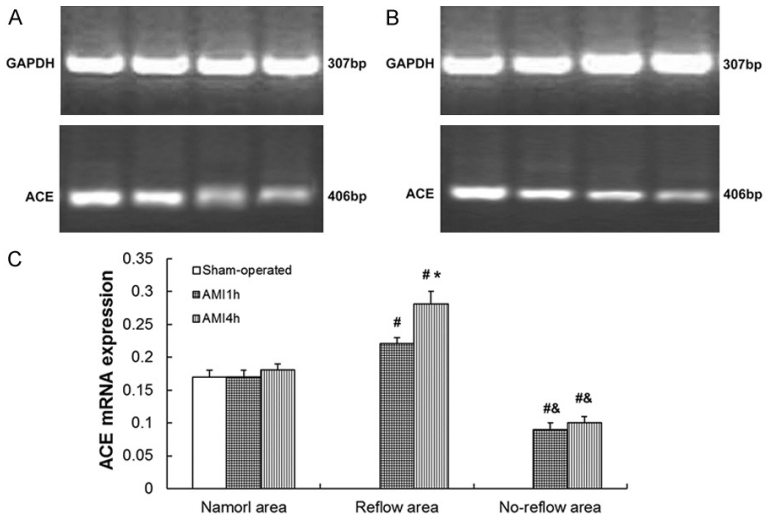

Figure 3. ACE mRNA expression in the myocardium of different regions in the three groups. A. Reflow and normal regions of the AMI 1 h group and the sham-operated group; no-reflow region of the AMI 1 h group from the left to the right. B. Reflow and normal regions of the AMI 4 h group and the sham-operated group; no-reflow region of the AMI 4 h group from the left to the right. C. ACE mRNA expression in myocardium of different regions in the three groups. #$P < 0.01$, compared with the normal region; &$P < 0.01$, compared with the reflow region; *$P < 0.01$, compared with the AMI 1 h group. AMI: acute myocardial infarction.

Results

Ischemia time was associated with an increased ANR and NA

No significant difference was observed in the pathological evaluation of LA between the AMI 4 h (36.87 ± 2.16%) and AMI 1 h (36.20 ± 1.67%; $P > 0.05$) groups. The mean ANR was significantly higher in the AMI 4 h group (85.67 ± 4.94% of LA) than in the AMI 1 h group (75.26 ± 3.27% of LA; $P < 0.01$). The NA also significantly increased in the AMI 4 h group (96.56 ± 2.26% of LA) compared with that in the AMI 1 h group (87.24 ± 4.95% of LA; $P < 0.01$) (**Figure 1**).

Ang II expression in myocardial tissue subjected to reflow and no-reflow

As shown in **Figure 2**, Ang II expression during reflow and no-reflow was significantly higher in the AMI 1 h and AMI 4 h groups than in normal myocardium or in the sham-operated group (all $P < 0.01$). In both groups, Ang II expression was more marked in no-reflow areas than in reflow areas of the myocardium (both $P < 0.01$). However, Ang II expression in the reflow and no-reflow myocardial areas was lower in the AMI 1 h group than in the AMI 4 h group (both $P < 0.01$).

ACE mRNA expression in myocardial tissue subjected to reflow and no-reflow

As shown in **Figure 3**, ACE mRNA expression during reflow was significantly higher in the AMI 1 h and AMI 4 h groups than in normal myocardium or in the sham-operated group (all $P < 0.01$). In both groups, however, ACE mRNA expression was less marked in no-reflow areas than that in reflow and normal areas of the myocardium (all $P < 0.01$). ACE mRNA expression only in the reflow myocardial areas was lower in the AMI 1 h group than that in the AMI 4 h group ($P < 0.01$).

Ischemia time was associated with increased expression of IFN-γ in myocardial tissue subjected to reflow and no-reflow

Immunohistochemistry clearly demonstrated positive expression of IFN-γ in vascular endo-

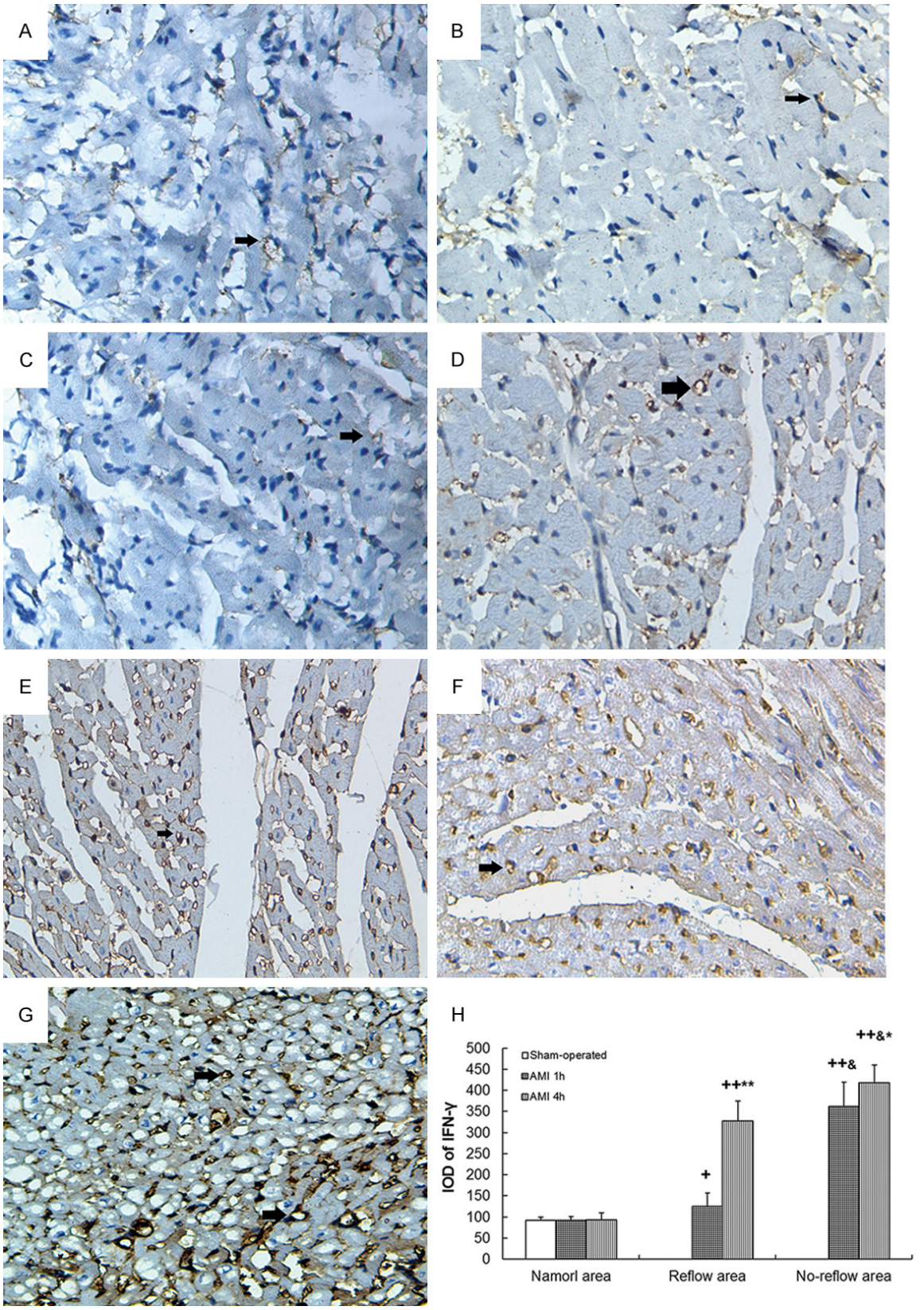

Figure 4. Expression of IFN-γ in different regions of the myocardium in the three groups after 1 h or 4 h occlusion and 2 h of reperfusion (IHC, DAB coloration, 200×). Positive IFN-γ expression was seen in vascular endothelial cells (as shown by arrows). A. Normal region in the sham-operated group. B. Normal region in the AMI 1 h group. C.

Normal region in the AMI 4 h group. D. Reflow region in the AMI 1 h group. E. No-reflow region in the AMI 1 h group. F. Reflow region in the AMI 4 h group. G. No-reflow region in the AMI 4 h group. H. IOD analysis of IFN-γ levels in the myocardium of different regions in the three groups. $^{+}P < 0.05$ or $^{++}P < 0.01$, compared with the normal region; $^{\&}P < 0.01$, compared with the reflow region; $^{*}P < 0.05$ or $^{**}P < 0.01$, compared with the AMI 1 h group. AMI: acute myocardial infarction.

thelial cells (**Figure 4**). IFN-γ expression during reflow and no-reflow was significantly higher in the AMI 1 h and AMI 4 h groups than in normal myocardium or in the sham-operated group ($P < 0.05$ or $P < 0.01$). In both the groups, IFN-γ expression was more marked in no-reflow areas than that in reflow areas of the myocardium (both $P < 0.01$). However, IFN-γ expression in the reflow and no-reflow myocardial areas was much higher in the AMI 4 h group than in the AMI 1 h group ($P < 0.05$ or $P < 0.01$).

Discussion

RAS can be divided into two categories: cyclic (classic) RAS and local tissue RAS, which can interact [22]. RAS is an important system for the regulation of blood pressure, body fluid volume, and electrolyte balance, and plays an important role in the pathophysiological process of cardiovascular disease. Several multicenter clinical trials, using ACE inhibitors or the newer angiotensin II (type 1) receptor blocking agents (ARBs), have confirmed the initial findings, and thereby demonstrating their beneficial effects on ventricular remodeling, reduction in the end-stage events of cardiac failure and the risk of mortality, and repeated myocardial infarction [29, 30]. The finding that these beneficial effects can occur independently on blood pressure supports the conclusion that the activation of local RAS contributes significantly to cardiovascular pathology [16].

This study demonstrated that both ANR and NA in the AMI 4 h group were significantly higher than those in the AMI 1 h group. This finding suggests that both ANR and NA increased with coronary occlusion time. It clearly demonstrated that both ischemia and reperfusion injury induced an increase in myocardial Ang II and IFN-γ expression, which was related to an increase of ANR and infarct size during AMI/R.

Ang II was the most important in RAS, and the physiological activities of RAS were mainly generated by Ang II. The contents of Ang II in the no-reflow region in the AMI group were significantly higher, which indicated that the synthesis of myocardial Ang II was increased by ischemia and reperfusion. Ang II can cause vascular endothelial cell injury and leukocyte adhesion to endothelial cells. It can also accelerate the formation of protein fibers and the damage of vascular endothelial cells to produce plasminogen activator inhibitor-1 (PAI-1) [31]. Previous studies [22, 31] have indicated that the local cardiac RAS plays an important role in the development of ischemic heart disease. The contents of Ang II in the reflow region, especially no-reflow region in the AMI 4 h group, were significantly higher than those in the AMI 1 h group ($P < 0.01$). The present findings indicated that with the extension of ischemic time, the synthesis of Ang II significantly increased. These results suggested that the occurrence of no reflow may be associated with elevated myocardial Ang II.

The expression of ACE mRNA in the reflow myocardial areas significantly increased in the AMI 4 h group than in the AMI 1 h group ($P < 0.01$). The results showed that myocardial ischemia can upregulate the mRNA expression of ACE in ischemia myocardial tissue, and its level was related to the severity and duration of ischemia. However, in the AMI groups, ACE mRNA expression was less marked in no-reflow areas than in reflow and normal areas of the myocardium (all $P < 0.01$). The reasons for the aforementioned results are unknown and need to be investigated further.

In both the AMI groups, IFN-γ expression was more marked in no-reflow areas than in reflow areas of the myocardium (both $P < 0.01$), and IFN-γ expression in the reflow and no-reflow myocardial areas was much higher in the AMI 4 h group than in the AMI 1 h group ($P < 0.05$ or $P < 0.01$).

The aforementioned findings indicated that both ischemia and reperfusion injury can induce inflammatory responses that promote IFN-γ production and release in the myocardium [23, 24]. In a study, the expression of mRNA of IFN-γ was detected by RT-PCR during myocardial ischemia and perfusion in rats, and the

results showed that the expression of mRNA of IFN-γ in ischemic myocardial tissue was significantly upregulated and its release has certain regularity [24]. Evidence showed that IFN-γ induces endothelial ACE transcription and regulates the activity of its enzyme [32]. The present findings indicated that both ischemia and reperfusion injury can induce inflammatory responses that promote IFN-γ production and release in the myocardium. The expression of IFN-γ was related to the severity and duration of ischemia and the occurrence of myocardial infarction and no reflow was related to the involvement of IFN-γ in the inflammatory reaction.

The results of the present study suggested that with the extension of ischemic time, activation of local RAS and expression of IFN-γ increased, and no-reflow and myocardial infarction areas expanded. Based on the present findings, it is believed that no-reflow phenomenon is closely related to local RAS and IFN-γ levels in the myocardium, and the activation of local RAS may be one of the important mechanisms of no-reflow phenomenon.

Acknowledgements

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Disclosure of conflict of interest

None.

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