Arginase-2 protects myocardial ischemia-reperfusion injury via NF-κB/TNF-α pathway

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Abstract. – OBJECTIVE: Arginase-2 exerts an anti-inflammatory potential. However, whether nuclear factor-kB (NF-κB)/TNF-α (tumor necrosis factor-α) pathway is involved in the anti-inflammatory effect of arginase-2 has not been fully elucidated. Our study aims to explore the regulatory role of arginase-2 on ischemia-reperfusion injury (IRI) in rats and its underlying mechanism.

MATERIALS AND METHODS: 24 male Sprague Dawley (SD) rats were randomly assigned into sham group, IRI group and arginase-2 group, with 8 rats in each group. Electrocardiogram was performed in each rat before and after animal procedures. Serum samples and heart samples of each rat were collected 10 days after animal procedures. Serum levels of CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) in each rat were detected using the relative commercial kits. Pathological lesions in rat myocardium were observed by hematoxylin and eosin (HE) staining. Cardiomyocyte apoptosis in rat heart was accessed by TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling) staining. Expression levels of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 in rat myocardium were detected by Western blot and immunohistochemistry.

RESULTS: Electrocardiogram showed slower heart rate, lower voltage of QRS wave and longer Q-T interval in rats of IRI group than those of sham group (p < 0.05). A few rats in IRI group even presented arrhythmia. On the contrary, rats in arginase-2 group presented higher heart rate and voltage of QRS wave, as well as shorter Q-T interval compared with those of IRI group (p < 0.05). Rats in arginase-2 group presented lower plasma levels of CK-MB and LDH than those of IRI group. Pathological lesions in rat myocardium and cardiomyocyte apoptosis were alleviated in arginase-2 group in comparison with those of IRI group. Western blot and immunohistochemistry indicated that arginase-2 pretreatment remarkably downregulated expressions of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 in rat myocardium.

CONCLUSIONS: Arginase-2 protects myocardial ischemia-reperfusion injury in rats through inhibiting the inflammatory response via suppression of NF-κB/TNF-α pathway.

Key Words: Arginase-2, NF-κB/TNF-α pathway, IRI, Myocardial injury.

Introduction

Ischemic heart disease is a common disease that seriously endangers human health. Reperfusion therapies effectively restore blood supply and nutritional support in ischemic myocardium, including drug thrombolysis, percutaneous transluminal coronary angioplasty, stent implantation and coronary artery bypass grafting1-3. However, traditional reperfusion therapy might lead to ischemia-reperfusion injury (IRI) when restoring blood supply in impaired myocardium4-5. IRI further aggravates pathological damage in myocardium and even results in irreversible cell death of cardiomyocytes6-7. Clinically, IRI patients manifest as reperfusion arrhythmia and persistent cardiac insufficiency induced by myocardial stunning8. With the widespread application of reperfusion therapy, the negative impacts of IRI on clinical outcomes of affected patients have been pronounced9.

Multiple factors may result in IRI, of which, the direct cell damage caused by inflammation and intracellular calcium overload is the major one10-12. Current researches13-15 have found that ischemic preconditioning, drug pretreatment, post-ischemic treatment and post-drug treatment all exert protective effects on IRI. Medication treatment is easily performed with exact drug target, presenting a high value in clinical application13. Nuclear factor-kB (NF-kB) pathway is a well-concerned pathway in many cardiovascular diseases16-18. As a pleiotropic transcription factor, NF-κB is widely present in eukaryotic organisms19-21. NF-κB consists of complex polypeptide subunits, which is closely related to immune...
response, oncogenesis, tumor development, cell apoptosis, and embryonic development\textsuperscript{15,20}. Researchers have found that NF-κB is involved in the pathological process of many chronic inflammatory diseases. External stress stimulates the activation of NF-κB pathway, which in turn promotes the release of cytokines, chemokines and adhesion molecules. NF-κB pathway is capable of regulating inflammatory response, immune response, cell differentiation and apoptosis\textsuperscript{22,23}.

Previous works\textsuperscript{24,25} have shown that arginase-2 directly inhibits oncogene synthesis and tumor growth by catalyzing arginine. It is currently believed that arginase-2 presents protective effects on ischemia-induced organ damage\textsuperscript{5}. This study aims to explore whether arginase-2 could alleviate IRI-induced myocardium damage by NF-κB/TNF-α (tumor necrosis factor-α) pathway. Our study provides novel directions for improving the prevention and treatment efficacies of IRI.

**Materials and Methods**

**Chemicals and Reagents**

Arginase-2 was obtained from Sigma-Aldrich (St. Louis, MO, USA); CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) determination kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China); Coarse balance, electronic thermometer and 721 type spectrophotometer were obtained from Inesa Analytical Instrument (Shanghai, China).

**Animal Experiments**

24 male Sprague Dawley (SD) rats weighing 200 ± 20 g (Model Animal Research Center of Xiamen University, Xiamen, China) were maintained in an environment with a 12 h/12 h light/dark cycle. Rats were given to free access to food and water. Rats were randomly assigned into sham group (saline administration), IRI group (saline administration) and arginase-2 group (20 µg/kg arginase-2 administration), with 8 rats in each group. After collecting rat blood sample from orbital vein, rats were sacrificed for the following experiments. Body weight and daily activities of each rat were regularly observed. This study was approved by the Animal Ethics Committee of Xiamen University Animal Center. For IRI model construction, rats were first anesthetized by intraperitoneal injection of 60 mg/kg pentobarbital sodium. After tracheotomy and mechanical ventilation, electrocardiogram was performed to diagnose rat IRI. Left internal jugular vein catheterization was performed and arterial pressure was monitored through right carotid artery cannula. After thoracotomy, 100% oxygen passed through general circulation from femoral artery allowed by atrial incubation. Myocardial ischemia was induced by ligation of the ascending aorta, following by the observation on ST-segment. 30 min later, the ligation was removed and reperfusion was allowed for 3 h. Tall peaked T wave in electrocardiogram and dark-red myocardium suggested the successful ligation.

**Myocardial Infarction Area Determination**

24 h after IRI, rat heart was harvested followed by injection of 1 mL Evans Blue (3%) into the left ventricle. Non-ischemic area and ischemic area were stained blue and red, respectively. Heart tissue was sliced into 5 pieces parallel to atrio-ventricular groove, with 5 µm in thickness. Heart tissue slice was incubated with TTC (pH 7.4) at 37°C for 15 min. The infarct size was determined by Image ProPlus 6.0 (Silver Springs, MD, USA).

**Hemodynamic Analyses**

A 20-G heparin-filled catheter (Spacelabs Medical, Inc., Redmond, WA, USA) was inserted from the right carotid artery to the left ventricle and a pressure transducer (Biolap 420F, Taimeng, Chengdu, China) was connected for hemodynamic parameters measurement. Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), and maximum rate of increase/decrease in left ventricular pressure (±dP/dtmax) of each rat were recorded.

**Cardiomyocyte Function Determination**

After animal procedures, 2 mL of blood sample were collected and centrifuged at 3500 g/min for 30 min. Serum levels of CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) were detected using relative commercial kits (eBioscience, San Diego, CA, USA).

**HE Staining**

Heart tissues were fixed with 10% paraformaldehyde and stained with hematoxylin and eosin (HE). Histological changes were assessed by semi quantitative detection of injury and necrosis of the myocardium. 5 randomly selected fields of each sample were observed and captured for evaluating IRI-induced pathological lesions.
**TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling) Assay**

CARDIOMYOCYTE apoptotic in heart tissue was detected by TUNEL assay according to the instructions of ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon, Millipore, Billerica, MA, USA). 5-µm paraffin section was counterstained with hematoxylin and TUNEL-positive cells were counted in 5 randomly selected fields (200×).

**Immunohistochemical Staining**

After deparaffin and hydration, heart tissue was incubated with the primary antibody (NF-κB, 1:100) at room temperature for 1 h. Slices were washed with phosphate-buffered saline (PBS) for three times, followed by incubation of secondary antibody at room temperature for 1 h. Diaminobenzidine (DAB, Beyotime, Shanghai, China) was added for image exposure and slices were then counterstained with hematoxylin for 2 min. Slices were hydrated, sealed and observed using an inverted microscope (Nikon, Tokyo, Japan).

**Western Blot**

Total protein was extracted using the RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) and quantified by BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA). Protein sample was separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Membranes were incubated with primary and secondary antibodies based on the standard protocols of Western blot. Chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) was used to expose the protein bands on the membrane.

**Statistical Analysis**

SPSS22.0 (Statistical Product and Service Solutions) statistical software package (IBM, Armonk, NY, USA) was used for data analysis. Data were expressed as \( \bar{x} \pm s \). t-test was used to analyze the difference between two groups. Repeated two-way ANOVA was used to analyze the data among different groups, followed by post-hoc LSD (Least Significant Difference). \( p < 0.05 \) was considered statistically significant.

**Results**

**Effect of Arginase-2 on Electrocardiogram Changes**

Electrocardiogram was performed before and after animal procedures. We did not find significant differences in heart rate, voltage of QRS wave and Q-T interval among the three groups before the animal procedures (\( p > 0.05 \)). Compared with that of sham group, rats in IRI group showed reduced heart rate, decreased voltage of QRS wave and prolonged Q-T interval after procedures (\( p < 0.05 \)). A few rats in IRI group even experienced arrhythmia. On the contrary, rats in arginase-2 group showed higher heart rate and voltage of QRS wave, as well as shorter Q-T interval compared with those of IRI group (\( p < 0.05 \), Table I).

**Arginase-2 Pretreatment Improved Myocardial Function in IRI**

To evaluate cardiac function of rats after animal procedures, serum levels of CK-MB and LDH were accessed using the relative commercial kits. Remarkable increase in serum levels of CK-MB and LDH were observed in IRI group compared with those of sham group, suggesting that the IRI rat model has been successfully established (Figure 1A). Decreased CK-MB and LDH levels were observed in rats of arginase-2 group compared with those in IRI group, indicating the protective effect of arginase-2 on IRI (Figure 1B).

**Table I.** The effect of arginase-2 on the electrocardiogram of the model rats induced by myocardial ischemia reperfusion injury.

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart rate/min</th>
<th>QRS/mv</th>
<th>Q-T interval/ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>425.00 ± 15.82</td>
<td>0.580 ± 0.098</td>
<td>58.42 ± 5.79</td>
</tr>
<tr>
<td>IRI</td>
<td>376.00 ± 38.24*</td>
<td>0.409 ± 0.079*</td>
<td>72.05 ± 5.08*</td>
</tr>
<tr>
<td>Arginase-2</td>
<td>421.60 ± 27.56#</td>
<td>0.580 ± 0.066#</td>
<td>55.03 ± 5.03#</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. \( n = 8 \) in each group. *\( p < 0.05 \) vs. Sham, *\( p < 0.05 \) vs. IRI.
Arginase-2 Improved Hemodynamic Function in IRI

Under baseline conditions, no statistical differences in systemic hemodynamics were found among the three groups before the coronary occlusion ($p > 0.05$). However, reperfusion resulted in declined systolic function and diastolic function. The recorded data showed decreased LVSP, LVEDP and $\pm dP/dt_{\text{max}}$ compared with those of baseline values ($p < 0.05$) in IRI and arginase-2 groups. Repeated two-way ANOVA showed significant differences in LVSP and $\pm dP/dt_{\text{max}}$ between IRI and arginase-2 group over the period of measurement. The recoveries of cardiac function were more pronounced in arginase-2 group than those of sham group, manifested as elevated LVSP, $dP/dt_{\text{max}}$, and $-dP/dt_{\text{max}}$ ($p < 0.05$, Table II).

Arginase-2 Preserved Myocardial Histologic Structure and Alleviated Neutrophil Infiltration

Larger infarct area was observed in IRI group compared with that of sham group. Arginase-2 group showed a smaller infarct area than that

Table II. Hemodynamics during the experiment investigating the effect of arginase-2 on reperfusion injury.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRI</td>
<td>133 ± 5</td>
<td>118 ± 3***</td>
</tr>
<tr>
<td>Arginase-2</td>
<td>135 ± 4</td>
<td>128 ± 5*</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRI</td>
<td>-11 ± 3</td>
<td>-5 ± 1***</td>
</tr>
<tr>
<td>Arginase-2</td>
<td>-12 ± 2</td>
<td>-8 ± 3*</td>
</tr>
<tr>
<td>dp/dt_{max} (mmHg/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRI</td>
<td>4833 ± 262</td>
<td>2695 ± 234***</td>
</tr>
<tr>
<td>Arginase-2</td>
<td>4663 ± 244</td>
<td>3889 ± 301</td>
</tr>
<tr>
<td>dp/dt_{max} (mmHg/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRI</td>
<td>-3788 ± 225</td>
<td>-3088 ± 258*</td>
</tr>
<tr>
<td>Arginase-2</td>
<td>-3980 ± 345</td>
<td>-3766 ± 199</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. LVSP = left ventricular systolic pressure, LVEDP = left ventricular end-diastolic pressure; $\pm dP/dt_{\text{max}}$ = maximum rate of increase/decrease in left ventricular pressure. n = 8 in each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. baseline, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. IRI.
of IRI group ($p < 0.05$, Figure 2A and 2B). HE staining showed well-ordered myocardial cells with regular structure and only a small amount of focal infiltrating inflammatory cells in sham group. On the contrary, a large amount of inflammatory cells, disordered myocardial cells and pink protein mucus exudation in rat myocardium were observed in IRI group. Arginase-2 group showed remarkably alleviated pathological lesions in cardiomyocytes (Figure 2C).

**Arginase-2 Decreased Cardiomyocyte Tubular Cells Apoptosis and Enhanced Cell Proliferation After IRI**

The amount of TUNEL-positive cells in IRI group was much more than that of sham group. However, fewer TUNEL-positive cells were observed in heart tissues of arginase-2 group than those of IRI group ($p < 0.05$, Figure 2C and 2D).

**Arginase-2 Upregulated Expression of Nrf2 and its Downstream Genes by Increasing Nuclear Translocation of Nrf2**

Immunohistochemistry suggested that arginase-2 group presented a higher ratio of Nrf2-positive cells than that of IRI group. NF-κB staining showed an opposite results (Figure 3A-3C). Subsequently, we extracted cytoplasm and nucleus of rat cardiomyocytes in each group, respectively. Western blot results indicated that cytoplasmic levels of Nrf2 and its downstream factors were all

![Figure 2](image-url)

**Figure 2.** Arginase-2 prevented myocardial ischemia-reperfusion injury in cardiac morphology. *A*, Blue-stained areas represented normal tissue and unstained pale areas indicated infarcted tissue. Infarct size was expressed as a percentage of total volume; **B**, Myocardial sections were stained with hematoxylin and eosin and examined using a light microscopy (200×). HE staining of myocardial tissues in sham group, IRI group, and arginase-2 group; **C**, Representative images of TUNEL immunostaining in myocardial ischemia reperfusion injury (200×); **D**, TUNEL-positive cells per 10³ germ cells in the three groups. Data were expressed as mean ± SD; *Significant difference vs. sham group ($p < 0.05$); **Significant difference vs. IRI group ($p < 0.05$).
upregulated than those of nuclear levels, whereas cytoplasmic level of NF-κB was downregulated (Figure 3D). Furthermore, protein expressions of NF-κB, TNF-α, VCAM-1, ICAM-1 and MCP-1 in arginase-2 group were downregulated compared with those of IRI group (Figure 3G-3H).

**Discussion**

IRI occurs after a short period of low-perfusion ischemia and sudden recovery of blood supply. Ischemic tissue is further damaged and even experiences irreversible pathological changes. IRI was initially found in myocardium. With the in-depth researches on IRI, it has been found to occur in multiple important organs, such as brain, lung, liver, kidney and skin. The first study of IRI was conducted using coronary artery ligation reperfusion model in dog, confirming that IRI accelerates the necrosis process of cardiomyocytes. Meanwhile, IRI damages the normal cell ultrastructure in a short period. Researches reported that edema and injury of cardiomyocytes are observed in patients undergoing coronary artery bypass graft surgery for revascularization. They considered that pathological lesions in cardiomyocytes are related to IRI. Inflammatory response is another important factor leading to IRI. Neutrophils are activated and accumulated in infarct area, eventually resulting in inflammatory response. During IRI process, neutrophils are recruited to the damaged myocardium, where the number of neutrophils is 2.6
Arginase-2 protects myocardial ischemia-reperfusion injury via NF-κB/TNF-α pathway

NF-κB was first discovered in B-cell nuclear extracts that specifically binds to the enhancer κB sequence of the immunoglobulin k light chain. As an important pleiotropic transcription factor, NF-κB is widely distributed in eukaryotes. NF-κB is involved in the development of various cardiovascular diseases. For example, NF-κB is expressed in smooth muscle cells, macrophages and endothelial cells extracted from atherosclerosis patients. On the contrary, activated NF-κB is barely found in the normal vessel wall. Activated NF-κB is also detected in the coronary arteries of pigs fed with high-cholesterol diet. NF-κB activity is closely related to the disease progression of atherosclerosis. Patients with acute coronary syndrome express activated NF-κB. The expression level of NF-κB is correlated to C-reactive protein level, reflecting disease condition of coronary heart disease. In recent years, many studies have confirmed that NF-κB is involved in the pathological processes of cardiovascular diseases, such as IRI, cardiomyocyte apoptosis, heart failure, and ventricular remodeling. Based on previous studies, drugs that indirectly affect NF-κB pathway have been developed. Recent studies mainly focus on developing novel drugs directly targeting NF-κB in the treatment of heart diseases. It is reported that NF-κB inhibitor remarkably decreases expressions of MMP-2 and MMP-9 in myocardial infarction rats, thus alleviating the negative remodeling of the ventricle. However, it is noteworthy that systemic knockdown of NF-κB may lead to immune deficiency and abnormal apoptosis. Therefore, it is necessary to find a safe and tissue-target therapy to intervene in the NF-κB pathway, so as to effectively treat IRI. The protective effect of arginase-2 on myocardial cell energy during ischemia-reperfusion has not been fully elucidated. Current researches pointed out that arginase-2 can affect the transport of membrane particles and decrease Ca²⁺ influx. Arginase-2 is capable of inhibiting the release of growth hormone (GH), thyroid stimulating hormone (TSH), insulin, serotonin and calcitonin. Arginase-2 participates in a variety of physiological functions, such as anti-inflammatory, immune regulation, anti-oxidation, and development of the nervous system and retina. Animal experiments confirmed that arginase-2 protects renal, intestinal, hepatic, and brain tissues during IRI. In the present study, rats in IRI group showed significant histopathological lesions in myocardium and cardiomyocyte apoptosis, which were alleviated by arginase-2 pretreatment. Our study indicated that arginase-2 group showed lower expression of NF-κB in lesioned myocardium, suggesting that arginase-2 could inhibit IRI-induced inflammatory response via NF-κB pathway.

Conclusions

We found that arginase-2 protects myocardial ischemia-reperfusion injury in rats through inhibiting the inflammatory response via suppression of NF-κB/TNF-α pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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