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Effect of carvedilol on cardiac dysfunction 4 days after myocardial infarction in rats: role of toll-like receptor 4 and β-arrestin 2

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Abstract. – OBJECTIVES: To assess the beneficial effect of carvedilol treatment on infarct myocardium and the relation to the expression of nuclear factor-kappa B (NF-κB), Toll-like receptor 4 (TLR4), and β-arrestin 2.

MATERIALS AND METHODS: Rat myocardial infarction (MI) model was produced by ligating the left anterior descending coronary artery. Forty-eight rats were randomized to the following groups before surgery: sham-operated group (n=8), MI group (n=10), and three carvedilol-treatment groups (n=30, 2 mg/kg, 10 mg/kg and 30 mg/kg).

RESULTS: Four days after MI, carvedilol treatment could ameliorate left ventricular dysfunction by inhibiting the MI-induced increase of left ventricular end diastolic pressure and the decrease of left ventricle end systolic pressure and the changes to their maximum rates (+dp/dt$_{max}$ and -dp/dt$_{max}$). Histological examination showed that carvedilol attenuated myocardium necrosis and inflammatory cell infiltration. In parallel, the treatment also suppressed the expression of NF-κB and TLR4 induced by MI, but increased the expression of β-arrestin 2.

CONCLUSIONS: These results indicate that short term administration of carvedilol could improve early cardiac dysfunction in a rat model of MI. This beneficial effect may be attributed to inhibit the expression of NF-κB and TLR4, but induce the expression of β-arrestin 2 in the infarct region of the myocardium, which would suppress inflammation.

Key Words:
Carvedilol, Myocardial infarction, TLR4, NF-κB, β-arrestin 2.

Introduction

Myocardial infarction (MI) is a leading cause of morbidity and mortality. The predominant mechanism of cardiomyocyte death in the infarcted heart is coagulation necrosis, which initiates an inflammatory reaction. Activation of the nuclear factor-kappa B (NF-κB) system plays an essential role in the induction of proinflammatory mediators[1]. Toll-like receptors (TLRs) have been identified as the key recognition components of the innate immune system in mammals and are known to be also involved in cardiac inflammatory response in heart failure[2,3]. In particular, activation of the TLR4 pathway has been shown to directly impair the contractility of isolated myocytes[4]. In a murine MI model, it was found that TLR4 expression in ventricular muscle was sharply increased in MI group compared to sham mice after 4 days of MI and knockout of TLR4 could protect against myocardial inflammation injury[5].

Clinical studies[6] and animal experiments have shown that carvedilol prevents left ventricular (LV) remodeling, reduces infarct size, decreases inflammatory cytokines' expression and improves survivals[6]. Wisler et al[7] compared among 16 adrenoceptor antagonists, and identified one compound, carvedilol, that possesses the unique signaling profile of negative efficacy for Gs-dependent adenylate cyclase activation but positive efficacy for β-arrestin-dependent ERK 1/2 (extracellular signal-regulated kinase 1 and 2) activation[7]. Thus, the distinct positive effect on β-arrestin may explain carvedilol's unique clinical effectiveness in heart failure and other cardiovascular diseases.

Long term treatment of carvedilol has been shown to positively improve cardiac function, but there is no report on the outcomes of short term treatment. The latter coincides with earlier stage of post-infarction recovery, and timely resolution and containment of inflammatory response of the infarcted area is critical for optimal healing. We hypothesized that carvedilol may act early to bring a beneficial effect to cardiac function fol-

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lowing MI. In the present study, we examined the effect of short term administration of carvedilol on improving the early cardiac dysfunction induced by MI in a rat model, and in parallel, investigated its effect on the expression of NF-κB, TLR4, and β-arrestin 2 proteins to gain insight into the potential mechanisms.

Materials and Methods

Animal Model

Six to eight week old male Sprague-Dawley rats weighing 200 g to 300 g were kept under the standard condition of 12h to 12h dark cycle with free access to food and tap water. All animal experiments were performed with the permission from the Medical Ethics Committee in Anhui Medical University and followed the protocol outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Rat MI model was generated according to a previously described method[8]. Briefly, after anesthetization by intra-peritoneal administration of ethyl carbamate (1.0 g/kg, i.p.), all animals underwent endotracheal intubation. Mechanical ventilation was provided with room air at 60 to 70 breaths per minute using a Rodent Respirator (Taimeng Company, Chengdu, China). A standard lead-II electrocardiogram was recorded via subcutaneous stainless steel electrodes. After a left thoracotomy was performed to expose the heart at the fifth intercostal space, the left anterior descending coronary artery (LAD) was ligated with a 5-0 silk suture. Ischemia was confirmed by the elevation of ST segment in electrocardiogram and cardiac cyanosis. After these surgical procedures, rats were allowed to stabilize for 15 min, and then the thoracic cavity was closed. The sham-operated rats underwent the same operative procedure, but the suture was loosely tied to avoid coronary artery occlusion.

Experimental Design

Forty-eight rats were randomized to the following groups before surgery: 1) sham-operated group (n=8), 2) MI group (n=10), 3) 2 mg/kg carvedilol-treatment group (n=10), 4) 10 mg/kg carvedilol-treatment group (n=10), 5) 30 mg/kg carvedilol-treatment group (n=10). Sham and MI groups were given vehicle, and carvedilol groups[9] received different doses of carvedilol by direct gastric gavage for 7 days, respectively. On the fourth day of drug or vehicle administration, forty rats (excepting Sham group) were rendered MI by ligation of LAD.

Hemodynamic Measurement

Four days later, rats were anesthetized ethyl carbamate (1.0 g/kg, i.p.) for hemodynamic measurement[10]. Briefly, to record LV (left ventricular) pressure, a polyethylene catheter (0.58-mm internal diameter, PE-50) was inserted into the right carotid artery and advanced into the left ventricle. The catheter filled with water was connected to the tube that was in turn connected to a water-filled pressure transducer. While rats were allowed to breathe spontaneously, the pressures were recorded on a physiological recorder (Taimeng Company, Chengdu, China). All hemodynamic data were obtained by averaging the values from 10 heart-beats.

Myocardial Infarct Size, LV Relative Weight and Histology Measurement

After hemodynamic measurement, rats were euthanized with an overdose of ethyl carbamate, and hearts were quickly removed and weighed. The left and right ventricles were then separated and weighed. Finally, parallel to the atrioventricular groove, the left ventricle was cut into 2 parts. One part was fixed with 4% paraformaldehyde for histology hematoxylin and eosin (HE) staining, Masson trichrome staining and immunohistochemistry. The other was dissected into infarcted and non-infarcted zones. The infarcted zones were immediately frozen and stored at –80°C until later use. Myocardial infarct size was measured as the percentage of the left ventricle circumference[11]. LV relative weight was calculated by dividing the weight of the left ventricle by the body weight.

Immunohistochemistry

Immunohistochemistry (Streptavidin-biotin-peroxidase complex staining) was performed as described previously[12]. Transverse myocardial sections (4-μm-thick) were deparaffinized and treated with 0.3% hydrogen peroxide, and then blocked with 10% normal goat serum for 30 min at the room temperature. The sections were incubated with the primary antibody rabbit anti-NF-κB subunit p50 (Santa Cruz, CA, USA) overnight at 4°C. After washing in phosphate-buffered saline (PBS), the sections were incubated with the secondary antibody goat anti-mouse biotin IgG (Zymed Laboratories Inc., South San Francisco, CA, USA) for 30 min at room temperature.

Following 30 minutes' reaction with streptavidin-biotin-peroxidase complex, the immunoreactivity was determined by diaminobenzidine (Zymed Laboratories Inc., South San Francisco, CA, USA). The slides were then counterstained with Mayer's acid hematoxylin. Negative control sections were incubated with the secondary antibody alone. After staining, the average positive area was calculated by computer-assisted planimetry (Image-Pro Plus6.0, Media Cybernetics, Silver Springs, MD, USA).

Western Blotting

Protein extracts for TLR4, β-arrestin 2 and NF-κB P50 were made from myocardial tissues with an RIPA lysis buffer on ice for 30 min. The lysate was centrifuged at 4ºC for 5 min and the supernatant was collected according to the manufacturer's procedures. Samples of 50-μg of these proteins were separated on a 10% sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membrane. Nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20. The membranes were incubated with anti-human TLR4 immune serum (diluted 1:200, Bioworld Technology, St. Louis Park, MN, USA), anti-human β-arrestin2 (1:200, Santa Cruz Inc., Santa Cruz, CA, USA), and anti-NF-κB P50 (1:200, Santa Cruz Inc., Santa Cruz, CA, USA) overnight at 4°C. After three washings, membranes were incubated with secondary HRP-conjugated goat anti-rabbit IgG for 30 minutes. The protein was detected by chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Pierce Bio. Inc., Rockford, IL, USA). Chemiluminescence was detected using an ECL-Plus kit (Perkin Elmer Life Science, Shelton, CT, USA) and visualized by Kodak X-ray film. The bands were quantified by densitometry. Levels of each protein were expressed as the ratio to β-actin for each sample.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed with SPSS 13.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with LSD post hoc test was used for comparisons between the groups. A value of $p < 0.05$ was considered as statistically significant.

Results

Effects on Infarct Size and LV Relative Weight

The present model of left anterior descending coronary artery occlusion resulted in myocardial infarction. As shown in Table I, there was significant increase in the size of the infarcted areas 4 days after surgery. Compared with the MI group, the infarct size tended to be smaller in the carvedilol treatment groups, although the difference did not reach a statistical significance. LV relative weight was also evaluated in MI group; however, there were no clear differences between Sham, MI and treatment groups (Table I).

Effects on Left Ventricular Haemodynamic Parameters

Measurements of hemodynamic parameters by cardiac catheterization 4 days after MI are shown in Table II. Compared with Sham group, Left Ventricular End Diastolic Pressure (LVEDP) and Maximum Rate of Left Ventricular Pressure Changes (LV ±dP/dt max) were significantly decreased, but Left Ventricular End Systolic Pressure (LVESP) increased in MI group ($p < 0.05$). LVEDP was lower, but LVESP was higher in three groups of carvedilol-treated rats than those of MI group ($p < 0.05$), although with no clear dose-effect relationship. The decrease in LV $+dp/dt_{max}$ in MI rats was significantly reversed by carvedilol (2 mg/kg) treatment ($p < 0.05$), and

Table I. Effect on infarction size and LV relative weight in rats.

	Sham	MI	Car 2 mg/kg	Car 10 mg/kg	Car 30 mg/kg
n	8	8	7	7	7
Infarct size (%)	0	49 ± 11[#]	41 ± 6[#]	40 ± 14[#]	42 ± 5[#]
LV relative weight (g/kg)	2.08 ± 0.15	2.22 ± 0.3	2.17 ± 0.18	2.20 ± 0.39	2.11 ± 0.31

[#]$p < 0.05$ vs Sham.

Table II. Effect on hemodynamics parameters 4 days after MI in rats.

	Sham (n=8)	MI (n=8)	Car 2 mg/kg (n=7)	Car 10 mg/kg (n=7)	Car 30 mg/kg (n=7)
LVESP (mmHg)	131.83 ± 18.24	110.72 ± 13.27#	130.78 ± 7.94*	130.35 ± 11.47*	125.48 ± 12.74*
LVEDP (mmHg)	17.50 ± 8.44	27.48 ± 11.32#	12.34 ± 5.55*	15.04 ± 6.44*	17.54 ± 7.61*
+ dp/dt_{max} (mmHg/ms)	4.01 ± 1.82	2.62 ± 0.83#	4.18 ± 1.31*	3.65 ± 1.33	3.51 ± 1.19
– dp/dt_{max} (mm Hg/ms)	-3.29 ± 1.28	-2.11 ± 0.57#	-3.66 ± 1.09*	-3.16 ± 1.11*	-2.76 ± 0.56

#$p < 0.05$ vs Sham,*$p < 0.05$ vs MI.

the reversal effect on LV -dp/dt_{max} was also observed in carvedilol-treated (2 mg/kg and 10 mg/kg) group ($p < 0.05$). The significant changes of LVESP, LVEDP and LV ±dp/dt_{max} indicated that 4 days of MI could result in cardiac dysfunction, and carvedilol could improve the function of the post-infarcted heart.

Effects on Histopathology

Result from HE staining show that myocardial tissue in Sham group was normal with orderly striated heart muscle fibers, intercalated discs, and a clear nuclear staining. Four days after MI, the myocardial infarction resulted in muscle fibers lacking transverse band structure and showing shrinkage, fragmentation or disappearance of nucleus. Myocardium necrosis and inflammatory cell infiltration were marked in MI group. As shown in Figure 1, carvedilol ameliorated the myocardium necrosis and inflammatory cell infiltration.

Effect on the Expression of NF-κB

To understand the cellular basis of the protective effect by carvedilol, the expression of NF-κB 4 days after MI was measured by immunohistochemistry (Figure 2). Four days after MI, marked increase of NF-κB subunit p50 was observed mostly in nuclear staining of the infarcted region. Carvedilol treatment inhibited the p50 expression induced by MI ($p < 0.05$), although with no dose-effect in three treatment groups.

The expression of NF-κB was further measured by Western blot (Figure 3). Consistent with the immunostaining results, the enhanced expression of NF-κB seen in MI was also reduced by carvedilol (2 mg/kg) treatment ($p < 0.05$ vs MI).

Effect on the Expression of TLR4

To explore the possible involvement of TLR4 in carvedilol-mediated protective effect, the expression of myocardial TLR4 was assessed by Western Blot analysis (Figure 4). TLR4 expression was found in Sham-operation myocardium and in-

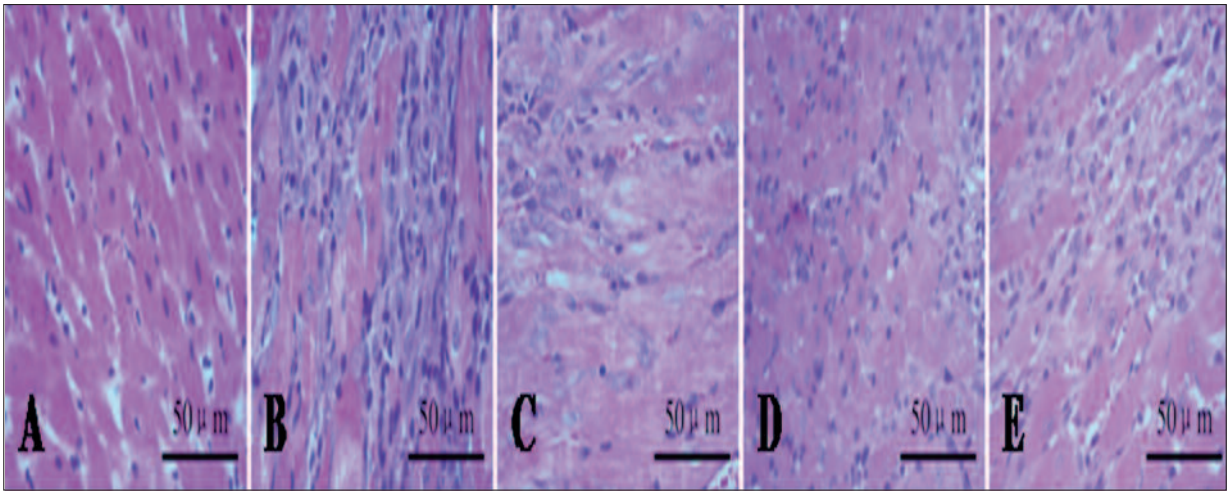

Figure 1. Effect on histopathological changes in the myocardium in different groups (Magnification × 400). ***A,*** Sham group. ***B,*** MI group. ***C,*** Car 2 mg/kg group. ***D,*** Car 10 mg/kg group. ***E,*** Car 30 mg/kg group.

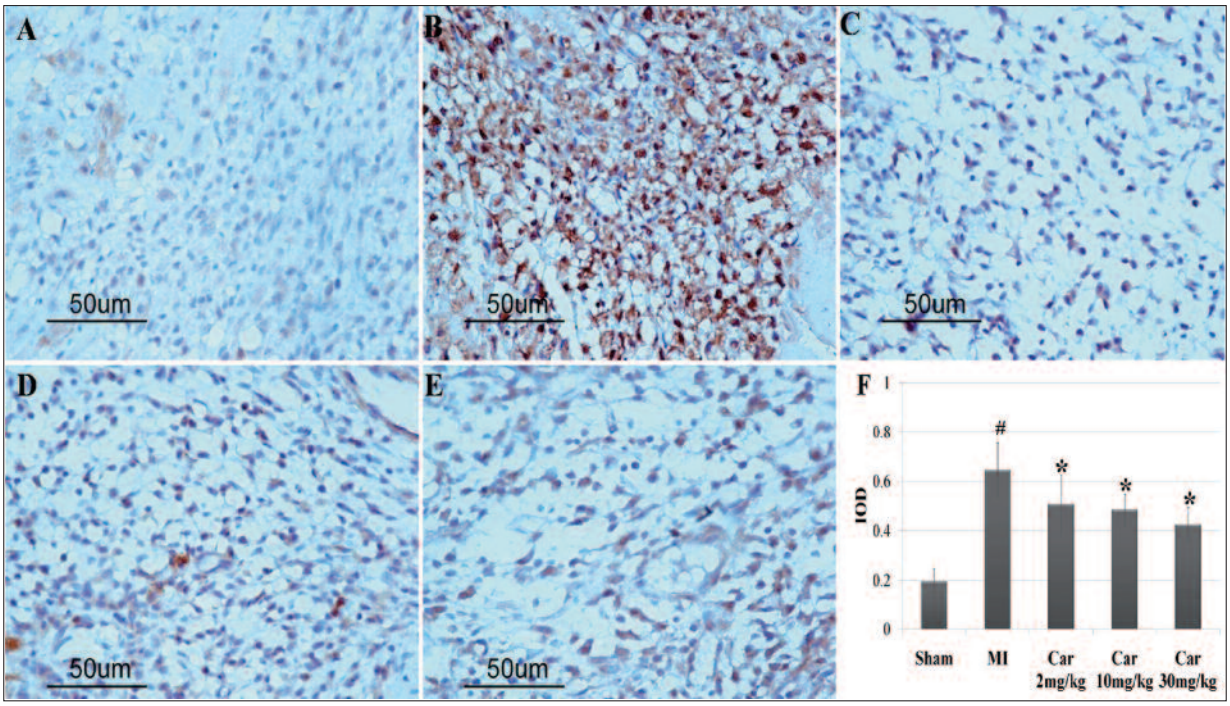

Figure 2. Effect on the expression of NF-κB p50 in different groups by immunohistochemistry (Magnification × 400). ***A,*** Sham group. ***B,*** MI group. ***C,*** Car 2 mg/kg group. ***D,*** Car 10 mg/kg group. ***E,*** Car 30 mg/kg group. ***F,*** Data are expressed as mean ± SD. $^{\#}p < 0.05$ vs Sham,$^{*}p < 0.05$ vs MI.

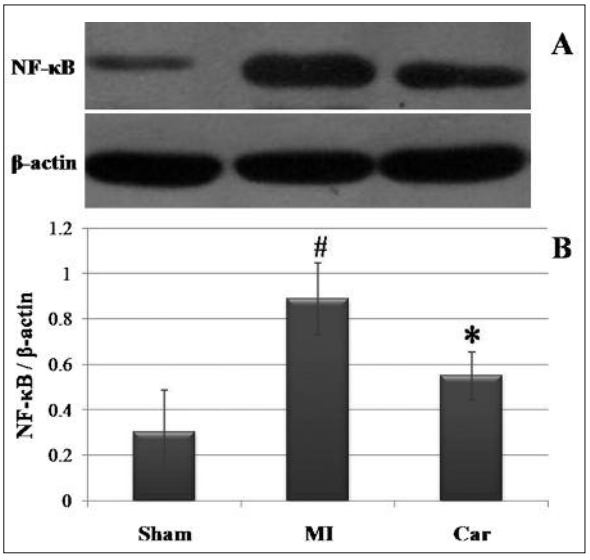

Figure 3. Effect on the expression of NF-κB p50 by Western blot. ***A,*** Four days after myocardial infarction, the myocardium issue lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-NF-κB or β-actin antibody. β-actin was used as internal control. ***B,*** Quantification of NF-κB protein expression was performed by densitometric analysis. Data are expressed as mean ± SD. $^{\#}p < 0.05$ vs Sham,$^{*}p < 0.05$ vs MI.

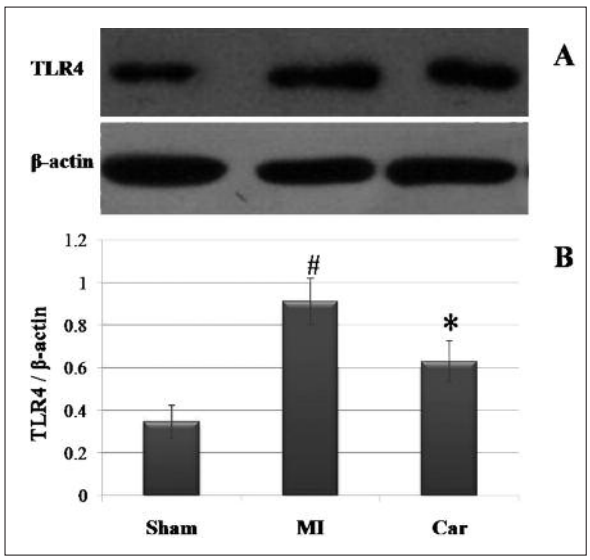

Figure 4. Effect on the expression of TLR4 by Western blot. ***A,*** Four days after myocardial infarction, the myocardium issue lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-TLR4 or β-actin antibody. β-actin was used as internal control. ***B,*** Quantification of TLR4 protein expression was performed by densitometric analysis. Data are expressed as mean ± SD. $^{\#}p < 0.05$ vs Sham,$^{*}p < 0.05$ vs MI.

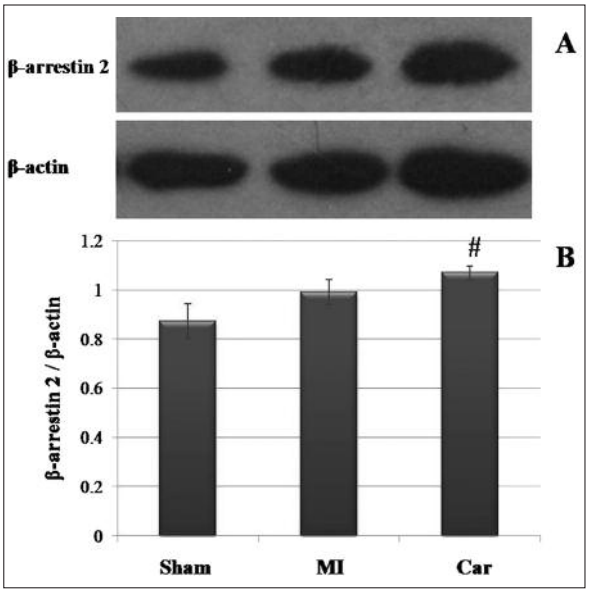

Figure 5. Effect on the expression of β-arrestin 2 by Western blot. ***A,*** Four days after myocardial infarction, the myocardium issue lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-β-arrestin 2 or β-actin antibody. β-actin was used as internal control. ***B,*** Quantification of β-arrestin 2 protein expression was performed by densitometric analysis. Data are expressed as mean ± SD. $^{\#}p < 0.05$ vs sham.

creased 4 days after MI ($p < 0.05$). Carvedilol (2 mg/kg) treatment significantly reduced TLR4 expression ($p < 0.05$ vs MI).

Effect on the Expression of β-arrestin 2

The expression of myocardial β-arrestin 2 was further assessed by Western Blot analysis (Figure 5). The expression of β-arrestin 2 showed no differences between sham group ($p > 0.05$) and 4 days after MI. Carvedilol (2 mg/kg) treatment, however, significantly increased the expression of β-arrestin 2 ($p < 0.05$ vs Sham).

Discussion

In the present study we have investigated the effect of carvedilol on improving the early cardiac dysfunction in a rat model of MI that closely mimicks human anatomy and physiology. The main results of the present work are: (1) short term administration of carvedilol significantly inhibited the inflammation in the infarcted myocardial tissue and improved early cardiac dysfunction. (2) compared with MI group, carvedilol treatment could inhibit the expression of NF-κB and TLR4 protein, but increase the expression of β-arrestin 2 protein in infarcted region of myocardium.

Many clinical trials and animal model experiments of MI report that long term treatment of carvedilol can positively improve cardiac function, but there is no study concerning the outcome of short term treatment. In the present study, we observed that 7 days administration of carvedilol could improve early cardiac dysfunction induced by MI. Histological results show that carvedilol treatment could limit the myocardial necrosis and inflammation, which suggests that the inhibition of inflammation may underlie its amelioration of cardiac dysfunction 4 days after MI.

Activation of the NF-κB system plays an essential role in the induction of proinflammatory mediators in the ischemic myocardium[13], possibly by regulating diverse genes including those involved in the inflammatory response[14]. Transgenic mice with cardiac-specific expression of a dominant-negative IκB resulting in inhibition of cardiomyocyte NF-κB activation, exhibited significantly decreased infarct size in a model of reperfusion infarction[15], and NF-κB p50 null mice had improved early survival and reduced left ventricular dilatation after myocardial infarction[16]. The present study showed that carvedilol could inhibit the excessive expression and nuclear translocation of NF-κB induced by myocardial infarction. This may contribute to the inhibition of inflammation and improved early cardiac dysfunction by carvedilol treatment.

TLRs have been identified as the key recognition components of the innate immune system in mammals and are known to be also involved in cardiac inflammatory response in heart failure. In early stage of MI, cells dying from necrosis release their intracellular contents and initiate an intense inflammatory response by activating innate immune mechanisms. Evidence suggests that TLR-mediated pathways play a significant role in triggering the post-infarction inflammatory response by activating the NF-κB system[1]. The TLRs represent a family of pattern recognition receptors that serve to recognize molecular patterns associated with pathogens and induce activation of several kinases and NF-κB. To date, 13 members of the TLR family have been identified in mammals; however, their role in cardiac pathology remains poorly understood. TLR4 is expressed in the heart and is markedly induced in mouse and rat infarcts and in samples obtained

from cardiomyopathic hearts[17]. Recent investigations have demonstrated that TLR4 deficient mice have decreased infarct size and suppressed inflammation[18], and exhibit attenuated adverse remodeling following myocardial infarction[19]. These studies suggested that TLR4 signaling may critically affect the inflammatory response in myocardial infarction progression. Clinical studies have further shown that TLR4 expression on peripheral blood mononuclear cell was markedly elevated in acute myocardial infarction (AMI) patients[20]. An increase in monocytic TLR4 expression is seen not only in the systemic circulation, but also at the site of plaque rupture, which even higher than system blood. A six-month follow-up study indicated that local and systemic levels of TLR4 were higher in patients with AMI with cardiac events than in those without[21]. A suppression of TLR4 expression observed in the present study suggests that TLR4 is probably involved in the carvedilol effect.

Taking together, our study consistently demonstrated that myocardial infarction could result in the activation of TLR4-NF-κB signaling pathway in the myocardium and, thus, induce inflammation and cardiac dysfunction. Treatment with carvedilol could inhibit the TLR4-NF-κB pathway and inflammation, hence improving cardiac function. The findings of present study may provide a scientific basis for the clinical application of carvedilol.

A further finding was the altered β-arrestin 2 by carvedilol treatment. This protein is a member of arrestin proteins family, originally identified as signal terminators for G protein-coupled receptor signaling[22]. Of particular interest, β-arrestin 2 may directly interacts with Iκβa, which masks the nuclear localization signal of NF-κB, and prevents the phosphorylation and degradation of Iκβa, and thus modulates activation of NF-κB and expression of NF-κB target genes[23]. Stimulation of β_2AR (β2-Adrenergic receptors) could enhance β-arrestin 2-Iκβa interaction and prevent Iκβa degradation, thus inhibit NF-κB activation[24]. β-blockers are recognized to improve cardiac function and long-term prognosis, but not all β-blockers have the same effect and carvedilol's beneficial effect appears to be outstanding. The distinct effect on cellular signaling involving the positive efficacy on β-arrestin dependent kinases[7] means that this molecule may underlie carvedilol's unique clinical effectiveness in heart failure and other cardiovascular diseases. The present study was the first experiment to demonstrate the specific effect of carvedilol on β-arrestin expression in myocardium. In the present study, administration with carvedilol increased the expression of β-arrestin 2 in parallel with the inhibition of the expression and nuclear translocation of NF-κB. These data suggest that β-arrestin 2-NF-κB signaling pathway may also be involved in the early effect of carvedilol on cardiac function.

Conclusions

Short term administration of carvedilol could improve early cardiac dysfunction in a rat model of MI, possibly mediated by inhibiting the expression of NF-κB and TLR4 proteins and increasing the expression of β-arrestin 2 proteins in infarct region of myocardium, which results in reduced inflammation. The mechanism for the inhibition of the TLR4 expression by carvedilol is unclear at present and we also don't know the relationship between TLR4 and β-arrestin 2. In cultured macrophage cells[25], the levels of β-arrestin 2 were reduced in RAW 264.7 cells after stimulation with LPS (lipopolisaccaride), a TLR4 activator. Together with the present observation, this suggests that there may exist a negative feedback between TLR4 and β-arrestin 2, which warrants further study.

Conflict of Interest

None.

Acknowledgements

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