Inhibition of TNF-α by Cyclophosphamide Reduces Myocardial Injury after Ischemia-Reperfusion

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Purpose: The purpose of this study was to determine whether cyclophosphamide (CP) can decrease myocardial and systemic TNF-α expression and thus protects myocardial I/R injury.

Methods: Open chest rats were subjected to 30 min of ischemia followed by 3h, 12h or 24h of reperfusion. Rats were divided into sham group, I/R group and CP group, and each group included 3 timepoint subgroups (3h, 12h and 24h). Plasma TNF-α was measured by cytometric bead array (CBA) and immunohistochemistry was used to detect TNF-α in myocardium.

Results: Compared with I/R group, rats treated with CP showed a significant difference with decreased plasma TNF-α (13.31 ± 2.62 vs 14.13 ± 5.95 pg/mL at 3 h reperfusion, 10.1 ± 2.73 vs 12.54 ± 5.00 pg/mL at 12 h reperfusion, 10.38 ± 5.59 vs 13.00 ± 3.59 pg/mL at 24 h reperfusion, p <0.05 respectively). Immunostaining was less intense with CP injection at each reperfusion time. The score of the intensity of myocardial TNF-α staining was down regulated.

Conclusions: TNF-α is expressed in the myocardium and plasma after myocardial I/R injury. CP might be a feasible strategy for anti-TNF-α to protect myocardial I/R injury.

Keywords: cyclophosphamide, TNF-α, ischemia/reperfusion

Introduction

The systemic inflammation and the local inflammation in myocardium contribute greatly to functional damage after myocardial ischemia/reperfusion (I/R) in vivo. A recent study demonstrated that prolonged use of treatments such as methotrexate, sulfasalazine, leflunomide, glucocorticoids, and tumor necrosis factor-alpha blockers appeared to be associated with a reduced risk of cardiovascular disease.1 We recently also disclosed that cyclophosphamide (CP) could improve myocardial function in rats after ischemia reperfusion injury. These findings could help to gain some insight into therapeutic potential of CP in myocardial I/R injury.2 However, the mechanism remains unclear. As we have known TNF-α is released during myocardial I/R from storage sites in tissue mast cells within the myocardium3 and possibly also from cardiomyocyte itself4 and the plasma inflammatory cells.5 Presently, we used open-chest ischemia-reperfusion rat model with minimal surgical trauma to further demonstrate whether down-regulation with TNF-α-related inflammatory pathways by CP could reduce myocardial injury after I/R.

Materials and Methods

The Animal Care and Use Committee of Zhejiang University approved all procedures. All animals received

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humane care in compliance with the guidelines in the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by National Academy Press.

**Experimental protocol (Fig. 1)**

Rats were subjected to intraperitoneal injection of 750 mg/m² cyclophosphamide (Jiangsu Hengrui Medicine Co. Ltd., Nanjing, China) or saline before surgery, followed by 30 min of the left ventricle normothermic ischemia, and 3h, 12h or 24h of reperfusion (n = 8 in each subgroup). In the sham group, saline was intraperitoneally injected before surgery. The silk suture was crossed without ligation, and the rat did not receive ischemia-reperfusion. In the I/R group, saline was intraperitoneally injected before surgery, and the rat received ischemia-reperfusion. In CP group, cyclophosphamide was intraperitoneally injected before surgery, and the rat received ischemia-reperfusion. Eighty-four rats have been subjected to surgery, and all surviving 72 rats were assigned to three groups. Hemodynamic parameters were detected at the end of reperfusion. The animals were sacrificed by bleeding from polyethylene tube, the blood and hearts were collected for TNF-α detection and later for histopathological study.

**Experimental preparation**

Briefly male Sprague-Dawley rats were anesthetized with chloral hydrate before surgery. Myocardial ischemia was produced by occlusion of the left anterior descending coronary artery (LAD) for 30 minutes, following reperfusion for 3, 12 or 24 hours. The ischemic area was distinguished from the area, not at risk by Evans blue dye staining, and the infarct portion of the myocardium was determined by the triphenyl tetrazolium chloride method as described previously. A polyethylene tube (PE 50) was passed through the right carotid artery into the left ventricle to detect LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), positive and negative maximal values of the first derivative of left ventricular pressure (± dP/dt).

**Detection of plasma cytokine with cytometric bead array (CBA)**

Analysis of TNF-α and IL-4 was conducted by using a rat inflammation cytometric bead array kit (CBA; Bender Med Systems Products, Vienna, Austria) and was conducted on a FACS Calibur flow cytometer (Becton Dickinson, Bedford, MA). Standard curve for each cytokine from each kit was generated by using the reference cytokine concentrations with a range of 27–20000 pg/ml (for TNF-α) or 3.0–2000 pg/ml (for IL-4). According to the manufacturer, the minimum of detection for CBA, is 4.3 pg/ml (for TNF-α) and 0.3 pg/ml (for IL-4). For CBA raw data exported from the FACS Calibur flow cytometer was analyzed by Flow Cytomix Pro2.1 software (Bender MedSystems GmbH).

**Immunohistochemistry**

For histological study of cardiac tissue, sections taken from endocardium to epicardium were fixed in 4%
phosphate-buffered formalin, 2% paraformaldehyde and embedded in paraffin. Sequential 2 to 5 µm sections were cut by microtomy. Immunostaining was performed according to the manufacturer's instructions. The following primary antibodies were used: goat polyclonal antibody to rat TNF-α (at 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) known to crossreact with canine TNF-α. The secondary antibody for TNF-α antibody was detected with a peroxidase-based system using diaminobenzidine (DAB) as a substrate. The slides were washed, dehydrated, and mounted for light microscopy. Appropriate controls were performed with rabbit serum substituted for the primary antibody.

TNF-α staining was quantified per high power field, by examining corresponding TNF-α stained sections from three separate animals per group (mean of five counts per animal) by two independent observers. 6) TNF-α staining was then examined, and each counted staining was graded as absent staining (score 0), mild staining (score 1–3), moderate staining (score 4–6) or intense staining (score 7–9).

**Statistical analysis**

All data were expressed as mean ± SD. Analysis was carried out by using the SPSS statistical package (version 14.0). Univariate analysis was used to test the differences of all measurements. When a significant p value, p < 0.05, was shown for certain variables, post hoc test (LSD and Bonferroni correction) was followed. A value of p < 0.05 was considered statistically significant.

**Results**

Compared with I/R group, rats treated with low dose CP showed a significant recovery in myocardial function with improved LVSP (88 ± 11 vs 69 ± 11 mmHg of 3h; 92 ± 11 vs 64 ± 14 mmHg of 12h; 90 ± 11 vs 64 ± 14 mmHg of 24h; p < 0.01 respectively). The ± dp/dtmax also had the similar trends. The myocardial infarct size was reduced in CP group compared to that in I/R group.

**Attenuated plasma TNF-α**

CP-treated rats showed a reduced plasma TNF-α compared to I/R group (p <0.05) (Fig. 2).

**Immunohistochemistry of TNF-α in myocardium**

The predominant area of TNF-α immunostaining localized to the infarct site and was present at each reperfusion time (Fig. 3). The staining was more intense after a 12-hour reperfusion without CP (Fig. 3C), and less intense with CP injection at each reperfusion time compared to that in the I/R group. TNF-α was mainly localized in vascular endothelium, the cardiomyocyte and the connective tissues within the infarct zone and peri-infarct zone. Myocytes in the contralateral non-infarct zone have the least intensive staining of TNF-α at all time points after reperfusion. TNF-α was undetectable in normal control hearts (data not shown).

The intensity of TNF-α staining was down regulated with CP injection in each reperfusion timepoint between CP and I/R group (2.42 ± 0.38 vs 3.33 ± 0.36 at 3h reperfusion, 3.75 ± 0.52 vs 5.08 ± 0.58 at 12h reperfusion, 6.50 ± 0.45 vs 8.08 ± 0.8 at 24h reperfusion p <0.01 respectively) (Fig. 4).
Fig. 3  Photomicrographs of immunostaining of TNF-α in rat heart after I/R. Intensive TNF-α staining was observed in infarct and peri-infarct areas. Arrows point to TNF-α staining within myocytes, vascular endothelium and connective tissue (at ×400 magnifications.)
A: Section was taken from rat heart tissue after 3 hour reperfusion without CP.
B: Section was taken from rat heart tissue after 3 hour reperfusion with CP.
C: Section was taken from rat heart tissue after 12 hour reperfusion without CP.
D: Section was taken from rat heart tissue after 12 hour reperfusion with CP.
E: Section was taken from rat heart tissue after 24 hour reperfusion without CP.
F: Section was taken from rat heart tissue after 24 hour reperfusion with CP.
G: Section was taken from rat heart tissue without ischemia/reperfusion as negative staining.
CP: cyclophosphamide

Fig. 4  TNF-α staining score in different groups, * p <0.01 compared to I/R group.
Discussion

It is well known that reperfusion of ischemic hearts is associated with pathophysiological events such as edema, metabolic abnormalities, myocardial necrosis and contractile dysfunction (the extreme of which is the “stone heart”), which have long been suspected of contributing significantly to the overall morbidity and mortality of percutaneous coronary intervention (PCI) in acute myocardial infarction patients. The hypothesis was put forth by early experimentation, that reperfusion was a potential contributor to lethal injury of previously ischemic myocardium. Protective pharmacologic agents aimed to different mechanisms administered during the reperfusion phase might be capable of limiting tissue necrosis, which was demonstrated by some early experimental evidence in nonsurgical models of coronary artery occlusion–reperfusion as well as surgical models of myocardial protection. However, key aspects of inflammation and effect of antiinflammation therapy in cardiac disease are still not illustrated clearly.

Many studies on blocking transcription and/or biological activity of TNF-α are discussed, as each suggests potentially therapeutic objections. TNF-α synthesized greatly after myocardial I/R. Especially after myocardial reperfusion, the concentrations of TNF-α might have a more robust effusion, and the damage on the myocardium aggravated. The inflammatory state permits a fairly rapid deterioration in myocardial function after I/R injury. Thus, the reperfusion of myocardium may not evoke any improvements in myocardial function because of inflammation injury. The production of TNF-α by cardiac myocytes is sufficient to cause myocarditis, myocardial dysfunction, cardiac failure, and premature death and, therefore, supports a causal role for TNF-α in the pathogenesis of myocardial I/R injury. Indeed, locally produced TNF-α in myocardium may be an important contributor to post-ischemic myocardial injury. On the other hand, many clinical studies also discussed elevated plasma TNF-α was associated with a large decrease in survival of cardiovascular disease. Systemic administration of recombinant TNF-α has consistently resulted in myocardial depression of myocyte contractility and has been associated with the development of cardiomyopathy. In the present study, we introduce CP as antiinflammation strategy in rats with myocardial ischemia reperfusion. We confirmed the effects of CP on TNF-α production in cardiac myocytes by immunohistochemistry staining and that TNF-α staining was less intensive with CP treatment. Thus, the antiinflammation effect on TNF-α, both in myocardium and plasma by CP, obviously would be a benefit to myocardial I/R injury, which might fundamentally change the inflammatory state, as well as the inflammation in myocardium.

Mann stated that TNF-α levels are elevated in patients with ischemic cardiomyopathy, and early preclinical and clinical studies suggested that interfering with TNF-α synthesis was beneficial. Indeed, a plenty of researches had established that myocardial I/R injury could be attenuated by pharmacologic strategies applied at or just before the onset of reperfusion using strategies targeting the numerous mechanisms of reperfusion injury. Experimental studies have shown that the first minutes of reperfusion are absolutely critical for the effective introduction of reperfusion therapy. In this regard, control destructive role of inflammation in cardiovascular disease represents a realistic goal for clinical medicine. Proinflammatory cytokines such as TNF-α is released during myocardial ischemia–reperfusion, which happened during primary percutaneous coronary intervention (PCI). As the guideline suggests, prompt reperfusion treatment is essential for patients who have myocardial infarction with ST-segment elevation. It also recommends that the interval between arrival at the hospital and intracoronary balloon inflation (door-to-balloon) during PCI should be 90 minutes less, according to the ACC/AHA Guideline for Percutaneous Coronary Intervention. If we introduce CP together with dual antiplatelet and other therapy in patients of acute myocardial infarction at the time when they contact to health care providers, the antiinflammation effect might effectively help to protect myocardial I/R injury. We presently introduced CP injection just a little before ischemia, after 30 min ischemia and the reperfusion surgery (totally about 1 hour), the peak concentration of CP in plasma arrived 1 hour after injection, which might be currently capable of myocardial protection as long as I/R injury began. Accordingly we concluded that CP might be a feasible strategy for anti-TNF-α in myocardial I/R injury after PCI.

Conclusion

Ultimately cardioprotective pharmaceuticals of CP might be an integrated strategy of antiinflammation therapeutics after myocardial ischemia-reperfusion by its down regulation of TNF-α. CP may be an important approach to decrease the mortality after PCI and limiting the incidence and severity of heart injury.
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Disclosure Statement

None to declare.

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