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Research Paper

Dephosphorylation of cardiomyocyte Cx43 is associated with myocardial ischemia and reperfusion injury ☆

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Abstract

Objective: Myocardial ischemia/reperfusion(I/R) injury is the leading cause of death in the world. However, the details of the mechanism of its pathophysiology are still unknown. The present study was designed to investigate the role of connexin 43(Cx43) in acute models of myocardial I/R injury. **Methods:** Male C57BL/6 mice were subjected to myocardial ischemia(45 min) followed by reperfusion(4 hrs) in vivo. The whole operation was monitored using a two-lead ECG. Hearts were harvested and the level of protein was assessed by western blot analysis. Haematoxylin and Eosin(HE) staining was used to detect the extent of neutrophil infiltration. The expression level of IL-6 was detected by ELISA. **Results:** A murine myocardial I/R injury model was constructed successfully. Phosphorylated Cx43 decreased 83.45% while non-phosphorylated Cx43 increased 1.62- fold in the myocardium after I/R injury. Neutrophil infiltration and the expression of the inflammatory cytokine IL-6 increased in the myocardium following I/R. **Conclusion:** During myocardial I /R injury, cardiomyocyte Cx43 is dephosphorylated, and this may be associated with an inflammatory response.

Keywords: myocardium; I/R injury; connexin 43; inflammatory response

INTRODUCTION

Myocardial ischemia/reperfusion(I/R) injury is a major cause of morbidity and mortality worldwide. Advances in understanding the pathophysiological mechanisms of this injury are necessary to make progress in its treatment.

It is well known that myocardial I/R injury is associated with an inflammatory reaction[1-3]. A series of proinflammatory, as well as anti-inflammatory cytokines play critical roles during the process[4,5]. Despite extensive investigations, the cellular and molecular mechanisms of the initiation and propagation of the inflammatory response during I/R injury have not been fully elucidated.

Gap junctions are specialized membrane structures consisting of arrays of intercellular channels that directly connect adjacent cells by providing chemical and electrical communication[6]. Connexin 43(Cx43) is the predominant protein forming gap junctions and non-junctional hemichannels in the ventricular myocardium [7-9]. Recently, it was reported that Cx43 antisense oligodeoxynucleotide treatment down-regulates the inflammatory response in an *in vitro* interphase organotypic culture model of optic nerve ischemia[10]. It is possible that Cx43 also participates in regulating the inflammatory response during myocardial I/R injury.

In the present study, we found a decrease of phosphorylated Cx43 in the presence of myocardial I/R injury in our mouse model of ischemia reperfusion. Our results also suggest that Cx43 may contribute to the inflammatory reaction during myocardial ischemic injury.

MATERIALS AND METHODS

Experimental animals

Male C57 BL/6 mice, age of 6-7 weeks, were purchased from the laboratory animal centre of The Academy of

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Military Medical Sciences. All aspects of the animal care and experimental protocols were in accordance with the guidelines for the "Principles of Laboratory Animal Care" and approved by the Nanjing Medical University Committee on Animal Care.

Experimental model of myocardial I/R injury

Male C57 BL/6 mice were subjected to myocardial I/R injury as described in our previous study[11,12]. Briefly, the mice were anesthetized and ventilated with room air using a rodent ventilator. After a left thoracotomy and exposure of the heart, the left anterior descending coronary artery was ligated with a 6-0 silk ligature over a 1-mm polyethylene tube(PE-10). After completion of 45 min of occlusion, the coronary artery was reperfused for 4 hrs. During the whole operation, we monitored the mice with a two lead electrocardiogram to verify whether the model was a success. On completion of the experiments, hearts were harvested, immediately frozen in liquid nitrogen, and then stored at -80℃. Sham surgically operated mice served as controls.

Isolation of cellular protein from heart samples

Cytoplasmic and nuclear proteins were isolated as described in our previous studies[11,12]. Briefly, heart samples were homogenized in ice-cold hypotonic buffer. The homogenates were maintained on ice for 20 min, vortexed for 10 s after adding 10% Nonidet P-40, and the preparations were centrifuged for 5 min at 9,000 × *g* at 4℃.

Supernatants containing cytoplasmic proteins were collected. The pellets were suspended in ice-cold hypotonic salt buffer, maintained on ice for 30 min, mixed frequently, and centrifuged for 10 min at 9,000 × *g* at 4℃. The supernatants containing nuclear extracts were collected. The concentrations of proteins were determined with the Pierce 550nm reagent (Thermo Fisher Scientific Inc., USA).

Western blot analysis

Cytoplasmic proteins (100× μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham, USA) using a Bio-Rad semidry transfer system (Bio-Rad Laboratories, Inc. USA) as described previously. The membranes were probed using the following primary antibodies: Connexin 43 (1:500 dilution, Zymed Laboratories, Inc., USA), β-Tubulin (1:1000 dilution, Cell Signaling Technology Inc., USA). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG secondary antibody. The signals were detected with a Pierce ECL Western Blot Detection Kit, and band density was analyzed using ImageJ software (NIH)[13,14].

Histology

Heart samples were rapidly excised, cross-sectioned and fixed in 10% buffered formalin. Fixed tissue was embedded in paraffin, sectioned in a standard fashion, and 5 μm thick paraffin embedded sections were stained with hematoxylin and eosin(HE). Four different areas of every section were observed.

ELISA

Expression of the pro-inflammatory cytokine IL-6 was measured in cardiac lysate samples by a commercial ELISA kit(R&D Systems, USA). The protein samples(10 μg) were diluted 1:4 in dilution buffer, and the assay conducted according to the manufacturer's instructions.

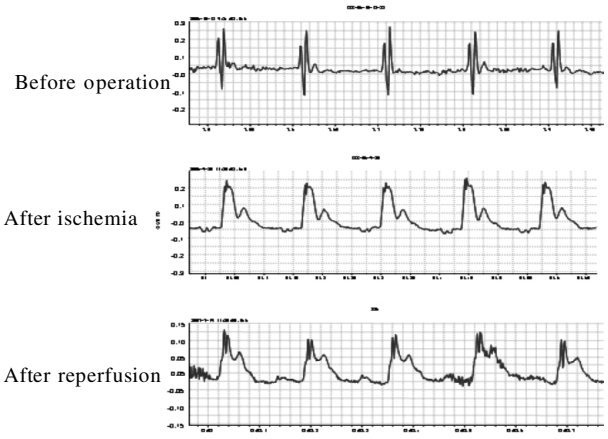
Statistical analysis

Data were presented as mean ± SD. Comparisons between groups were performed using student-t test. A *P*-value of $^{*}P < 0.05$ was considered to be significant.

RESULTS

ECG verified that the mice were subjected to myocardial I/R injury

As exemplified in ***Fig. 1***, before surgery the ECG recordings of the mice were unremarkable. After 45 min ischemia we observed ST-elevation, which became even higher after 4hrs reperfusion, suggesting that myocardial I/R injury had been successfully produced.



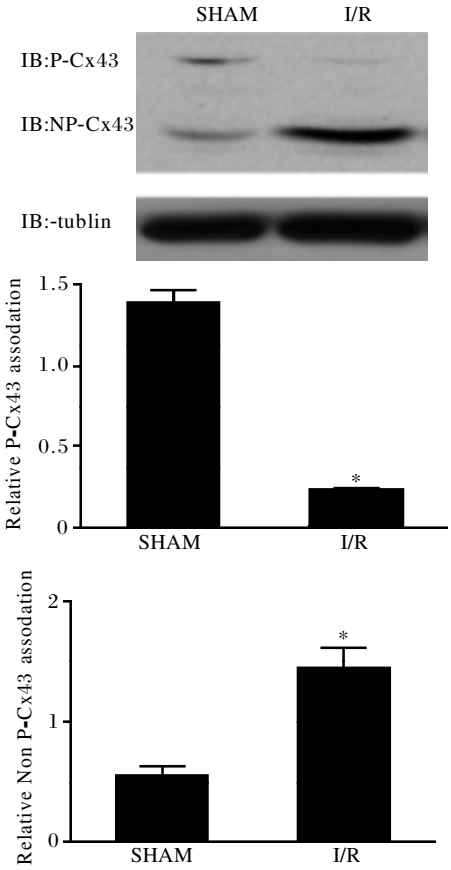
A two leads electrocardiogram was used to monitor the mice during the entire operation. Before the operation, a normal ECG recording was obtained. After ischemia for 45min, ST-elevation was observed, and after 4hrs reperfusion, a higher ST-elevation was detected.

Fig. 1 Representative ECG recordings of mice subjected to *in vivo* myocardial I/R injury

Phosphorylated Cx43 decreased whereas non-phosphorylated Cx43 increased in the myocardium after I/R injury

The gap junction protein connexin-43(Cx43) exists

mainly as the phosphorylated form in the normal heart[8]. After 45 min ischemia and 4h reperfusion, phosphorylated Cx43 and non-phosphorylated Cx43 in the cytoplasm were assessed by Western blot analysis. As illustrated in ***Fig. 2***, the density of non-phosphorylated Cx43 was increased 1.62- fold(0.55 ± 0.08 vs 1.44 ± 0.19), while phosphorylated Cx43 was reduced 83.45% (1.39 ± 0.08 vs 0.23 ± 0.01) after myocardial I/R injury (n=6 mice/group, $^{*}P < 0.05$).



C57 BL/6 mice were subjected to myocardial I/R(45 min/4 h). Hearts were harvested and the protein extracts were separated by SDS-PAGE and assessed by Western blot analysis with the specific antibody. Representative results of P-Cx43(phosphorylated) and NP-Cx43(non-phosphorylated) in cytoplasmic proteins were shown in the panel. The relative levels of myocardial P-Cx43 and NP-Cx-43 were shown in the panel. Data were expressed as mean ± SD (n=6 mice/group, $^{*}P < 0.05$).

Fig. 2 The expression of phosphorylated Cx43 and non-phosphorylated Cx43 in the myocardium after I/R injury

Neutrophil infiltration increased in the myocardium following I/R

We examined myocardial neutrophil infiltration in HE stained sections. ***Fig. 3*** showed that the number of neutrophils infiltrating the myocardium significantly increased after I/R.

I/R increased the expression of the pro-inflammatory cytokine, IL-6, in myocardium lysates

ELISA analysis showed a significant increase in the expression of the key myocardial inflammatory cytokine IL-6 after 45 min ischemia and 4 h reperfusion when compared with sham animals(795.45 ± 31.04 vs. 443.45 ± 26.28 pg/mg protein, $^{*}P < 0.05$)(***Fig. 4***).

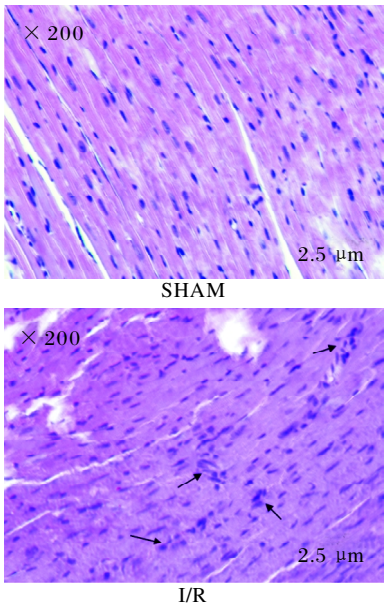
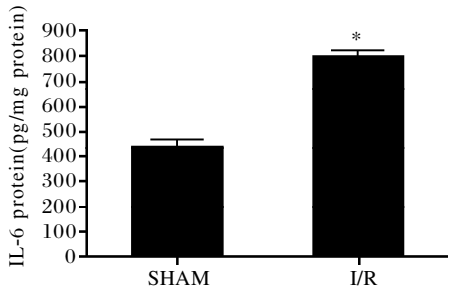


Fig. 3 An increase in infiltrating neutrophils in the myocardium following I/R Mice were subjected to I/R(45 min/4 h). Representative HE-stained histological images were shown(× 200). The arrows indicated infiltrating neutrophils.



The expression of the inflammatory cytokine IL-6 was increased after I/R injury. The level of IL-6 expression in the myocardium was measured by ELISA in animals after sham operation and after I/R(45 min/4 h). Data were expressed as the mean ± SD(n=6 mice/group, $^{*}P < 0.05$).

Fig. 4 The expression of the inflammatory cytokine IL-6

DISCUUSION

In the present study, we observed that phosphorylated Cx43 decreased and non-phosphorylated Cx43 increased after I/R injury, while the number of infiltrating neutrophils in the myocardium significantly increased and the expression level of a major pro-inflammatory cytokine in the myocardium also increased. Our data suggested that dephosphorylation of cardiomyocyte Cx43 was associated with myocardial ischemia and reperfusion injury.

Innate immune and inflammatory responses play

important roles in the pathophysiology of myocardial ischemia/reperfusion(I/R), which is a prerequisite for healing and scar formation[1]. Neutrophil depletion in animals undergoing reperfused myocardial infarction led to a marked decrease in the infarct, suggesting that a significant amount of the myocardial injury induced by coronary artery occlusion followed by reperfusion may be neutrophil-dependent[15, 16]. Neutrophils may release oxidants and proteases and possibly express mediators capable of amplifying cell recruitment[17]. In our study, we also observed that infiltrating neutro-phils in the myocardium significantly increased, and the expression level of an inflammatory cytokine in the myocardium also increased after I/R injury. However, the details of the mechanisms of the innate immune and inflammatory responses during I/R injury have yet to be fully determined.

It has been reported that gap junction-mediated intercellular communication(GJMIC) may play a role in the progression and spread of cell injury and death during myocardial ischemia-reperfusion. During reperfusion, GJMIC has been shown to mediate cell-to-cell propagation of hypercontracture and cell death[18]. There is also evidence that gap junctions or their protein components are involved in the genesis of the protective effect observed with ischemic preconditioning[19]. The gap junction protein Cx43 is the major connexin protein in ventricular myocardium, and downregulation of Cx43(mRNA and protein) has been demonstrated in some experimental heart failure models and in the failing human heart[20]. During myocardial ischemia, Cx43 is dephosphorylated in vitro, and the subsequent opening of gap junctions formed by two opposing Cx43 hexamers was suggested to propagate the ischemia/reperfusion injury[21]. A marked reduction of junctional conductance, loss of dye coupling, loss of Cx43 protein, and down-regulation of Cx43 mRNA expression have been demonstrated in astrocyte cultures after IL-1beta treatment[22]. Furthermore, it has also been showed that activation of the innate immune response in astrocytes is associated with functional loss of GJIC through a pathway involving NF-κB[23,24]. However, the role of Cx43 in myocardial I/R injury is still unknown. In this study, we demonstrated that after I/R injury, dephosphorylation of cardiomyocyte Cx43 may be associated with an inflammatory response. Still more investigation is needed to elucidate the mechanisms involved in myocardial I/R injury.

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