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Original Article

Change of mitochondrial function in the early stage after cardiac ischemia-reperfusion injury in mice

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Abstract: Objective: To detect the change of mitochondrial function in the early stage after myocardial ischemia-reperfusion injury in mice. Methods: C57BL/6 mice were divided randomly to five groups. Hearts in the time control group (TC) were perfused for 45 min in identical Krebs-Henseleit buffer without any treatment. In ischemia-reperfusion groups, hearts were given different reperfusion time including 5 min, 10 min, 15 min or 30 min followed the same ischemia period 25 min. Mitochondria were extracted from left ventricular after reperfusion. Then respiratory function, mitochondrial calcium retention capacity and inner mitochondrial membrane integrity were measured. Results: The results revealed that R3, RCR and P/O of mitochondria were decreased significantly after ischemia and reperfusion 10 min, 15 min and 30 min ($P<0.05$), but not in reperfusion 5 min group compared with time control group using glutamate + malate as substrates. And in different ischemia-reperfusion groups respiratory function decreased significantly compared with time control group using succinate, and TMPD-ascorbate as substrates ($P<0.05$). Meanwhile, both mitochondrial calcium retention capacity and inner mitochondrial membrane potential decreased gradually following prolonged reperfusion. Conclusions: Mitochondrial respiratory function changes differently in different complex at early stage after ischemia and reperfusion. So we should choose different ischemia-reperfusion time to detect different mitochondrial complex changes after heart injury.

Keywords: Cardiac ischemia and reperfusion injury, mitochondria, oxidative phosphorylation, mitochondrial permeability transition pore

Introduction

Cardiac ischemia-reperfusion leads to myocardial infarction and compromises cardiac function [1-3]. Mitochondrial dysfunction contributes to myocardial injury during ischemia-reperfusion [4, 5]. Cardiac injury is decreased by interventions applied at the onset of reperfusion by ischemic postconditioning [6] and pharmacologic therapy [7, 8]. Ischemic postconditioning is effective by modulating the pathologic biochemical behavior of mitochondria that have been damaged by preceding ischemia, including attenuating the onset of mitochondrial permeability transition pore (MPTP) opening during early reperfusion [9, 10]. MPTP opening is a key mechanism of injury during ischemia reperfusion [4, 5]. We use the Langendorff isolated heart perfusion model, extraction of myocardial mitochondria of mice, observed mitochondrial respiratory chain complex of oxidative phosphorylation function, mitochondrial calcium retention capacity and inner mitochondrial membrane potential changes of mitochondrial respiratory chain complex in myocardial reperfusion injury mechanism at different time points during the early stage of reperfusion.

Methods

Preparation of mouse hearts for perfusion

The Animal Care and Use Committee of Tongji Hospital of Huazhong University of Science and Technology approved all protocols of this study. Male C57BL/6 mice [2-3 months of age (22-28 g)] were individually housed in standard plastic mouse cages with *ad libitum* access to water and food. The cages were housed inside isolation chambers, which provided controlled lighting (12:12 light-dark cycle), room temperature of 22±1°C, ventilation, and visual isolation.

The animals were anesthetized with pentobarbital sodium (100 mg/kg intraperitoneal [i.p.]) and anticoagulated with heparin (1000 IU/kg i.p.). Hearts were excised and perfused retrograde via the aorta in the Langendorff mode with modified Krebs-Henseleit (K-H) buffer (115 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.1 mM $MgSO_4$, 0.9 mM KH_2PO_4, and 5.5 mM glucose), gassed with 95% O_2-5% CO_2 to adjust pH to 7.35-7.45 [11]. Hearts were paced at 420 beats per min. The cardiac function was monitored with a balloon inserted into the left ventricle, and data were recorded digitally with Powerlab (AD Instruments, Colorado Springs, CO).

In ischemia-reperfusion groups, hearts were given different reperfusion time including 5 min, 10 min, 15 min or 30 min followed the same ischemia period 25 min. Mitochondria were extracted from left ventricular after reperfusion.

Isolation of mouse heart mitochondria

Mouse hearts were quickly placed in cold buffer A (composition in mM: 100 KCl, 50 MOPS [3-(N-morpholino)propanesulfonic acid], 1 EGTA, 5 $MgSO_4$, and 1 mM ATP). After removal of blood by washing with buffer A, the heart was blotted dry, weighed, and homogenized using a polytron tissue homogenizer at 10,000 rpm for 2.5 seconds in the presence of trypsin (5 mg/g tissue). The homogenate was incubated for 15 min at 4°C, then the same volume of buffer B [buffer A + 0.2% bovine serum albumin (BSA)] was added and the mixture was centrifuged at 500 g for 10 min. The supernatant was again centrifuged at 3000 g to pellet mitochondria. The mitochondrial pellet was first washed with buffer B, resuspended in KME (100 mM KCl, 50 mM MOPS, 0.5 mM EGTA), and centrifuged at 3000 g to yield the final mitochondrial pellet. Mitochondria were re-suspended in KME for study. This method yielded approximately 3-4 mg of mitochondrial protein per 100 μg murine heart. Protein content was measured using the Lowry method. The isolation procedure required approximately 2 h. Mitochondria were kept on ice and used within 4 h [12].

Mitochondrial oxidative phosphorylation

Oxygen consumption in mitochondria was measured using a Clark-type oxygen electrode at 30°C as previously described [12]. Mitochondria were incubated in 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH_2PO_4, and 1 mg defatted, dialyzed bovine serum albumin/ml at pH 7.4. Glutamate (20 mM) + Malate (10 mM) (complex I substrate), succinate (20 mM) plus 7.5 μM rotenone (complex II substrate), and TMPD (N, N, N', N' tetramethyl p-phenylenediamine, 1 mM)-ascorbate (10 mM, complex IV substrate) + rotenone were used.

Calcium retention capacity (CRC) in isolated mitochondria

CRC was used to assess calcium-induced mitochondrial permeability transition poreopening in isolated mitochondria [9]. CRC was evaluated in mitochondria (125 μg/ml) incubated in medium containing 150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinic acid in 20 mM Tris/HCl, pH 7.4 by sequential pulses of a known amount of calcium (5 nmol). Extramitochondrial Ca^{2+} concentration was recorded with 0.5 μM Calcium Green-5N (Life Technologies) and fluorescence monitored with excitation and emission wavelengths set at 500 and 530 nm, respectively.

Relative estimation of mitochondrial inner membrane potential

Mitochondrial inner membrane potential (Δψ) was assessed using the fluorogenic indicator TMRM (tetramethylrhodamine, methyl ester) [10]. Freshly isolated mitochondria (0.2 mg/ml) were incubated in a single cuvette with continuous stirring at 30°C with sequential additions of glutamate (10 mM) + malate (5 mM), ADP (2 mM), oligomycin (1 μM), and DNP (0.3 mM) in buffer (100 mM KCl, 50 mM MOPS, 5 mM KPi, 1 mM EGTA). Relative changes in Δψ were assessed by the change in the 573 nm/546 nm fluorescence ratio of TMRM [10]. Relative membrane potential was measured at 30°C in order to parallel conditions under which oxidative phosphorylation was measured.

Statistical analysis

Data are expressed as the mean ± standard error. For all analyses, differences between groups were compared by one-way ANOVA. When a significant F value was obtained, means were compared using the Student-Newman-Keuls test of multiple comparisons. Statistical significance was defined as a value of $P<0.05$.

Table 1. Myocardial weight and mitochondrial protein of mice heart after ischemia-reperfusion

	TC	IR5 min	IR10 min	IR15 min	IR30 min
Weight of left ventricular (g)	0.110±0.003	0.124±0.002*	0.117±0.002*	0.119±0.001*	0.127±0.003*
Mitochondrial protein (mg)	33±1	29±1*	28±1*	28±0.9*	26±1*

$\bar{x}$±s, n = 8, compared with TC, *P<0.05.

Table 2. Change of mitochondrial Oxidative phosphorylation after ischemia-reperfusion injury (nAO/min/mg)

	TC	IR5 min	IR10 min	IR15 min	IR30 min
Glutamate + malate					
R3	324±9	313±10	261±9*	168±14*	144±10*
R4	26±1	28±3	34±1*	34±5*	37±2*
RCR	11.1±0.5	10.3±0.4	7.8±0.2*	7.5±0.4*	3.9±0.3*
ADP/O	2.96±0.07	3.01±0.03	3.24±0.03	3.85±0.18*	3.80±0.07*
2 mM ADP	371±11	368±13	323±7*	218±8*	175±15*
Succinate					
R3	448±30	311±12*	325±8*	289±34*	211±20*
R4	139±7	114±3*	124±4	108±4*	120±5*
RCR	3.3±0.2	2.7±0.1*	2.6±0.1*	2.8±0.2*	1.8±0.2*
ADP/O	1.46±0.06	1.57±0.02	2.14±0.09*	2.27±0.01*	1.33±0.05*
2 mM ADP	489±18	380±10*	386±6*	346±9*	322±31*
TMPD					
2 mM ADP	1296±52	1019±24*	1123±19*	874±71*	854±48*

$\bar{x}$±s, n = 8, compared with TC, *P<0.05.

Results

Myocardial weight and mitochondrial protein of mice heart after ischemia-reperfusion

In comparison with TC group, the left ventricular myocardium weight gradually increased (P<0.05, **Table 1**), and the content of mitochondrial protein was decreased (P<0.05, **Table 1**), indicating that the increase of myocardial tissue swelling and mitochondrial damage, mitochondrial protein extraction decreased with the prolongation of ischemia and reperfusion.

Change of mitochondrial Oxidative phosphorylation after ischemia-reperfusion injury

Compared with the TC group, the IR 5 min group had no significant change in the respiratory function of the complex I using glutamate + malate as substrates (P > 0.05, **Table 2**), and R3, RCR and P/O of mitochondria were decreased significantly after ischemia and reperfusion 10 min, 15 min and 30 min (P<0.05, **Table 2**, **Figures 1** and **2**). Respiratory function of complex II and IV decreased significantly using succinate, and TMPD-ascorbate as substrates in different ischemia-reperfusion groups compared with time control (P<0.05, **Figures 1** and **2**), and the inhibition of respiratory function was gradually increased with the reperfusion time prolonged.

Change of susceptibility to MPTP opening during early reperfusion

Calcium retention capacity (CRC) was used to assess the susceptibility to MPTP opening in isolated mitochondria (**Figure 3**). The CRC of mitochondria from IR hearts was gradually decreased following prolonged reperfusion time compared to TC hearts (**Figure 3**).

Change of mitochondrial inner membrane integrity following more prolonged periods of reperfusion

MPTP opening increases the permeability of both inner and outer mitochondrial membranes. Permeation of the inner mitochondrial membrane leads to the loss of inner membrane potential. A relative esti mation of inner mem-

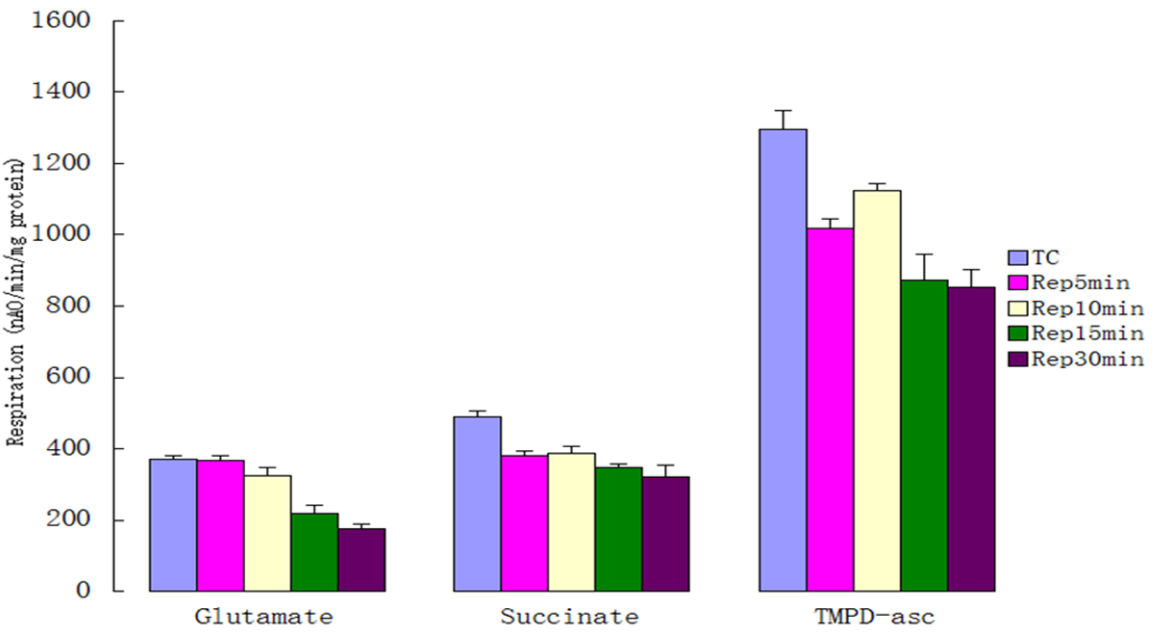

Figure 1. Oxidative phosphorylation stimulated with 2 mM ADP for the substrates glutamate (complex I), succinate (complex II), and TMPD-ascorbate (complex IV) are decreased in ischemia reperfusion cardiac mitochondria compared to time control. $\bar{x}\pm s$, $n = 8$, compared with TC, $^*P<0.05$.

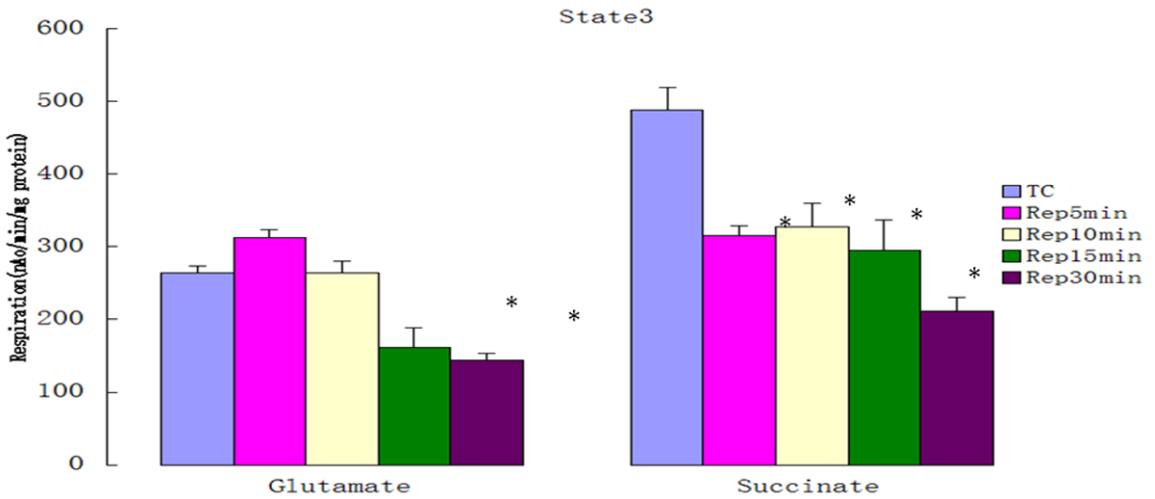

Figure 2. State 3 respiration rate of oxidative phosphorylation is decreased in ischemia reperfusion cardiac mitochondria compared to time control. $\bar{x}\pm s$, $n = 8$, compared with TC, $^*P<0.05$.

brane potential was used to further assess the functional integrity of the inner membrane. Inner membrane potential was more depolarized in mitochondria studied at the end of reperfusion compared with time controls in the presence of ADP, oligomycin, and DNP (**Figure 4**), indicating increased permeability of the inner membrane following prolonged reperfusion time.

Discussion

Mitochondrial ischemia injury is the key mechanism of myocardial injury during reperfusion. Paradies et al found that decrease of complex I activity after ischemia reperfusion injury in the heart of rats, and decreased with the decrease of the State 3 respiration [13]. Regulation of complex II and IV activity improved myocardial

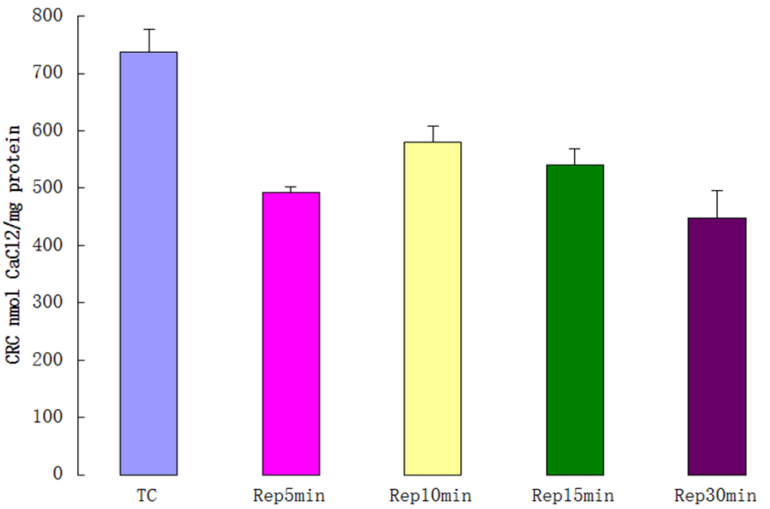

Figure 3. The calcium retention capacity (CRC) is decreased in ischemia reperfusion cardiac mitochondria compared to time control. $\bar{x}$±s, n = 8, compared with TC, *P<0.05.

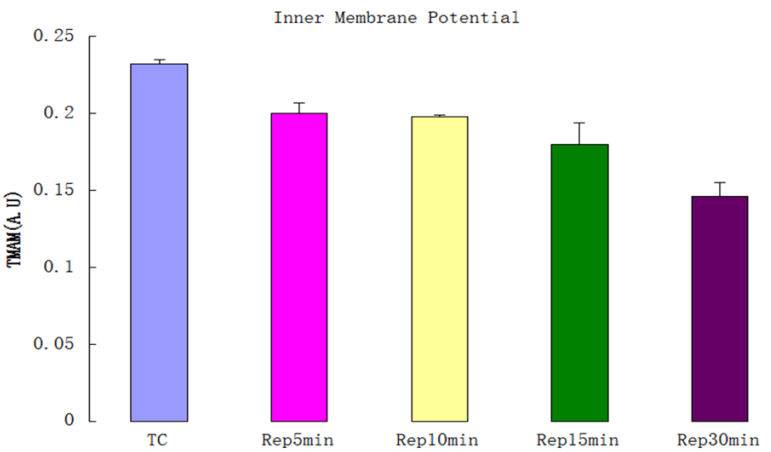

Figure 4. The inner membrane potentia calcium retention capacity (ψ) is decreased in ischemia reperfusion cardiac mitochondria compared to time control. $\bar{x}$±s, *n* = 8, compared with TC, **P*<0.05.

ischemia reperfusion injury [14, 15]. The results of our experiments showed that the oxidation of complex I, II and IV decreased significantly in the early stage of myocardial ischemia reperfusion in mice, suggesting that ischemia and reperfusion resulted in the damage of mitochondrial respiratory chain complex components.

As soon as possible to restore the myocardial blood supply and reperfusion therapy such as thrombolysis and PCI become the key to the treatment of myocardial infarction. However, there is still 10%~30% patients appear myocardial ischemia reperfusion injury even if the best reperfusion therapy. It has become a hot research topic in recent years that post-conditioning can prevent and reduce the new myocardial injury caused by ischemia. In the early stage of ischemia and reperfusion, blockade of mitochondrial complex I could effectively reduce myocardial ischemia reperfusion injury [10, 16]. Our study found that oxidative phosphorylation of mitochondrial respiratory chain complex with the prolongation of reperfusion is different after myocardial is chemia in mice given different time of reperfusion. Complex I of the mitochondrial oxidative phosphorylation in perfusion for 5 min without significant changes, suggesting that respiratory function of complex I can still maintain normal levels during ischemia and early reperfusion, and decreased with the prolongation of reperfusion damage aggravated gradually. The respiratory function of complex II and IV in the composite was decreased significantly during reperfusion, which indicated that the II and IV were the main injury in the early stage of ischemia and reperfusion and lead to oxidative phosphorylation of mitochondria decreased.

Therefore, we should choose the different time of reperfusion when studying the effect of different mitochondrial complex after ischemia reperfusion injury.

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Disclosure of conflict of interest

None.

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