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Dynamics of interstitial calcium in rat myocardial ischemia reperfusion injury *in vivo*

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Intracellular calcium ([Ca++]ic) overload is the key factor for myocardial ischemia reperfusion injury(IR), however there was no report for interstitial Ca++ concentration ([Ca++]ex) dynamics. This research aims to plot the [Ca++]ex dynamics in rat myocardial IR *in vivo*. A microdialysis system was composed and the time delay of the system, recovery time, was introduced and tested with a fluids switching method. Twelve SD rats were divided into IR or control group. Myocardial IR was induced by ligating (20 min) or releasing (60 min) the suture underlying LAD. Mycrodialyisis probe was implanted into the left ventricular myocardium perfusion area to be occluded. Dialysate samples were collected every 10 min. Blood samples were drawn at the beginning and at the end of the procedures. Dialysate calcium concentration ([Ca++]i) was detected with an atomic absorption spectrophotometer. Serum calcium and cTnT were detected. Results revealed recovery time for the microdialysis system was 20 min, recovery rate was 16%. [Ca++]i showed no changes during ischemia and descended immediately after reperfusion, reached the lowest level at 20 min after reperfusion, then escalated slowly while keeping lower than control with significant difference. There was no difference in serum calcium at the beginning (control vs IR, mmol/L: 2.35±0.31 vs 2.63±0.2, p=0.093) and ending point (control vs IR, mmol/L: 2.38±0.34 vs 2.66±0.15, p=0.095). Serum cTnT of both groups showed no difference at the beginning (control vs IR, ng/mL: 0.47±0.38 vs 0.74±0.46, p=0.287) and rose respectively at the 60 min after reperfusion (control vs IR, ng/mL: 1.62±0.6 vs 4.29±2.22, p=0.031). Given the recovery time and recovery rate, baseline of rat myocardial [Ca++]ex was estimated as 1.26±0.22 mmol/L and the [Ca++]ex was speculated steady in ischemia, descending 33% at the start of reperfusion, then escalating slowly. Thus it can be inferred that recovery time was an important parameter for mycrodialysis technique, which should not be neglected and need to be tested. Our data suggest that [Ca++]ex in rat myocardial IR *in vivo* kept steady in ischemia, descended rapidly at the initial reperfusion then rebounded slowly.

Key words: Myocardium, ischemia, reperfusion, calcium, microdialysis.

INTRODUCTION

Abnormal high level of intracellular calcium concentration ([Ca++]ic) is called calcium overload, which is a key factor in ischemia reperfusion injury(IR). [Ca++]ic rises before irreversible myocardial injury. Calcium overload during ischemia and reperfusion is an indicator for later injury (Giorgi et al., 2008). Drugs and interventions lowering [Ca++]ic alleviate or delay cell death. Emerging data show that abnormal opening of mitochondrial permeability transition pore (mPTP) in early reperfusion conducts later cell injury form and scope, and calcium is the key factor for mPTP opening (Javadov and Karmazyn, 2007). Presently fluorescent or radioisotopic indicator is used

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for intracellular calcium detection. These methods can only work with cultured cells or perfused whole heart but not with heart *in vivo*. Ischemia is defined as the lack of blood flow and can therefore only be studied in an intact organ, although measurements have been made of Ca++ during metabolic inhibition in isolated myocytes (Murphy and Steenbergen, 2008a). Two defects in the present calcium researches are: (1) non *in vivo* model (that is, cultured cell or perfused heart), (2) being confined to intracellular changes. Extracellular space is an important space for cell and blood substance exchange, where conventional techniques can hardly take effect. There are no reports on the myocardial interstitial Ca++ concentration ([Ca++]ex) changes. We speculate intracellular calcium overload may cause [Ca++]ex fluctuation and change with certain pattern during myocardial IR.

Microdialysis is a unique technique to monitor the chemistry of the extracellular space in living tissue (Lee et al., 2002; Lin et al., 2004). Microdialysis system is composed of microinjection pump, microdialysis probe and extending tubes with accessories. The microdialysis probe designed to mimic a blood capillary, has a semi-permeable membrane that when perfused with a physiological salt solution allows an exchange of compounds from an area of high concentration to an area of low concentration. A representative proportion of molecules from the extracellular fluid is then carried to the probe outlet for collection and analysis.

The process of target molecule transportation from detected fluid to dialysate could be taken as signal transferring, which has two important parameters: speed and intensity. Signal intensity shall decay during transferring, while only part of target molecules enter dialysate. Recovery rate is defined as the ratio of target molecule concentration in the dialysate and the primary (detected) fluid. Most reported recovery rate range is 12 to 30% (Obata, 2002; Miura et al., 2001; Kennergren et al., 2003; Multani et al., 2005). Speed is another important factor that influences the manifestation of signal transferring. A classic example is lightning and thundering. The process of target molecules entering the dialysate collector consists of two steps: (1) transmembrane infiltration and (2) movement in the outlet and extending tubes, if exist. The latter could be calculated as:

Dead volume / perfusion rate (1)

Dead volume means the outlet and extending tubes internal volume.

So a certain time delay should occur before the dialysate concentration can reflect the primary changes. We define the concept of recovery time (RT): time gap between the start of target molecule changes and corresponding stable detected concentration formation in the dialysate. Interestingly, there were no reports mentioning this factor when applying microdialysis technique (Lee et al., 2002; Lin et al., 2004; Obata, 2002; Miura et al., 2001; Kennergren et al., 2003; Multani et al., 2005; Pokela et al., 2003). Can we neglect this issue in microdialysis? If not, can we estimate/replace the time delay with dead volume/perfusion rate?

To answer the questions, the leading experiment was conducted before the animal study.

METHODS

Leading experiment: Microdialysis system recovery parameters plotting

In this part of experiment, we determined the specific recovery parameters of microdialysis system to use.

Components of microdialysis system

This system included one microinjection pump (CMA 402 Syringe Pump with accessory kit, 800310, CMA Microdialysis AB, Sweden), one microdialyis probe (CMA20 Elite, 8010435, CMA Microdialysis AB, Sweden. 20K Daltons cut-off, Membrane length 4 mm, Inlet tube length 200 mm, internal volume 3.6 µL,Outlet tube length 200 mm, internal volume 3.6 µL) and the extending tubes with accessories (FEP Tubing, 3409501 and Tubing Adaptors, 3409500, CMA Microdialysis AB, Sweden).

Two FEP Tubing (internal volume is 1.2 µL/10 cm length) were cut into 40 cm and connected with inlet and outlet tube respectively. During the whole experiment the same extending tubes were kept in use and not changed, assuring the stable microdialysis system.

Microdialysis perfusing and diluting strategy

The microdialysis probe was perfused with normal saline at a rate of 2.0 µl/min. A flushing and diluting method was used as mentioned previously (Lee et al., 2002). Briefly, a dilution tube was connected with a peristaltic pump to flush the tip of outlet tube with NS at a rate of 1 ml/min synchronously.

Time delay estimation

The dead volume in this system was calculated as:

(Outlet tubing internal volume) + (Outlet FEP tubing internal volume) = 3.6µL + 4×1.2µL = 8.4µL (2)

Perfusing rate is 2 µl/min and the estimated time delay should be:

8.4 µl/(2 µl/min) = 4.2 min (3)

Microdialysis signal switching

The detected fluids were standard calcium solution ([Ca++] set to 1.25 mmol/L) and normal saline (NS) ([Ca++] was about 1 to 3 µmol/L).

Signal switch procedures were: (1) Put the microdialysis probe into NS for 20 min stabilization, then switch the probe to standard calcium solution. Start the experiment counting and continuous sampling with 10 min interval. (2) 40 min later, switch back the probe to NS, continue sampling for 40 min. (3) Diluent (NS) was collected along with dialysate samples at the end each time. (4) Repeat steps 1 and 2 four times.

[Ca++] detection and calculation

All diluted dialysate and diluent samples were analyzed for [Ca++] by atomic absorption spectrophotometer (Z5000 Polarized Zeeman Atomic Absorption Spectrophotometer, HITACHI, Japan) at 422.7 nm wavelength. Dialysate [Ca++] ([Ca++]i) was calculated as:

$$[Ca^{++}]i = ([Ca^{++}]d - [Ca^{++}]NS) \times 50 \quad (4)$$

[Ca++]i: dialysate [Ca++]; [Ca++]d: diluted dialysate [Ca++]; [Ca++]NS: diluent(NS) [Ca++]

Animal experiment: Myocardial [Ca++]i detecting

All experiments were performed in accordance with the approval of the Ethical Committee for Animal Experiments of Sun Yat-sen University.

Twelve male SD rat weighing 200 to 250 g (Sun Yat-sen University, Guangzhou, China) were randomly divided into IR or control group, 6 for each group. Adult male SD rats weighing between 200 and 250 g were anaesthetized (urethane 1 to 1.5 g/kg ip), tracheotomized, and ventilated with room air at 70 breaths/min (Rodent 683, Harvard Apparatus, USA) with a tidal volume of 20 mL/kg. No positive end-expiratory pressure was applied. The right carotid artery was cannulated (22 gauge) for blood sampling and to record mean arterial blood pressure (MAP) via a pressure transducer connected to a biological data acquisition and analysis system (BL-420E+, TME Technology, Chengdu, Sichuan, China). Lead II ECG was also monitored with the same system. The heart was exposed through a parasternal thoracotomy and incision of the pericardium. A 7-0 prolene suture was looped under the left descending coronary artery. With the aid of a needle, the microdialysis probe (CAM20 Elite, CMA Microdialysis AB, Sweden) was implanted from the epicardial surface into the left ventricular myocardium perfusion area to be occluded. Because the extending tubes were long enough to make the probe move up and down with heart beating, there was no additional fixation for the probe. After implantation, perfusion and synchronous dilution kept going for 20 min before sampling started. The microdialysis system components, perfusing and sampling strategy were the same as leading experiment. In the IR group, myocardial ischemia (20 min) was performed by making a single node in the looped prolene suture and reperfusion (60 min) was induced by its release. Myocardial ischemia was confirmed by ST-segment elevation, visible regional cyanosis or pale color. 1 ml blood sample was collected at the start and end of experiment respectively and detected for serum calcium and cardiac troponin T (cTnT). The procedures were the same for the control rats without IR process. Animals were excluded from the study if bradycardia (< 300 bpm) or hypotension (< 80 mm Hg) were observed during the experiment period.

Statistical analysis

Data analyses were performed using SPSS for Windows 11.0. Results are expressed as mean±s.d.. Differences between groups were assessed by t test. $P<0.05$ was considered to be statistically significant.

RESULTS

Leading experiment: Microdialysis system recovery parameters plotting

Corresponding stable concentration in the dialysate lagged 20 min behind the detected fluid changes (Figure 1). In other words, recovery time for this microdialysis system is 20 min, longer than the estimated value (4.2 min). Here we could see recovery time is not neglectable and shall not be estimated with (dead volume/perfusing rate) value. Peak plateau concentration is 200 µmol/L, so corresponding recovery rate is 16%.

Animal experiment: Myocardial [Ca++]i detecting

[Ca++]i of IR group showed no changes during ischemia, descended after reperfusion, reaching the lowest level at 40 min(20 min after reperfusion) then escalated slowly while keeping lower than control group with significant difference (Table 1, Figure 2).

There were no difference in serum calcium at the beginning (control vs IR, mmol/L: 2.35±0.31 vs 2.63±0.2, $p=0.093$) and the ending point (control vs IR, mmol/L: 2.38±0.34 vs 2.66±0.15, $p=0.095$). Serum cTnT of both groups showed no difference at beginning (control vs IR, ng/mL: 0.47±0.38 vs 0.74±0.46, $p=0.287$) and rose respectively at the end (control vs IR, ng/mL: 1.62±0.6 vs 4.29±2.22, $p=0.031$). There were no differences in HR and BP between both groups.

DISCUSSION

Recovery time

To the best of our knowledge，this is the first research focusing on the time delay issue for the microdialysis technique. We coined the term recovery time, theoretically which should equal to the time cost sum of both transmembrane infiltration and outlet movement process. So, factors influencing the two processes will alter RT. Time for the target molecule movement in the outlet and extending tubes could be calculated as dead volume/perfusion rate. However, factors interfering with the transmembrane dialysis such as target molecule nature (size, electricity, stereochemical structure), semi-permeable membrane characters (material, pore diameter), temperature, and concentration gradient may change RT with a complicated pattern and hard to be estimated precisely. In the leading experiment, tested RT (20 min) was larger than the estimated value (4.2 min) and implied the great portion of the transmembrane dialysis accounted for the RT. Dialysate sampling interval might be another, if not the last, influencing factor. Smaller interval could detect the stable concentration more quickly then might yield a shorter RT as RT might vary with different microdialysis system, sampling strategy and detected environments. Testing the RT under nearly same conditions as following experiment makes sense. In this experiment, RT was tested with the same microdialysis system, sampling strategy and the

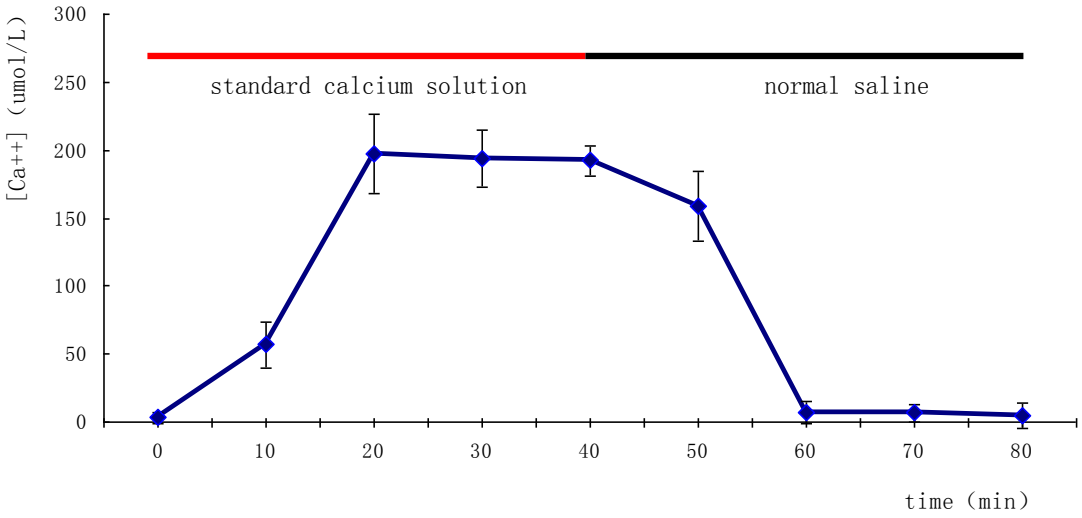

Figure 1. Dialysate [Ca++] changing with different detected fluids. Red or black bar indicated the microdialysis probe merged in different fluids, standard calcium solution or normal saline. The steady level of dialysate [Ca++] was plotted 20 min after detected fluids switching in both phases. Values are means±SD. of each four detections.

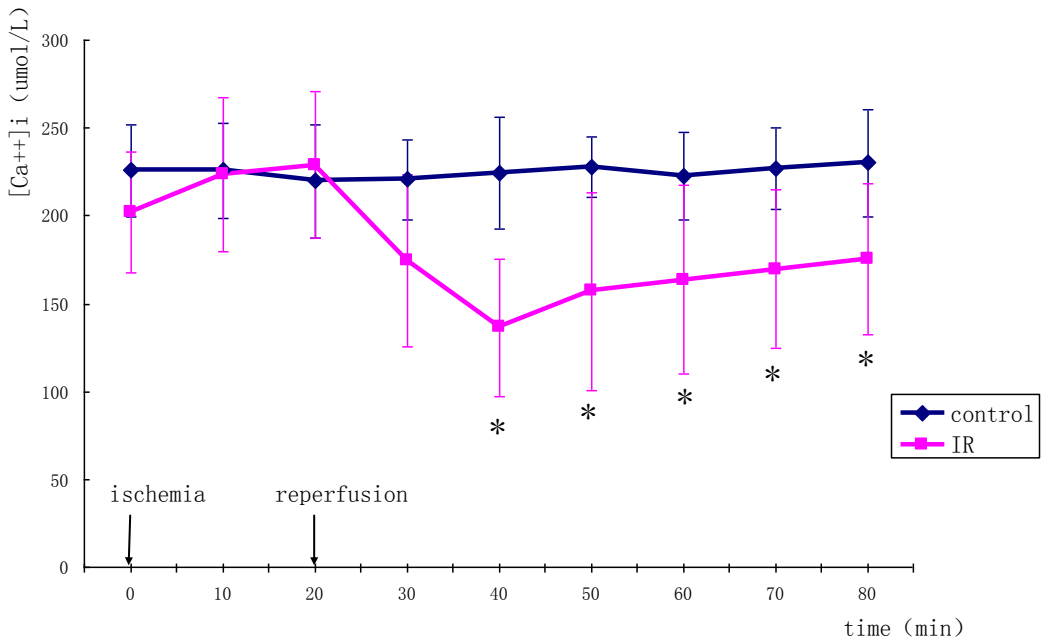

Figure 2. Myocardial [Ca++]i in IR . IR group (pink) was compared with the control group (blue). Asterisk (*) indicated the significant difference between groups. Values are means±SD. of each group (six rats) at different time points.

standard calcium solution was set to similar concentration with detected level. Given 20 min (RT) lag and [Ca++]i dynamics, the [Ca++]ex was speculated steady in ischemia, suddenly descending 33% at the start of reperfusion, then escalating slowly. Here great impact of recovery time was seen on the result explanation from [Ca++]i to [Ca++]ex dynamics. Long extending outlet tube of the microdialysis system, relatively short sampling interval and rapid change of [Ca++]ex emphasized the influence of RT. However, this impact might be minor when long sampling interval or long time scale change occurred and that might partially explain why it was omitted in previous researches. We think specific RT should be tested when using microdialysis technique ordinarily, especially under conditions like short sampling interval or target molecules fluctuating severely.

Table 1. Dynamics of [Ca++]i (µmol/L).

Time (min)	Group		P value
	Control	IR	
0	225.75±26	202.25±34.5	0.213
10	225.75±27.25	223.25±44	0.91
20	220±32.25	229±41.5	0.36
30	220.5±22.75	174.25±49	0.063
40	224.25±31.75	136.25±39.5	0.002
50	227.75±17.5	157±56.5	0.026
60	222.75±24.75	163.75±54	0.035
70	226.75±23	169.5±45.25	0.027
80	230±30.25	175±43	0.028

*In IR group, myocardial ischemia was set at 0 min and reperfusion at 20 min.

Myocardial [Ca++]ex

For more than thirty years, Microdialysis has been used to study brain neurophysiology. Microdialysis research in heart accounts for less than 5% total articles, aiming energy metabolites, neurotransmitters and drugs. There are no reports in inorganic ion monitoring. This is the first study on the myocardial [Ca++]ex *in vivo*. With 16% recovery rate, rat myocardial baseline [Ca++]ex was estimated as 1.26±0.22 mmol/L. Recent studies have demonstrated that intracellular Ca++ and H+ fluctuate in the first few minutes after reperfusion and these changes determine the following injury (Murphy and Steenbergen, 2008a, b; Inserte et al., 2008; Fujita et al., 2007). During ischemia [Ca++]ic rises slightly, which dramatically elevates after reperfusion due to sarcolemma NCX reverse transport. High [Ca++]ic, normal pH and ROS open the mitochondrial permeability transporting pore (mPTP), initiating the irreversible cell injury (Murphy and Steenbergen, 2008a, b). Our result corresponded to the hypothesis. Rapid decline of [Ca++]ex at the start of reperfusion may be the result of extracellular calcium influx. Further investigation is necessary to verify the Ca++ transmembrane flux, for instances, using the specific NCX or other calcium channels antagonist in the study model.

Serum calcium consists of free Ca++ and combined Ca++ with a fixed (near 1:1) ratio. The two forms of Ca++ concentration will change with same rate (Goldstein, 1990). In this experiment serum calcium was kept steady, so the free Ca++ should stay constant too. Myocardial [Ca++]ex fluctuation just reflected local dynamic distribution and these changes was buffered by the huge calcium pool, or in other words, whole body serum calcium balance was not affected. Obviously [Ca++]ex is a sensitive and selective mark to detect the specific organ metabolic changes. Methodology in the present study could also extend to other interstitial inorganic ion detection.

In the present study, 10 min sampling interval was used to plot the dynamics of myocardial [Ca++]ex. Narrower interval will be better because more decisive changes of [Ca++]ic might occur in just few minutes during the initial of reperfusion. There was only one IR strategy (20 min ischemia and 60 min reperfusion) was introduced in the study, however [Ca++]ex dynamics may change with longer ischemia/reperfusion periods.

Conclusion

In this study, we introduced the concept of recovery time for microdialysis technique and provided a simple testing method. Recovery time is an important parameter for mycrodialysis technique, which should not be neglected or estimated with (dead volume/perfusing rate) value. Continuous monitoring myocardial interstitial inorganic ion concentration *in vivo* could be achieved with microdialysis technique. Our data suggest that [Ca++]ex in rat myocardial IR *in vivo* remained steady in ischemia, descended rapidly at the beginning of reperfusion then rebounded slowly.

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