Yonsei Medical Journal
Vol. 42, No. 3, pp. 333-337, 2001

Original Article

Hypoxia Delays the Intracellular Ca^{2+} Clearance by Na^{+}-Ca^{2+} Exchanger in Human Adult Cardiac Myocytes

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Transient myocardial ischemia during cardiac surgery causes a loss of energy sources, contractile depression, and accumulation of metabolites and H+ ion resulting in intracellular acidosis. The reperfusion following ischemic cardioplegia recovers intracellular pH, activates Na^{+}-H^{+} exchange and Na^{+}-Ca^{2+} exchange transports and consequently produces Ca^{2+} overload, which yields cell death. Among the various Ca^{2+} entry pathways, the Na^{+}-Ca^{2+} exchanger is known to play one of the major roles during the ischemia/reperfusion of cardioplegia. Consequently, information on the changes in intracellular Ca^{2+} activities of human cardiac myocytes via the Na^{+}-Ca^{2+} exchanger is imperative despite previous measurements of Ca^{2+} current of human single myocytes. In this study, human single myocytes were isolated from the cardiac tissues obtained during open-heart surgery and intracellular Ca^{2+} activity was measured with cellular imaging techniques employing fluorescent dyes. We report that the Na^{+}-Ca^{2+} exchanger of adult cardiac myocytes is more susceptible to hypoxic insult than that of young patients.

Key Words: Human, Na^{+}-Ca^{2+} exchanger, hypoxia, adult cardiac myocytes

INTRODUCTION

Cardioplegic solutions are used to arrest the heart during open-heart surgical procedures and to protect the heart tissue from ischemic injury.[1] Ischemia due to cardioplegic arrest causes a loss of energy sources, contractile depression, and accumulation of metabolites and H^{+} ion resulting in intracellular acidosis.[2] The reperfusion following ischemic cardioplegia recovers intracellular pH, activates Na^{+}-H^{+} exchange and Na^{+}-Ca^{2+} exchange transports and consequently produces Ca^{2+} overload, which yields cell death.[3] Although these experimental findings have provided an understanding of the myocardial protection mechanism, more detailed information on the changes in intracellular ionic activities during open-heart surgery is necessary along with the characteristics of the cellular mechanism of Ca^{2+} movements of human cardiac myocytes.

Meanwhile, the cardiac surgical procedure for infants and children demands even greater care than that for adult patients.[4,5] Studies on the effects of aging and cardioplegia have also shown that the aged myocardium is more sensitive to ischemia and has accumulated significantly more cytosolic calcium than the newborn or the mature myocardium.[6,7]

Among the various pathways of intracellular Ca^{2+} accumulation, the Na^{+}-Ca^{2+} exchanger operating in reverse mode has been suggested as a causative mechanism for the calcium paradox.[8] The contribution of the Na^{+}-Ca^{2+} exchanger to the hypoxia-induced and reoxygenation-induced calcium overload has been demonstrated previously.[9,10] And also the depressant effect of anoxia on the Na^{+}-Ca^{2+} exchanger current has been observed in guinea-pig myocytes.[11] However, age-related differences in the Na^{+}-Ca^{2+} exchanger in human cardiac myocytes and their responses to the hypoxic condition of cardioplegia has not

Received January 22, 2001
Accepted May 7, 2001

This study was supported by a Research Grant (HMP-97-M-5-0054) from the Health Technology Planning and Evaluation Board (HPEB).

previously been studied.

In this study among different age groups, we measured the changes in intracellular Ca^{2+} activity via the Na^{+}-Ca^{2+} exchanger of human cardiac myocytes and found that the Na^{+}-Ca^{2+} exchanger of adult patients is more susceptible to hypoxic insult than that of young patients.

MATERIALS AND METHODS

Human single cardiac myocytes were isolated from tips of atrial appendages discarded during open-heart surgery and the donor patient characteristics are listed in Table 1. The tissues were transferred to the laboratory in nominally Ca^{2+}-free Tyrode solution [NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1 mM, NaH_2PO_4 0.3 mM, glucose 10 mM, N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES) 10 mM, pH 7.4] to which 30 mM butanedionemonoxime was added and through which 100% oxygen was continuously bubbled. The cardiac tissues were cut to about 1 mm^3 size, washed in Ca^{2+}-free Tyrode solution with continuous 100% oxygen supply for 12 minutes and then enzymatically digested with Ca^{2+}-free Tyrode solution containing 400 IU/ml collagenase type I and 4I U/ml protease VII. After washing the digested myocytes by centrifugation at 20 × g for 2 minutes, the isolated single cardiac myocytes were stored in KB solution (taurine 10 mM, oxalic acid 10 mM, glutamic acid 70 mM, KCl 25 mM, KH_2PO_4 10 mM, glucose 11 mM, EGTA 0.5 mM, HEPES 10 mM, pH 7.3~7.4) for about an hour.

The cell suspensions were then loaded with the membrane-permeant fluorescent calcium indicator fura-2 AM (3 μM, Molecular Probe, Eugene, Oregon, USA) in an Erlenmeyer flask placed in a shaking water bath for 40 minutes at 37℃.

Table 1. Patient Characteristics

Sex	Age	Weight (Kg)	Cardiac diagnosis
F	66	53	ASD-MR
F	64	48	CAOD
M	62	61	CAOD
F	44	62	ASD
M	43	57	MR
F	24	51	MR
F	24	56	MR
F	6	21	VSD
M	5	13	C-AVSD
M	4	15	VSD+DCRV
M	4	11	TOF
M	4	13	ASD
M	3	19	VSD
M	2	11	ASD
F	2	8.1	VSD
M	9m	6.5	TOF
M	8m	7	UVH
M	7m	5.9	VSD+ASD
F	5m	5	TAPVR
M	4m	3.5	VSD
M	2m	4.9	TAPVR
F	13m	7.9	C-AVSD

ASD, atrial septal defect; VSD, ventricular septal defect; C-AVSD, complete atrioventricular septal defect; TOF, tetralogy of fallot; TAPVR, total anomalous pulmonary venous return; MR, mitral regurgitation; CAOD, coronary artery occlussive disease; DCRV, double chambered right ventricle; UVH, univentricular heart.

After loading, the cells were washed by centrifugation for 5 minutes at 600 × g, resuspended in the Tyrode solution and transferred to a 300 μl glass-bottomed recording chamber on an epifluorescence inverted microscope (Nikon Diaphot 300, Tokyo, Japan). Fluorescence was measured at an excitation wavelength of 340 and 380 nm and an emission wavelength of 510 nm with a cooled CCD camera (Photometrics PXL37, Tucson, Arizona, USA) and cellular Ca^{2+} imaging was processed with Axon Imaging Workbench v.2.1 (Axon Instrument, Foster city, CA, USA). Experimental solutions were superfused at a flow rate of 2 ml/minute. NaCl was isotonically replaced with N-methyl-D-glucamine (NMG) to reduce the Na^+ concentration in the superfusing solutions. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Contribution of Na^+-Ca^{2+} exchange to the changes in intracellular Ca^{2+} activity

To examine the contribution of Na^+-Ca^{2+} exchange to the changes in intracellular Ca^{2+} activity, the Na^+-Ca^{2+} exchange process was isolated from other Ca^{2+} movements, by the addition of 1 μM thapsigargin (ER Ca^{2+}-ATPase inhibitor), 5 mM caffeine (ryanodine receptor inhibitor), and 0.25 mM La^{3+} (sarcoplasmic Ca^{2+}-ATPase inhibitor) to the superfusing solutions. Under these conditions, the Na^+ and Ca^{2+} ion concentrations in the superfusing solution were lowered to 0 mM to elicit the intracellular Ca^{2+} accumulation followed by superfusing with 140 mM Na^+, 0 Ca^{2+} solution, which resulted in the decline of Ca^{2+} activity due to Ca^{2+} efflux via Na^+-Ca^{2+} exchange (Fig. 1).

There was no observable difference between adult and young age groups during the rising phase of intracellular Ca^{2+} activity. However, the decay rate of Ca^{2+} activity (-ratio change/min) showed a significant difference between child and adult cardiac myocytes, 0.232 ± 0.039 (n=11) and 0.091 ± 0.010 (n=5), respectively (Fig. 1). The decline of Ca^{2+} activity in adult myocytes was slower, compared to that in child myocytes, to a statistically significant degree. The observed variation in Ca^{2+} decay rate could be due to differences in membrane potentials, intracellular Na^+ activities and membrane fluidity due to aging, with further studies being required to elucidate the underlying mechanisms.

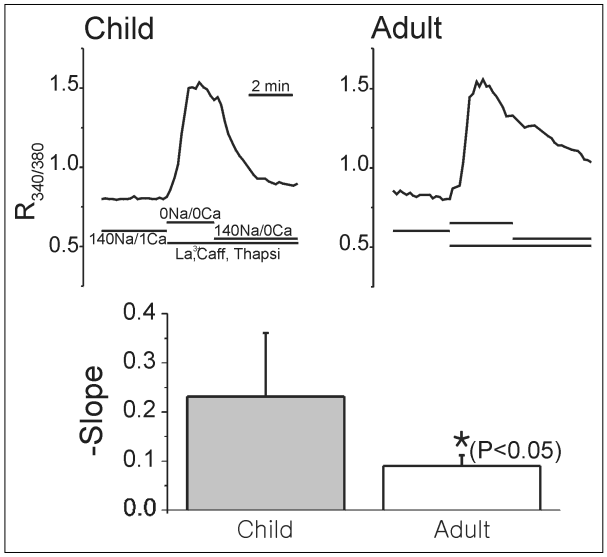

Fig. 1. Contribution of Na^+-Ca^{2+} exchange to the changes in intracellular Ca^{2+} activity. To isolate the Na^+-Ca^{2+} exchange process from other Ca^{2+} movements, 1 M thapsigargin (ER Ca^{2+}-ATPase inhibitor), 5 mM caffeine (ryanodine receptor inhibitor), and 0.25 mM La^{3+} (sarcoplasmic Ca^{2+}-ATPase inhibitor) were added to the superfusing solutions. Under these conditions, the Na^+ ion concentration in the superfusing solution was lowered to 0 mM to elicit the intracellular Ca^{2+} accumulation followed by superfusing with 140 mM Na^+ solution, which resulted in the decline of Ca^{2+} activity due to Ca^{2+} efflux via Na^+-Ca^{2+} exchange. Note that the decay rate (calculated as $R_{340/380}$/sec) of Ca^{2+} activity in adult myocytes was slower than that in young myocytes, to a statistically significant degree ($p<0.05$).

Effect of hypoxia on Ca^{2+} clearance by Na^+-Ca^{2+} exchange

To simulate the ischemic condition of cardioplegia, superfusion solutions were bubbled with 100% N_2 gas and the hypoxic effect on two parameters of intracellular Ca^{2+} changes, the decay rates and latency of Ca^{2+} efflux were measured (Fig. 2). Under the hypoxic condition, both the degree and rate of Ca^{2+} accumulation were decreased. This reduction was expected as anoxia decreases the Na^+-Ca^{2+} exchanger inward current (reverse mode), resulting in the decrease of Ca^{2+} influx.[11] It has also been reported that the

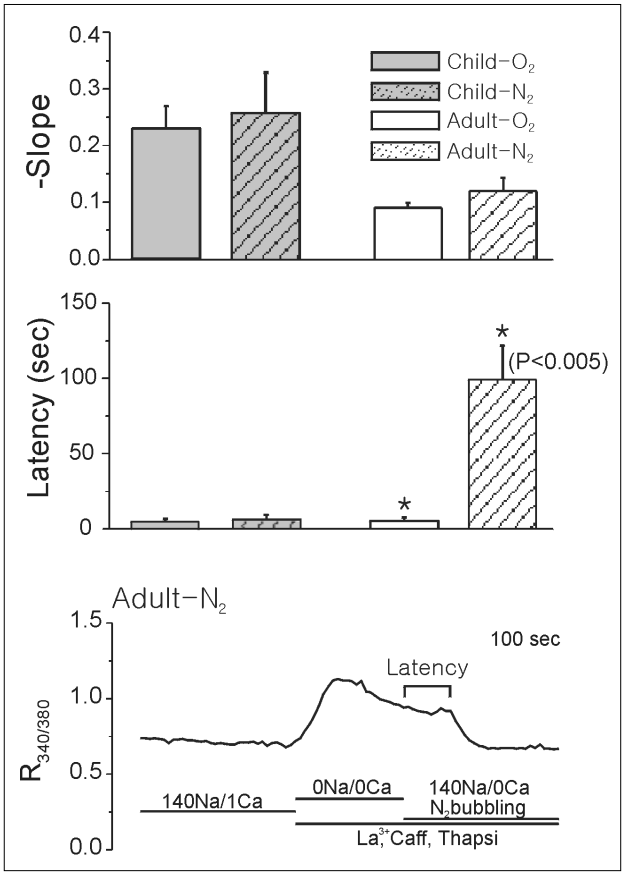

Fig. 2. Effect of hypoxia on Ca^{2+} clearance by Na^{+}-Ca^{2+} exchange in human cardiac myocytes. The hypoxic condition was simulated with N_2 gas bubbling of superfusing solutions. Although the decay rates of intracellular Ca^{2+} activity were not affected under the hypoxic condition, the onset of decline was delayed significantly, compared to the normoxic condition ($p<0.005$).

degree of suppression of Na^{+}-Ca^{2+} exchanger current by anoxia varied between the inward and the outward current in such a manner that the Ca^{2+} clearance via the Na^{+}-Ca^{2+} exchanger was more strongly suppressed than the degree of Ca^{2+} accumulation.[11,12] The decay rates of intracellular Ca^{2+} were slowed down by the hypoxic condition in cardiac myocytes of both adult and young patients (0.259 ± 0.072, n=6 vs. 0.232 ± 0.039, n=11 in young patients; 0.119 ± 0.024, n=6 vs. 0.091 ± 0.010, n=6 in adults), but not to a statistically significant degree between two groups. The Na^{+}-Ca^{2+} exchanger reportedly depends upon the cytosolic level of ATP as cytosolic pH decreases.[13-15] Under our experimental conditions, the degree of anoxia might not have been effective enough to enable the decrease in intracellular ATP and pH to yield significant suppression of the intracellular Ca^{2+} decay rate. However, while the clearance of intracellular Ca^{2+} by Ca^{2+} efflux in 140 Na^{+}/0 Ca^{2+} solutions was delayed significantly in adult cardiac myocytes (99.2 ± 22.5 sec), a corresponding delay in the onset of intracellular Ca^{2+} clearance under hypoxic condition was not observed in the cardiac myocytes of children. Although the phosphorylation of the Na^{+}-Ca^{2+} exchanger is not necessary for its operation,[16] ATP sensitivity and pH effects on Ca^{2+} binding and Na^{+}-Ca^{2+} exchanger kinetics may be involved in age-dependent responses to anoxia. The hindrance of Ca^{2+} binding to Ca^{2+} regulatory site under increased H+ activity has neen suggested to delay the onset of intracellular Ca^{2+} clearance by the Na^{+}-Ca^{2+} exchanger,[17,18] an effect observed in this study. However, a complete understanding of the age-dependence of the observed delay in Ca^{2+} clearance will require further study on the molecular regulatory mechanism of the Na^{+}-Ca^{2+} exchanger along with its interaction with the myocytes cell membranes of different age.

In conclusion, this study discovered a delay in intracellular Ca^{2+} clearance by the Na^{+}-Ca^{2+} exchanger of adult cardiac myocytes under the hypoxic condition. We also determined that the activity of the Na^{+}-Ca^{2+} exchanger is age-dependent and that it decreases in the adult human cardiac myocytes. These findings strongly suggest that the Na^{+}-Ca^{2+} exchanger plays one of the major roles in modulating the intracellular Ca^{2+} activities of human cardiac myocytes and that the age-dependent activities of the Na^{+}-Ca^{2+} exchanger must considered in clinical applications of the principle of myocardial protection such as cardioplegia.

ACKNOWLEDGEMENTS

We thank Drs, SR Hong and JH Kim, the directors of Sejong Hospital, for their valuable input into discussions on experimental design.

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