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Alteration of the expression of ion channels, ion exchangers and Ca^{2+}-handling proteins in ischemic cardiomyocytes

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Alteration of the expression of ion channels, ion exchangers and Ca^{2+}-handling proteins in ischemic cardiomyocytes

Directed by Professor Byung Chul Chang

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This certifies that the Doctoral Dissertation of Do-kyun Kim is approved.

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This paper is dedicated to my loving wife, Lucia, who gives me unwavering support, inspiration, and love in all of my endeavors, and our children, Heejin, Kyungmin, and Bumjin. They provide us with limitless pleasure and humor and give us our purpose in life.

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Abstract

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Myocardial infarction results in inability to supply adequate oxygen and cause damage to the cardiac muscle and has been associated with conditions of pathological stress that impair myocardial homeostasis. It has been known the effect of calcium in myocardium but there are few studies for calcium regulation of early ischemic cardiomyocytes. Early ischemia was induced in 3 to 6 hours and membrane/cytosol ratio of PKC was up-regulated in this condition. Early ischemia was suppressed the phosphorylation levels of Erk and Akt as well as anti-apoptotic proteins, Bcl-2, and induction of pro-apoptotic protein, Bax. Calcium-related gene expression in ischemic cardiomyocytes was investigated by microarray. Early ischemic condition induced reactive oxygen species (ROS), intracellular Ca^{2+}, and Na^{+} overload. In early ischemic condition, the expression of Ca^{2+}-handling protein (calsequestrin, calmodulin, and calreticulin), ion exchanger(NCX) and ion channel (RyR2), ion pump (Na^{+}-K^{+}-ATPase, SERCA2a and PLB) and stress markers(ANP and BNP) were significantly altered. In conclusion, early ischemic condition alters Ca^{2+}-related gene expression in cardiomyocytes. This results indicate that early ischemia have may provide strategies to define calcium regulation.

Key words : cardiomyocytes, calcium, early ischemia

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I. Introduction

Cardiovascular disease, such as heart attack, stroke and heart failure (HF), are the leading cause of death and has been associated with conditions of pathological stress that impair myocardial homeostasis.[1] Among the cardiovascular disease, ischemic heart disease is the leading cause of death in the developed world.[2] Ischemic heart disease (IHD) occurs when blood flow to the myocardium is obstructed. This process results in ischemia, inability to supply adequate oxygen to cardiac muscle which causes damage to the myocardium.[3,4] Complete occlusion of the coronary artery leads to a myocardial infarction.[5]

Excitation-contraction coupling is the main process of cardiac contraction by which complex mechanism of electrical conversion. Cardiac muscle is activated by two sources of Ca^{2+}. One is an influx of Ca^{2+} from the extracellular humor, principally via the L-type Ca^{2+} channel the other is a release from the sarcoplasmic reticulum(SR) through a Ca^{2+} discharge channel known as the ryanodine receptor (RyR). When cell is in a normal condition, Ca^{2+} released from the SR is much more than that entering via the L-type current. Relaxation occurs by $[Ca^{2+}]_i$ being lowered to resting levels via a combination of pumping out the Ca^{2+} from the cell largely by Na^+/Ca^+- exchange (NCX) (with a little contribution from the plasma membrane Ca^{2+}-ATPase, PMCA) and being taken

back up into the SR via the SR Ca^{2+}-ATPase (SERCA). In the steady state the amount of Ca^{2+} pumped out of the cell must equal that which entered from extracellular fluid and the amount taken back into the SR by SERCA must equal that released via the RyR (Figure 1). [6,7,8]

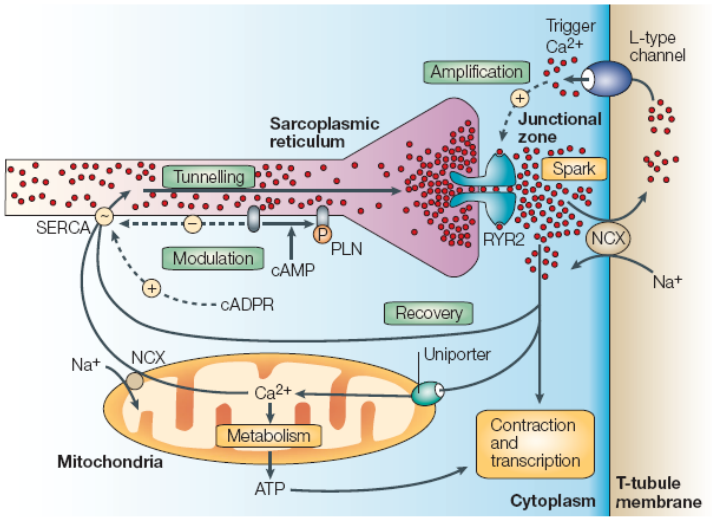


Figure 1. Cardiac calcium signal module(Taken from reference 9)

Alteration in intracellular Ca^{2+} signaling causes pathological hypertrophic heart and fetal gene activation. Extracellular stimulation regulate intracellular Ca^{2+} levels by promoting Ca^{2+} release from intracellular organelles or entry of Ca^{2+} across the plasma membrane via agonist- or receptor-mediated Ca^{2+} entry channels, voltage-gated Ca^{2+} channels (VGCC), or ligand-gated cation channels.[10]

Protein kinase C (PKC) also correlates closely with cardioprotection. Numerous studies have documented a central role of PKC in the cardioprotection through various mechanisms like ischemic preconditioning (IP).[11,12] The PKC family consists of at least 10 isoforms, among which PKCα, PKCε, and PKCδ are the prominent isoforms expressed in the heart. Upon

stimuli, PKC isoforms translocate from the cytosol to subcellular membrane regions, a process associated with their activation. Such translocation has been considered as a hallmark of PKC activation.[13] However, it is completely unknown whether hypoxic condition can stimulate PKC activation and what the even downstream effects are. PKC activation has been reported to play a role in regulating intracellular calcium handling.[14] Under the physiological condition, intracellular calcium concentration ($[Ca2^{+}]_i$) is sophisticatedly regulated by several proteins present in the sarcolemmal and SR membranes. Upon the arrival of action potential, Ca^{2+} influxes through the L-type Ca^{2+} channel and triggers the opening of the RyR, resulting in further release of Ca^{2+} from the SR, which accomplishes the sharp $[Ca^{2+}]_i$ elevation required for myofibril contraction.[15] In the rat cardiomyocytes, over 90% of the Ca^{2+} after contraction is immediately reuptaken by SR via SERCA, whereas the remaining Ca^{2+} is pumped out of the cell via NCX.[16] A rise in intracellular Ca^{2+} in cardiomyocytes during ischemic heart has been regarded to be a pivotal event in the activation of the calpain leading to myocardial death and structural damage of the heart by breakdown of cytoskeletal proteins such as myosin heavy chain (MHC), troponin T (TnT) and I (TnI), tropomyosin A and α-actinin.[4] And, under the assumption that Ca^{2+} entry into myocytes through cardiac Ca^{2+} channels during myocardial ischemia is the initiating signal in triggering Ca^{2+} overload, researcher investigated the effects of a chronic Ca^{2+} channel blockade on the Ca^{2+}-dependent cardiac calpain system.[5] Ca^{2+} channel antagonists have been shown to exert beneficial myocardial effects via their inhibiting action on the Ca^{2+} inward current through L-type Ca^{2+} channels into cardiac cells. And, cardiac T-type Ca^{2+} channels have been shown to be up-regulated in the failing myocardium, suggesting an influence of this channel type on intramyocardial Ca^{2+} levels.[17-21] However, no studies have been performed to clarify whether alterations in expression of Ca^{2+}-related molecules such as ion channels, ion

receptors, ion exchangers and Ca^{2+}-handling proteins in ischemic stress, except myocardial hypertrophy.

To analyze correlation between Ca^{2+}-related gene expression and ischemic Ca^{2+} overload in cardiomyocytes, this study was compared to the change of Ca^{2+}-related and other intracellular gene expression between negative control (normal) and experimental group (early ischemia), *in vitro*. Mainly, this study will focus on change of calcium regulation via Ca^{2+}-related and other intracellular gene expression level in ischemic cardiomyocytes.

II. Material and Methods

1. Materials

1) Animals

Neonatal rat ventricular cardiomyocytes (NRVCMs) were isolated from the hearts of 1~2-day-old Sprague-Dawley fetal rats. All animal procedures were carried out according to a protocol approved by the Yonsei University Animal Care Committee.

2. Methods

1) Isolation and primary culture of rat cardiomyocytes

Neonatal rat ventricular cardiomyocytes were isolated from neonatal rat hearts, as previously described. This heart tissue was washed with pH 7.4 Dulbecco's phosphate-buffered saline (D-PBS; Gibco BRL, Grand Island, NY, USA) in order to remove red blood cells. After depletion of the atria, the hearts were sliced up approximately 0.5 mm^3 sized pieces and treated with 4 ml collagenase Ⅱ (1.4 mg/ml, 270 units/mg; Gibco BRL) solution for five minutes. The supernatant was removed, and the pellet was suspended in collagenase Ⅱ. After cells were incubated in a 37°C humidified atmosphere

chamber containing 5% CO_2 for five minutes, gained supernatant was mixed with alpha minimum essential medium (α-MEM; Gibco BRL) then centrifuged at 2000 rpm for 2 minutes. The remaining tissue was treated with fresh collagenase Ⅱ solution for five minutes. This incubation procedure was repeated until the tissue was completely digested. The gathering cell pellet was resuspended in α-MEM and attached to a culture dish at 37°C in 5% CO_2 chamber. Two hours later, adherent cells turned into fibroblasts and non-adherent cells became NRVCMs. The NRVCMs were washed twice and replated on 100 mm plates with α-MEM plus 10% FBS at 37°C in 5% CO_2 chamber. To eliminate fibroblast expansion, we used 0.1 mM 5-bromo-2'-deoxyuridine (Brd-U; Sigma-Aldrich, St. Louis, MO,USA).

2) Cell viability assay

Cardiomyocytes were plated in triplicate wells of 96 well plates at a density of 5 x 10^4 per well, and put in a hypoxic chamber for 1 hour. Culture plates containing cardiomyocytes in α-MEM were subjected to hypoxic stress in an anaerobic chamber (ThermoForma, Waltham, MA, USA) maintained at 37°C in which ambient oxygen was replaced with a mixture of 5% CO_2, 5% H_2 and 90% N_2. Cell viability was determined by the MTT assay. After the incubation period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well to a final concentration of 0.5 mg/ml and the cells were incubated at 37°C for 3 hours to allow MTT reduction. Formazan crystals were dissolved by adding dimethylsulfoxide (DMSO, Sigma) and absorbance was measured at the 570 nm with a spectrophotometer.

3) Measurement of intracellular reactive oxygen species generation

Neonatal rat cardiomyocytes were labeled with 2'7'-dichlorodihydro-fluorescein diacetate (H2DCFDA; Invitrogen, Carlsbad,

CA, USA). The probe H2DCFDA (10 μM) enters the cell, and the acetate group on H2DCFDA is cleaved by cellular esterases, trapping the nonfluorescent 2'7'-dichlorodihydro-fluorescin (DCFH) inside. Subsequent oxidation by reactive oxygen species yields the fluorescent product DCF. The dye, when exposed to an excitation wavelength of 480nm, emits light at 535nm only when it has been oxidized. Labeled cells were examined using a luminescence spectrophotometer for the oxidized dye.

4) Microarray analysis [22-28]

(A) RNA preparation

Total RNA was extracted using Trizol (Invitrogen), purified using RNeasy columns (Qiagen, Valencia, CA, USA) according to the manufacturers' protocol. After processing with DNase digestion, clean-up procedures, RNA samples were quantified, aliquot and stored at -80°C until use. For quality control, RNA purity and integrity were evaluated by denaturing gel electrophoresis, OD 260/280 ratio, and analyzed on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

(B) Labeling and purification

Total RNA was amplified and purified using the Ambion Illumina RNA amplification kit (Applied Biosystems Inc., Foster City, CA, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 550 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo(dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP(Nitroblue-tetrazolium salt). After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE, USA).

(C) Hybridization and data export

750 ng of labeled cRNA samples were hybridized to each rat-12 expression bead array for 16-18 hours at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, CA, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions. Array data export processing and analysis was performed using Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8).

(D) Raw data preparation and Statistic analysis (example)

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina BeadStudio v3.1.3 (Gene Expression Module v3.3.8). Array data were filtered by detection p -value < 0.05 (similar to signal to noise) in at least 50% samples (we applied a filtering criterion for data analysis; higher signal value was required to obtain a detection p -value < 0.05). Selected gene signal value was transformed by logarithm and normalized by quantile method. 1-way analysis of variance (ANOVA) was applied to determine the group effects. If the group effect is exist, Tukey's Honestly Significant Differences test was applied to determine the difference between groups. Statistical significance was adjusted by the Benjamini-Hocbherg multiple-testing correction with false discovery rate (FDR).

Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. The analysis was performed K-means clustering for time dependent effect.

All data analysis and visualization of differentially expressed genes was

conducted using ArrayAssist® (Stratagene, La Jolla, USA) and R statistical language v. 2.4.1.

Biological ontology-based analysis was performed by using Panther database (http://www.pantherdb.org).

5) Measurement of intracellular Ca^{2+} overload in early ischemic cardiomyocytes

The measurement of cytosolic free Ca^{2+} was performed by microscopic analysis using confocal microscopy. NRVCMs were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL) and 0.1 μM BrdU. Cardiomyocytes were put in a hypoxic chamber for established ischemic time. After incubation, the cells were washed with modified Tyrode's solution containing 0.265 g/l $CaCl_2$, 0.214 g/l $MgCl_2$, 0.2 g/l KCl, 8.0 g/l NaCl, 1 g/l glucose, 0.05 g/l NaH_2PO_4 and 1.0 g/l $NaHCO_3$. Cells were then loaded with 5 mM of the acetoxymethyl ester of Fluo-4 (Fluo-4 AM, Invitrogen, USA) for 20 minutes, in the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Carl Zeiss Inc., Germany). This fluorochrome is excited by the 488 nm line of an argon laser and emitted light is collected through a 510-560 nm bandpass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

6) Measurement of sodium overload in early ischemic cardiomyocytes

Intracellular sodium was measured by corona green (CoroNa™ Green, Invitrogen). Cells (5 x 10^6) were cultured for 1 day in 100 mm plate coated with 1.5% gelatin. For intracellular sodium measurement, Corona green stock was individually added to 1 ml of cells at a final concentration of 5 μM for 1 hour

prior to the time of examination. Incubation was continued at 37°C, 5% CO_2 atmosphere. Before flow cytometric examination, propidium iodide (PI) (sigma) was added to final concentration (10 μg/ml). The 1 x 10^4 cells were analyzed by sequential excitation of the cells containing corona green and PI at 492-516 nm and 488 nm, respectively, using a FACS Caliber system (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuest^{TM} software.

7) Immunoblot analysis

Cells were washed once in PBS and lysed in a lysis buffer (Cell Signaling Technology Inc., Danvers, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM PMSF. Protein concentrations were determined using the Bradford protein assay kit (BioRad, Laboratories Inc., Irvine, CA, USA). Proteins were separated in a 12% SDS-polyacrylamide gel and transferred to PVDF membrane (Millipore Co., USA). After blocking the membrane with Tris-buffered saline-tween 20 (TBS-T, 0.1% tween 20) containing 5% non-fat dried milk for 1 hour at room temperature, membrane was washed twice with TBS-T and incubated with primary antibodies for 1 hour at room temperature or for overnight at 4°C. The membrane was washed three times with TBS-T for 10 minutes, and then incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies. After extensive washing, the bands were detected by enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities were quantified using Photo-Image System (Molecular Dynamics, Sweden).

8) Screening of Ca^{2+}-related gene expression in normal cardiomyocytes

and ischemic cardiomyocytes

(A) Isolation of total RNA

Total RNA was extracted by 500 μl/ 60 mm plate Tri-reagent (Sigma). Chloroform was poured about 100 μl above Tri-reagent, vortex a sample about 10 seconds. Then, sample was centrifuged at 12,000 g, 4°C for 15 minutes. Three layers were appeared in the tube, transparency upper layer collected in new tubes. And, 2-propanol was poured about 250 μl over the sample, again vortex a sample about 30 seconds. Centrifuge was accomplished about 12000 g, 4°C and 10 minutes. Left the pellet, supernatant was discarded and washed by 75% ethanol (Duksan, Korea) –mixed with diethylpyrocarbonate (DEPC; Sigma) water. Centrifugation was also operated about 7,500 g, 4°C and 5 minutes. The supernatant was dismissed, and pellet was dried on room temperature about 7 minutes. Finally, 30 μl nuclease free water (NFW) was poured onto pellet. The quality and quantity of the RNA was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer (Effendorf, Germany).

(B) cDNA synthesis

Complementary DNA (cDNA) was synthesized with RT-&GO^{TM} (Qbiogene, Carlsbad, CA, USA). Quantitative 1 μg total RNA was added to 1 μl anchored primer $(dT)_{25}V$, 2 μl dithiothreitol (DTT) and NFW, totally 9 μl. To prevent secondary structures, mixture was incubated for 5 minutes at 70°C, and added 8 μl of RT-&GO^{TM} mastermix. Sample was incubated the assay at 42°C for 1 hour. At the conclusion, sample was inactivated the reverse transcriptase at 70°C for 15 minutes. Alike isolation of total RNA, sample was detected by OD_{260}/OD_{280} with DU 640 spectrophotometer.

(C) PCR analysis

Quantitative 1 μg cDNA, each 10 pmol primer (forward and backward; Table 1), 0.1 mM dNTP mixture, 1.25 U of Taq polymerase and 10 X reaction buffer were mixed with NFW, finally total volume of 25 μl. PCR condition was fixed as fellow. A cycle of denaturing at 94°C for 3 minutes followed by number of 35 cycles with denaturation at 94°C for 30 seconds, annealing at 48°C to 60°C for 30 seconds, and elongation at 72°C for 30 seconds. Then sample was kept up 72°C for 10 minutes. Primers were appeared Table 1. When PCR assay have finished, PCR product was separated by electrophoresis in a 1.2% agarose gel (Biorad) and Gel-Doc (Biorad) visualized after staining with ethidium bromide (EtBr; Sigma).

Table 1. PCR primers used in this study

primer	sequence	primer	sequence
Calsequestrin	TCAAAGACCCACCCTACGTC CCAGTCTTCCAGCTCCTCAG	SERCA 2a	TCCATCTGCCTGTCCAT GCGGTTACTCCAGTATTG
Calreticulin	ATGACCCCACAGATTCCAAG GCATAGGCCTCATCATTGGT	Phospholamban (PLB)	GCTGAGCTCCCAGACTTCAC GCGACAGCTTGTCACAGAAG
Calmodulin	GAAGCAGGCCAGTCAAAGAC CGAATTTGGAAGCCAACACT	Ryanodine receptor 2 (RyR2)	CCAACATGCCAGACCCTACT TTTCTCCATCCTCTCCCTCA
NCX-1	TGTCTGCGATTGCTTGTCTC TCACTCATCTCCACCAGACG	ANP	GCCGGTAGAAGATGAGGTCA GGGCTCCAATCCTGTCAATC
Na^+-K^+-ATPase	TGTGATTCTGGCTGAGAACG AGGACAGGAAAGCAGCAAGA	BNP	TCTGCTCCTGCTTTTCCTTA GAACTATGTGCCATCTTGGA
18s rRNA	GTCCCCCAACTTCTTAGAG CACCTACGGAAACCTTGTTAC		

9) Statistical analysis

Data are expressed as means ±SE. Statistical comparisons between the two groups were performed using the Student´s t-test. In addition, a one-way

ANOVA using a Bonferroni test was used when comparing more than two groups. Statistical significance was considered as p-value <0.05.

III. Result

1. Survival rate of ischemic cardiomyocytes in hypoxic condition

In order to investigate whether ischemic condition affects cardiomyocytes proliferation, cardiac myocytes were exposed in anaerobic chamber for hypoxic condition. The viability of hypoxic cardiomyocytes were more than 50% in 3 hours and 6 hours exposure to hypoxic condition exposure. But the survival rate of hypoxic cardiomyocytes were less than 50% in 9 hours (Figure 2). The result showed that early ischemic time is in 3 hours and 6 hours.

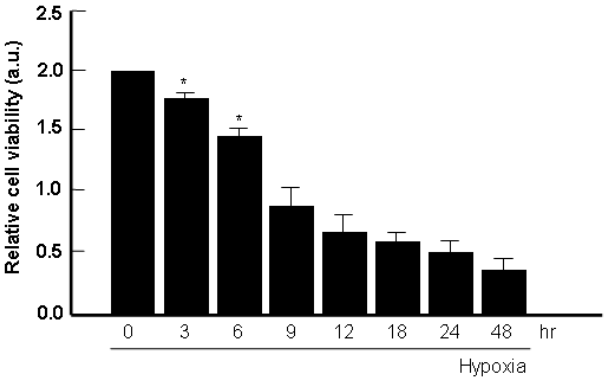


Figure 2. Time-dependently survival rate of ischemic cardiomyocytes. Cells (5 x 10^4 cells per well) were in hypoxic condition. After 6 hours incubation, cardiomyocytes were determined by MTT assay. * p <0.01 vs. hypoxia 0 hour.

2. Effects of early ischemic condition on PKC level

Figure 3 showes the translocation of PKC in ischemic condition of cardiomyocytes, respectively. To evaluate whether early hypoxia-induced translocation of PKC involves membrane translocation, we evaluated protein

expression of PKC in the membrane fraction as well as the cytosolic fraction. PKC cytosolic fraction was significantly declined and PKC membrane/cytosol ratio was increased about 1.8-fold in 6 hour of hypoxia.

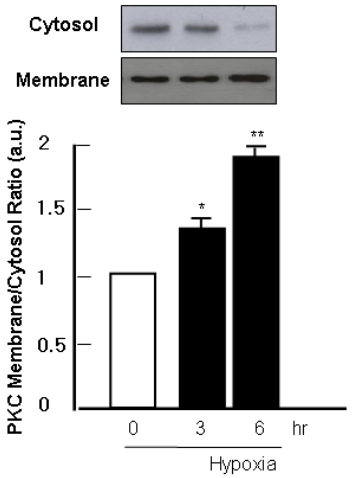


Figure 3. Alteration of PKC in ischemic cardiomyocytes. Western blot analysis of PKC in cytosol and membrane fractions from early hypoxic cardiomyocyte was determined. Representative experiment shown and was repeated 3-time. * p <0.05 vs. hypoxia 0 hour, ** p <0.001 vs. hypoxia 0 hour.

3. Effects of early ischemia on cardiomyocytes apoptosis

To prove the mechanism by which early ischemic condition operates as pro-apoptotic signaling, immunoblot was experimented with phosphorylation of ERK1/2 and Akt, Bcl-2 and Bax (Figure 4). The phosphorylation activities of both ERK1/2 and Akt were dramatically decreased in hypoxic cardiomyocytes (3 hours and 6 hours) compared with control cells. And, hypoxia at 3 hours and 6 hours induced the decreased ratio of Bcl-2/Bax than control condition.

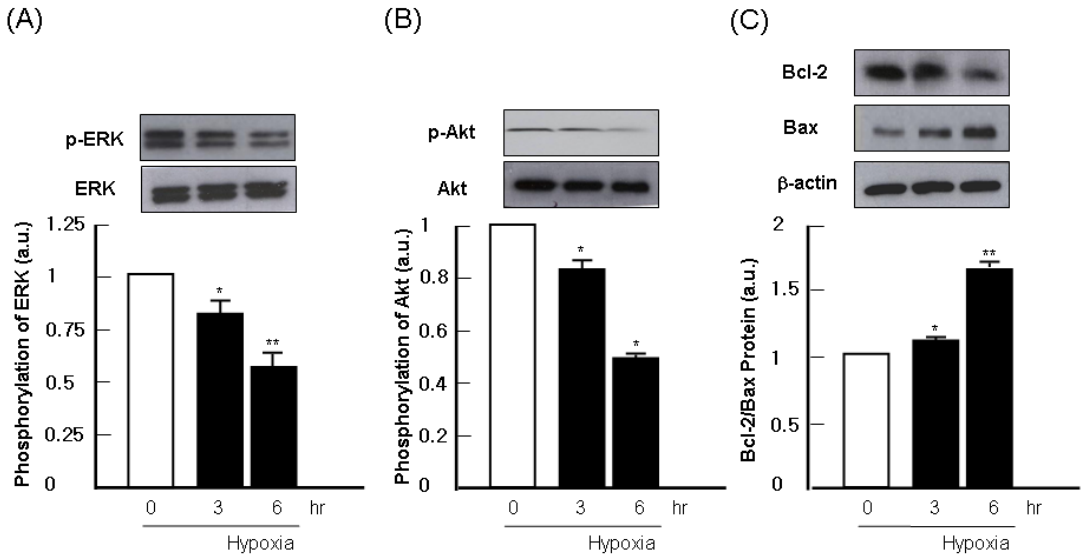


Figure 4. Pro-apoptotic effects under early ischemic condition. Effects of early ischemia on the activity of survival-related proteins under hypoxic conditions were monitored by western blot with antibodies specific for (A) ERK and p-ERK (**p* <0.05 vs. hypoxia 0 hour, ** *p* <0.001 vs. hypoxia 0 hour), (B) Akt and p-Akt (**p* <0.001 vs. hypoxia 0 hour), (C) Bcl-2, Bax, and β-actin (**p* <0.05 vs. hypoxia 0 hour, ** *p* <0.001 vs. hypoxia 0 hour).

4. Expression profile of differentially expressed genes in early ischemic cardiomyocytes

Microarray analysis was performed by generating ratio of each condition (hypoxic condition in 3 hours and 6 hours) to the normal and selecting for probe sets that showed consistent changes in the duplicate experiments. Total probe sets were identified that showed replication across both experiments at any of the given various condition (or time-point). When grouped, these genes fall into several distinct functional categories. For clustering, expression values for each target gene were normalized to emphasize trends in expression changes over the magnitude response. Within each time course, differential expression values were divided by the absolute maximum value to ensure that all values are

between -2 and 2 (Figure 5A). Expression patterns fell into a total of 9 different clusters in Figure 5B. A graphic representation of the clustering of all 630 differentially expressed genes is shown.

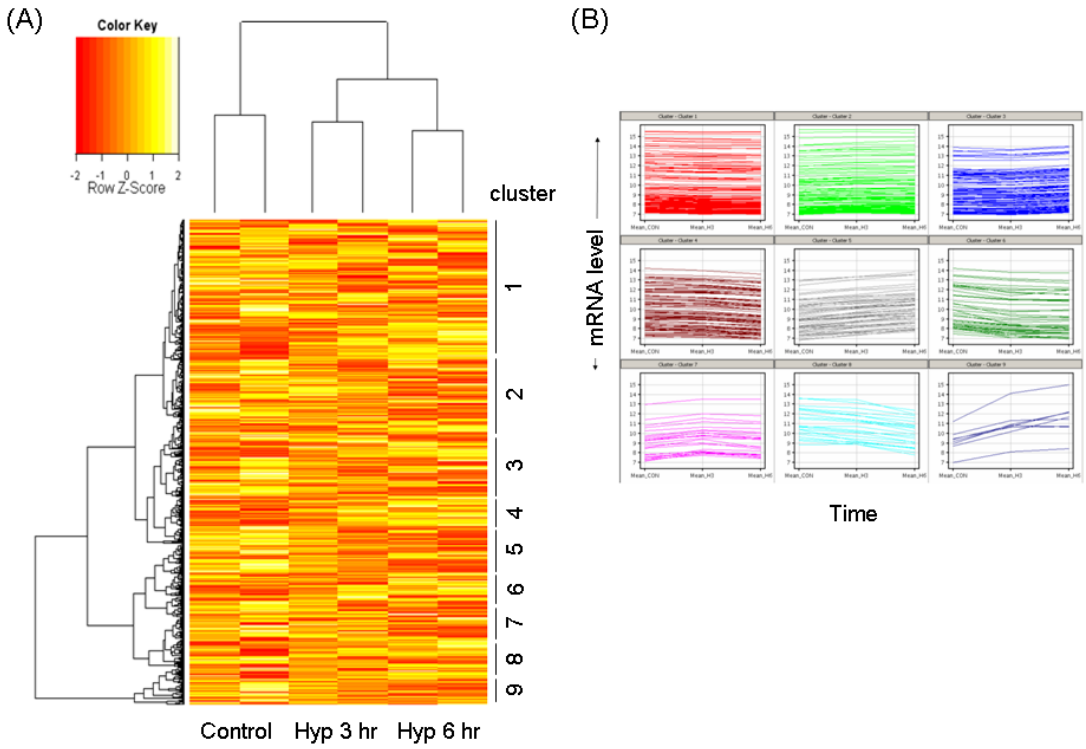
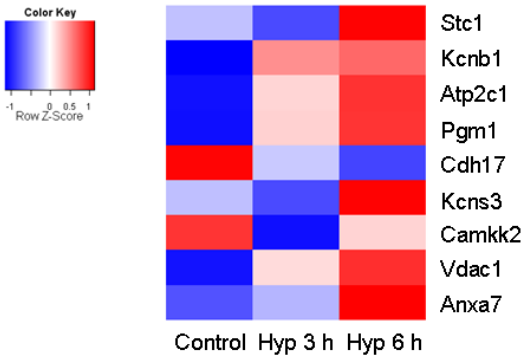


Figure 5. Clustered gene expression patterns of 630 clones that display differential expression in early ischemic cardiomyocytes. (A) Hierarchical clustering was expressed the row Z-score of -2 to 2. (p<0.02), (B) Genes with similar expression patterns clustered together in 9 different clusters of control, hypoxia 3 hours and 6 hours.

In order to identify the most statistically significant gene expression changes, a stringent filter was applied such that only calcium-related genes and showing a 2-fold change across both duplicates were selected (Figure 6A and 6B).

(A)



(B)

Gene no.	Score	Gene product	Function/description
D29646	2.0	ADP-ribosyl cyclase (CD38)	Calcium regulation
X52840	2.3	Myosin regulatory light chain	Contractility
D42145	3.0	ATP-sensitive potassium channel uKATP-1	Stress/IPC/cardioprotection
X68191	3.5	Sodium-calcium exchanger	Calcium regulation
U95157	2.5	Ryanodine receptor type II (RYR2)	Calcium regulation
U48247	3.2	Protein kinase C–binding protein enigma	Calcium channel binding
L18889	2.3	Calnexin	Calcium binding
X53363	2.1	Calreticulin	Calcium binding

Figure 6. Calcium-related cluster group of genes in early ischemic cardiomyocytes. (A) Expressed genes were categorized on calcium-related functional group, (B) High-scores for calcium-related genes. The relative level of expression is shown as fold increase over control as determined by microarray analysis. Expression levels were determined at the time point indicated from hypoxia 6 hours.

5. Intracellular ROS production in early ischemic condition

Cells subjected to early ischemia showed a significant increase in intracellular ROS production examined by H_2DCFDA fluorescence intensity compared to normal cardiomyocytes (Figure 7). The fluorescence intensity of early ischemic condition was 40% (3 hours) and 75% (6 hours) higher compared to the normal cardiomyocytes.

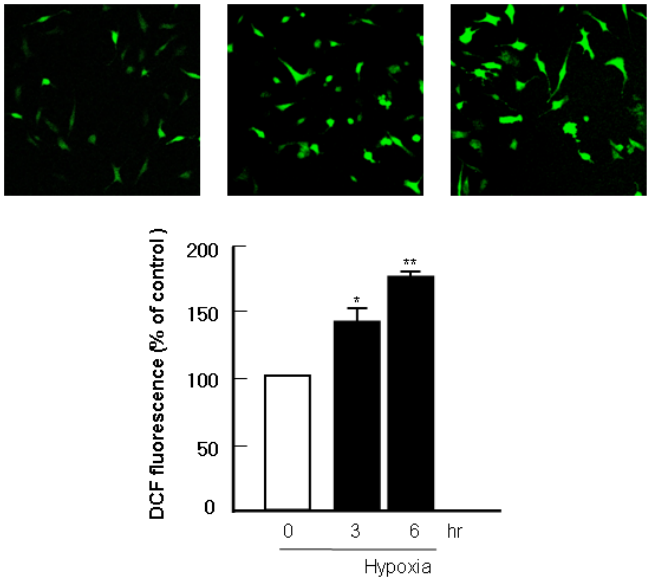


Figure 7. Alteration of intracellular ROS on early ischemic condition in cardiomyocytes. H2DCFDA(1-10 μM, Molecular Probes) enters the cells and can be oxidized into 2',7'-dichlorofluorescein(DCFH). Fluorescence intensity was detected on UV wavelength. * p <0.05 vs. hypoxia 0 hour, ** p <0.001 vs. hypoxia 0 hour.

6. Intracellular Na^+ overload in early ischemic cardiomyocytes

It was examined cardiomyocytes at the single cell level for changes in intracellular sodium using the fluorescent corona green (Na^+) dye and flow cytometry (Figure 8). Gating on only the normally sized or positive control, cardiomyocytes exposed in hypoxia for 3 and 6 hours was measured. At 3 hours exposed cardiomyocytes, sodium overload was increased about 2.9-fold compared to normal cells. Six hours exposed cells, intracellular sodium was overloaded about 3.2-fold.

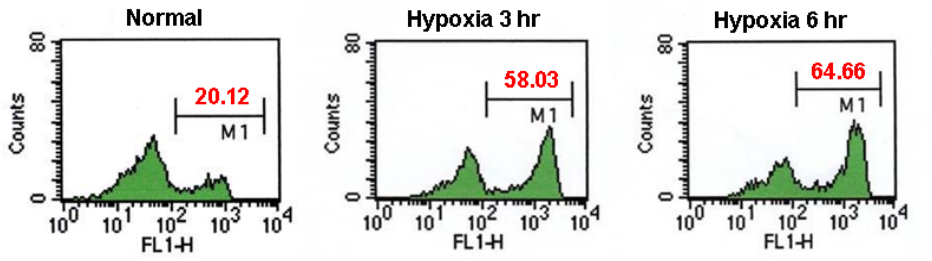


Figure 8. Effects of early ischemic condition on intracellular Na^+ overload in cardiomyocytes. Hypoxic condition of 3 hours and 6 hours was initially examined for changes in intracellular sodium by flow cytometry using corona green (Na^+). The cells were analyzed by gating on only the normal cells or hypoxia-induced cells on a side scatter histogram plot. Subsequently, this population of cells was then analyzed on a corona green fluorescence histogram.

7. Intracellular Ca^{2+} overload in early ischemic cardiomyocytes

To examine intracellular Ca^{2+} overload in hypoxic cardiomyocytes, the fluorescence intensity method was used in cardiomyocytes loaded with fluo-4 AM (Figure 9). Intracellular calcium level was increased 1.5-fold at 3 hours and 1.7-fold at 6 hours in hypoxic condition. These indicate that early ischemia can increase intracellular calcium overload in cardiomyocytes.

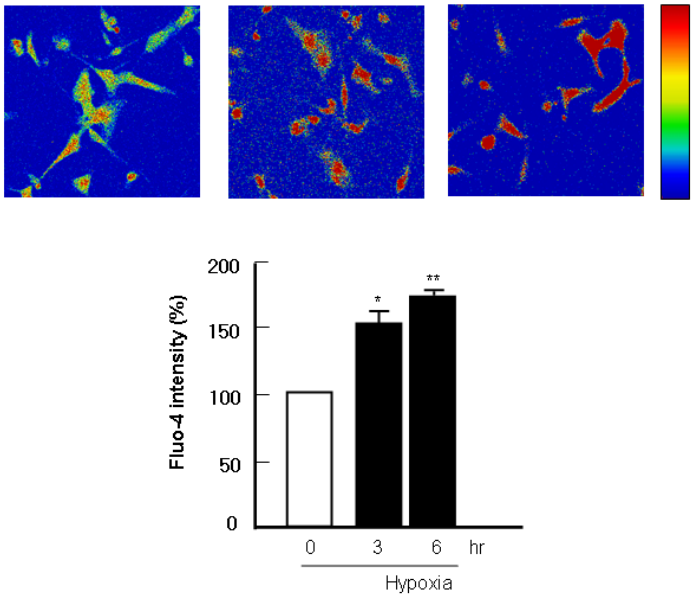


Figure 9. Effects of early ischemic condition on intracellular Ca^{2+} concentration in cardiomyocytes. Confocal fluorescent images of cardiomyocytes were obtained by loading with fluo-4 AM and its intensity was quantified in different cells in each condition and analyzed. * p <0.01 vs. hypoxia 0 hour, ** p <0.001 vs. hypoxia 0 hour.

8. Effects of early ischemia on gene coding for Ca^{2+}-handling proteins in cardiomyocytes

Compared with normal cardiomyocyte, expression of the Ca^{2+}-handling protein calsequestrin and calreticulin was nearly same with 3 hours exposed cell. At 6 hours exposure, gene expression was up-regulated about 1.5-fold and 1.75-fold, respectively. Gene expression of calmodulin was gradually increased in normal to 6 hours about 1.4-fold to 1.7-fold compared to control (Figure 10).

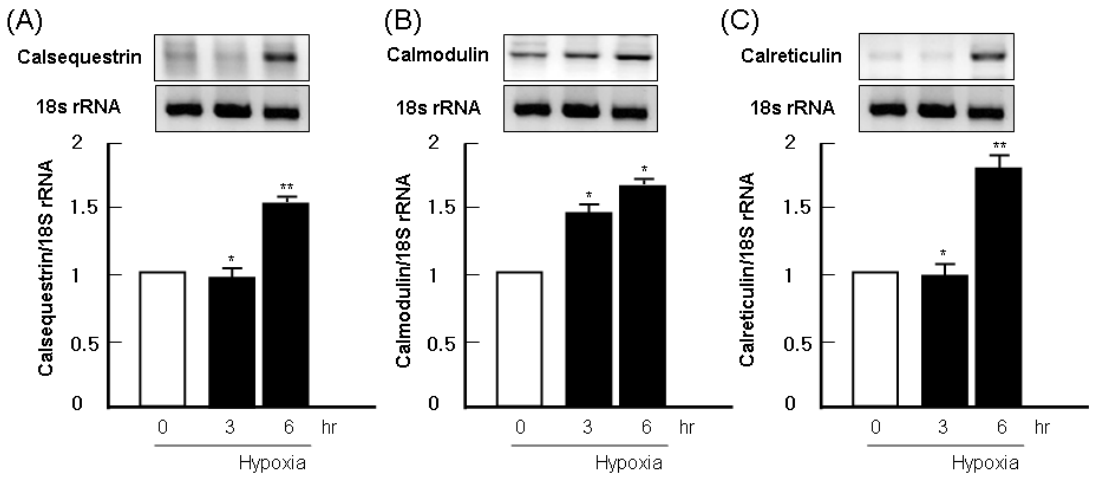


Figure 10. Analysis of Ca^{2+}-handling proteins in ischemic cardiomyocytes. (A) Calsequestrin; (B) Calmodulin; (C) Calreticulin. Amplication product were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Calsequestrin: *p<0.05 vs. hypoxia 0 hour, **p<0.001 vs. hypoxia 0 hr; Calmodulin: *p<0.001 vs. hypoxia 0 hour; Calreticulin: *p<0.05 vs. hypoxia 0 hour, **p<0.01 vs. hypoxia 0 hour.

9. Effects of early ischemia on gene coding for ion exchangers and associated partners

In NCX-1, gene expression in hypoxic cardiomyocytes was increased and 6 hours exposed cell was highly expressed about 1.4-fold. But, Na^+-K^+-ATPase, SERCA2a and phospholamban(PLB) was significantly down-regulated in 6 hours hypoxic condition-exposed cardiomyocytes. Compared with normal cell, Na^+-K^+-ATPase was decreased in 50%, SERCA2a was decreased in 80% and PLB was declined about 40% (Figure 11)

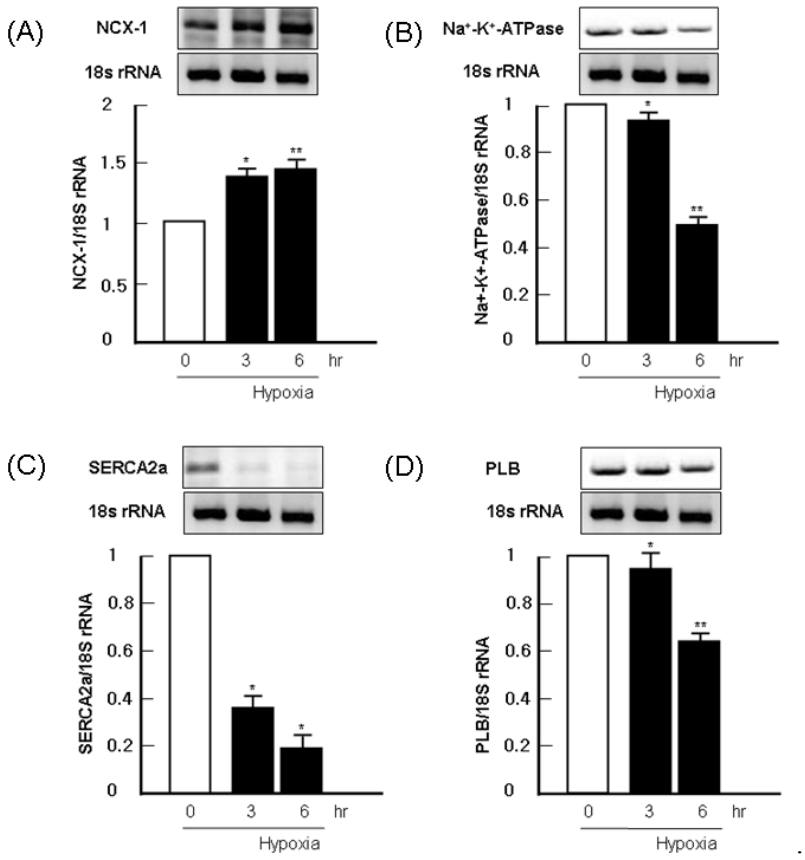


Figure 11. Analysis of ion exchanger in early ischemic cardiomyocytes. Amplification product were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (A) NCX: *p<0.05 vs. hypoxia 0 hour, **p<0.01 vs. hypoxia 0 hour; (B) Na^+-K^+-ATPase and (D) PLB: *p<0.05 vs. hypoxia 0 hour, **p<0.001 vs. hypoxia 0 hour; (C) SERCA2a: *p<0.001 vs. hypoxia 0 hour.

10. Effects of ischemia on the expression of RyR2 and stress markers

Gene expression of ryanodine receptor isoform 2 (RyR-2) was significantly risen in 6 hours exposed cells about 1.8-fold compared to normal cardiomyocytes. Expression level of ANP and BNP, stress marker, was highly increased in 3 hours each 1.2-fold (ANP) and 1.45-fold (BNP), and in 6 hours exposed cardiomyocytes about 2-fold (Figure 12).

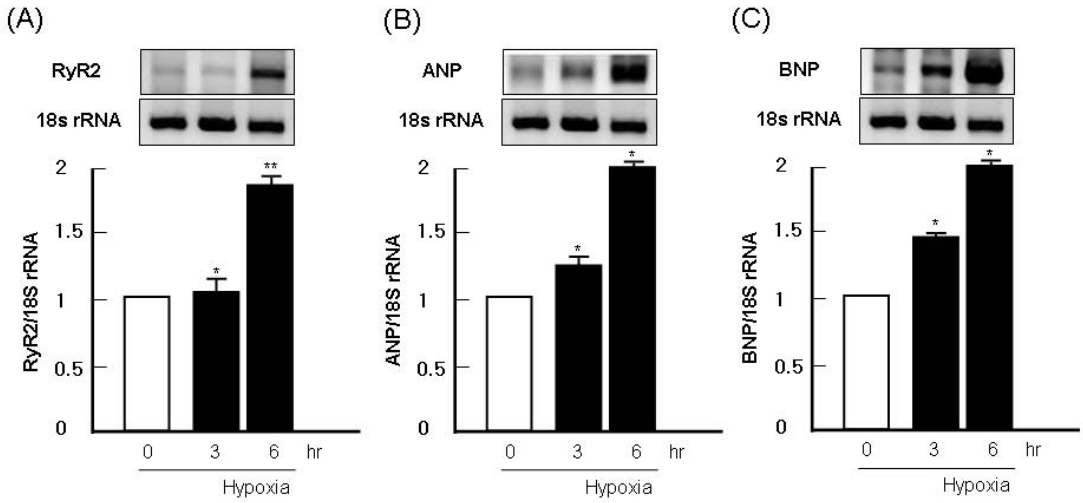


Figure 12. Effects of early ischemic condition on genes coding for ion exchangers of cardiomyocytes. Amplication product were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (A) RyR2: **p*<0.05 vs. hypoxia 0 hour, ***p*<0.001 vs. hypoxia 0 hour; (B) ANP and (C) BNP: **p*<0.001 vs. hypoxia 0 hour.

IV. Discussion

In this paper, it has established that early hypoxic condition at 3 hours and 6 hours affects many intracellular molecules and calcium-related proteins such as calcium-handling protein, ion exchanger, ion channel, and stress marker especially operates in cardiomyocytes.

In general, myocardial ischemia initiates a range of cellular events, which are progressively become damaging with increasing duration of ischemia and at last apoptosis occurs. Cardiomyocytes apoptosis is a major pathogenic mechanism underlying myocardial ischemia. Apoptosis can be initiated through two basic pathways. One is the mitochondrial or intrinsic pathway, which is

initiated by stress signals through the release of cytochrome c from the mitochondrial intermembrane space. This triggers cytosolic caspase-3 activation. The other is the so-called extrinsic pathway, which is a receptor-mediated cell death initiation process.[29] To gain more insight into the downstream signaling events during early ischemia, survival effect of cardiomyocytes which is exposed on early hypoxic condition was preliminarily measured. Potential sources of oxidative stress during ischemia include mitochondria, NADPH oxidase, and xanthine oxidase.[30] In the present study cardiomyocytes exposed to simulated early ischemic conditions showed an increased intracellular ROS production that, as indicated by DCF fluorescence. Under pathological condition of early hypoxia, weakened survival of cardiomyocytes is related to decreased activation of ERK and Akt provides potent pro-survival signals. Members of the ERK-MAPK family have been implicated in anti-survival signaling in response to ischemic condition. The phosphorylation status of ERK gradually decreases when cardiomyocytes are cultured in 1% O_2 at 4, 8, and 16 hours and stress-activated MAPKs induce down-regulation of Akt at hypoxia 4 hours.[31,32] Also, an increase of Bcl-2/Bax ratio which are a major signal mediator of cell apoptosis. In early hypoxic condition, cardiomyocytes rised the expression and activity of pro-apoptotic signals such as Bax and attenuated the expression of anti-apoptotic Bcl-2 in hypoxia.

The rise in intracellular Na^+ may then induce an increase in $[Ca^{2+}]_i$ via Na^+/Ca^{2+} exchange resulting in Ca^{2+} overload.[33] Under the assumption that the Ca^{2+} entry into myocytes through cardiac Ca^{2+} channels during myocardial ischemia is the initiating signal in triggering Ca^{2+} overload, acetoxymethyl ester of Fluo-4 and corona green was experiment for calcium, and sodium overload of time-dependent manner and microarray analysis was used to assess changes in gene expression in cardiomyocytes subjected to the early hypoxic condition for 3 hours and 6 hours. This result for studying early ischemia events was

exploited to look at gene expression changes for Ca^{2+}-related proteins.

It is well documented that SR is involved intimately in the regulation of Ca^{2+}-transport in cardiomyocytes. Depolarization of the cell membrane leads to Ca^{2+} entry through sarcolemmal Ca^{2+}-channels, which in turn induces the release of Ca^{2+} from the SR stores into the cytosol via the SR Ca^{2+}-release channel or ryanodine receptor (RyR) to cause cardiac contraction. For the occurrence of cardiac relaxation, the intracellular concentration of Ca^{2+} is restored to the diastolic level mainly by Ca^{2+}-uptake into the SR lumen via Ca^{2+}-pump ATPase (SERCA2a), which is regulated by phospholamban (PLB). In the SR lumen, Ca^{2+} is bound by the negatively charged protein calsequestrin and is available for the next cardiac contraction. Previous studies have shown altered gene expression of Ca^{2+}-handling proteins in the remote myocardium of failing hearts post-MI.[34] The impairment of myocardial contractility and relaxation has been associated with lower sarcoplasma reticulum Ca^{2+} content and reuptake rate, due to reduced SERCA2a activity. Guerra et al was experiment with SERCA2a, and PLB decrement after 30 and 120 minutes of ischemia in the rat[35] and NCX and calsequestrin expression were significantly higher at the mRNA level than control in neonatal post-ischemic hearts.[36] Ischemia-induced decrease in Na^{+}-K^{+}-ATPase activity was indicated in rat heart after oxidative stress.[37] The atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are predominantly expressed in the atrium and the ventricle of the heart, respectively. Previous study indicated that ANP and BNP were overexpressed in ischemic human myocardium.[38] ANP and BNP are reported as stress marker.[39] This study represented that Ca^{2+}-handling protein and stress marker were overexpressed in early ischemic condition. And, ion exchangers were downregulated in hypoxic condition of 3 hours and 6 hours except NCX-1.

V. Conclusion

Our study suggests that early ischemic condition of neonatal rat cardiomyocytes affects pro-apoptotic response and anti-survival signaling at 3 hours to 6 hours. Early ischemic response-induced cardiomyocytes induce ROS production, then intracellular Na^{+} and Ca^{2+} overload is produced. The Ca^{2+}-related proteins with handling protein, ion exchanger, and stress markers should operate via PKC regulation, and may be also mediated by hypoxic condition of cardiomyocytes. These findings extend the understanding of the important role of calcium homeostasis in cardiomyocytes of early ischemia.

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ABSTRACT (IN KOREAN)

허혈성 심근에서의 이온통로, 이온교환체와 칼슘조절 단백질 발현의 변화

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김도균

심근경색은 심근세포에 산소공급이 충분치 않음으로 인해서 심근의 항상성에 영향을 주고 병리적 결과를 초래하여 심근의 기능이상을 가져오는 질환이다. 지금까지 많은 연구에서 심근내 칼슘의 항상성이 심근의 기능에 밀접한 연관이 있음이 밝혀져 왔지만 초기 허혈성 심근세포내에서 단백카이네이스 C(protein kinase C, PKC)와 관련된 칼슘 조절에 관한 논문은 드물다. 초기 허혈기는 허혈후 3-6시간까지이며 이 조건하에서 단백카이네이스 C(PKC)의 세포막/세포질비가 상향조절 되고 있다. 초기 허혈기의 심근세포에서는 Erk와 Akt의 인산화가 억제되고 반세포소멸 단백질인 Bcl-2의 억제도 일어나고 세포소멸의 전구단백질인 Bax의 발현을 유도한다. 허혈성 심근세포 내에서 칼슘과 관련된 유전자는 유전자 미세배열기법(microarray)으로 검사한 결과 다르게 발현되었다. 초기 허혈상태는 활성산소부산물(reactive oxygen species)의 증가를 유도하고 세포질내 칼슘과 나트륨의 과부하를 초래한다. 초기 허혈기에는 칼슘조절 단백질들(calsequestrin, calmodulin, calreticulin)과, 이온교환체 및 통로들(NCX, Na^+-K^+-ATPase, SERCA2a, PLB, RyR2), 그리고 스트레스 표지자들(ANP,BNP)이 의미 있는 변화를 보였다. 결론적으로,

초기 허혈기에 심근세포에서 칼슘관련 유전자들이 의미 있는 변화를 보였고 이 결과로 초기 허혈기에 칼슘의 조절기전을 밝히는데 도움이 될 것으로 생각된다.

핵심되는 말 : 심근세포, 칼슘, 초기허혈기