Essential Role of Lipid Raft in Ischemic Preconditioning

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Key Words

Ischemia • Precondition • Heart • Lipid raft • Signal transduction

Abstract

Lipid rafts represent a subcompartment of the plasma membrane that coordinate and regulate varieties of signaling processes while caveolins are the integral membrane protein of the lipid raft. To study the role of lipid raft in ischemic preconditioning (PC) of the heart, rat hearts were perfused by working mode and then preconditioned in absence or presence of a lipid raft disintegrator, Methyl- β -cyclodextrin. As expected, precondition made the heart resistant to ischemia reperfusion (I/R) injury as evident by improved ventricular performance, reduced myocardial infarct size and cardiomyocyte apoptosis. Cyclodextrin abolished the cardioprotection. Transmission Electron Microscopy revealed severe degeneration, swelling of mitochondria, chromatin condensation and myofibril disarray in cyclodextrin treated PC heart similar to I/R heart. In the PC hearts, there was an increased association of the proapoptotic p38MAPK α with caveolin-1 while there was a reduced association of anti-apoptotic p38MAPK β with caveolin-3 indicating

that reduced amount of p38MAPK α and increased amount of p38MAPK β were available to the adapted hearts thereby generating a survival signal. In contrast, there was very weak caveolin-MAP kinase interaction in cyclodextrin treated heart. Myocardial damage was further confirmed by reduced or no expression of anti-apoptotic phospho-AKT, Bcl2, Bcl-xl and increased expression of pro-apoptotic JNK, BAX, and p53 in methyl- β -cyclodextrin (lipid raft disintegrator) treated heart. These results indicate that lipid raft play a pivotal role in the generation of survival signal in PC or adapted heart and disintegration of lipid raft completely abolish cardio-protection.

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Introduction

Lipid rafts are sphingolipid and cholesterol rich domains of the plasma membrane, which contain a variety of signaling and transport proteins. Different subtypes of lipid rafts can be distinguished according to their protein and lipid compositions. Caveolae are types of rafts that are rich in caveolin family (caveolin-1, caveolin-2, and

caveolin-3) proteins, and present as distinct signaling platforms [1]. Caveolae are receiving increasing attention as cellular organelles contributing to the pathogenesis of several human diseases. A large variety of *in vitro* and *in vivo* studies have implicated caveolae and caveolin in pathogenesis of cancer, atherosclerosis, vasculoproliferative disease, degenerative muscular dystrophy, cardiac hypertrophy and heart failure [1].

To date, a few reports are available on caveolin-1 and caveolin-3 gene ablation. Caveolin-1 knockout mice develop progressive cardiac hypertrophy as demonstrated by transthoracic echocardiography (TTE) and magnetic resonance imaging (MRI) [2]. In contrast, caveolin-3 knockout mice develop cardiomyopathy characterized by hypertrophy, dilation and reduced contractility as well [3]. Caveolin-1 and caveolin-3 double knockout mice completely lacking caveolae are deficient in all three caveolin proteins because caveolin-2 is degraded in absence of caveolin-1. The double knockout mice developed a severe cardiomyopathic phenotype with cardiac hypertrophy and decreased contractility [4].

Earlier investigation from our laboratory demonstrated differential interaction and/or translocation of p38MAPK α and p38MAPK β with caveolin-1 and caveolin-3, respectively, and such interaction functioned as a molecular switch for the conversion of ischemia/reperfusion (I/R) induced death signal into precondition (PC) induced survival signal [5]. To study the detail mechanism of lipid raft in generation of survival signal during PC, a lipid raft disintegrator was used in this present investigation and our results concluded that generation of survival signal during PC was completely abolished in presence of a lipid raft disintegrator.

Materials and Methods

Chemicals

Methyl- β -cyclodextrin and were obtained from Sigma, St Louis, MO. Antibodies against caveolin-1, caveolin-3, p38MAPK β , eNOS and Bcl-2 were purchased from Santa Cruz Biotechnology, CA. Antibodies against p38MAPK α , JNK, ERK, p53, AKT, phospho-AKT were purchased from Cell Signaling technology, Danvers, MA.

Animals

All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institute of Health (NIH Publication No. 85-23, revised 1996).

Experimental protocol

The study used isolated working rat hearts subjected to ischemia/reperfusion protocol. Isolated rat hearts were randomly divided into four groups: perfused with KHB buffer only (group I); the hearts were subjected to 30 min ischemia and 120 min reperfusion [I/R] (group II), the hearts were perfused with KHB buffer for 15 min in the absence (group III) or presence (group IV) of cyclodextrin [0.2 mM] and then subjected to 4 cyclic episodes of 5 min ischemia and 10 min reperfusion followed by 30 min ischemia and 120 min reperfusion. Control hearts (group I) after perfusion with KHB buffer for 15 min, were not exposed to ischemia and reperfusion. They were only subjected to 2 hr and 30 min continuous perfusion.

Isolated Working Rat Heart Preparation

Male Sprague Dawley rats of 250 gm body weight were used for this study. The rats were anesthetized with sodium pentobarbital (80 mg/kg b/w, ip.), (Abbott Laboratories, North Chicago, IL, USA) and anticoagulated with heparin sodium (500 IU/kg b.w, i.p) (Elkin-Sinn Inc., Cherry Hill, NJ, USA) injection. After ensuring sufficient depth of anesthesia, the hearts excised and then the isolated hearts were perfused in Langendorff mode (cannulation via aorta) against a constant perfusion pressure of 100 cm of water (10kPa) for 5 min stabilization [6]. Any heart that showed any cardiac disturbance (ventricle arrhythmia, and fibrillation) during the entire experiment was excluded from this study. After 5 min perfusion in Langendorff mode the hearts were converted in working mode (i.e. left ventricular preparation) and perfused by working mode according to the protocol as described earlier. To ascertain the normal function of the heart, heart rate, left ventricular developed pressure (the difference between the maximum systolic and diastolic pressure), and the first derivative of developed pressure were recorded by Gould p23XL transducer (Gould Instrument System Inc., Valley View, OH). The signal was amplified by using Gould 6600 series signal conditioner (Gould Instrument System Inc., Valley View, OH) and monitored on Cordat II real-time acquisition system (Triton technologies, San Diego, CA) [7]. The aortic flow was measured by flow meter. The coronary flow was measured by time-collection of the coronary effluent dripping from the heart.

Measurement of the infarct size

After ischemia/reperfusion (I/R) procedure, the heart was infused with 10 % solution of the Triphenyl tetrazolium (TTC) in phosphate buffer through the aortic cannula for 20 minutes [8]. The left ventricle was removed and sliced into 1-mm thickness of cross-sectional pieces and weighted. Each slice was scanned with computer-assisted scanner (Scanjet). The infarct zone remained unstained while the non-infarct part of myocardium was stained red by TTC. These were measured by using of computerized software (Scion Image); areas were multiplied by the weight of each section, and summed up to obtain the total infarct zone. The infarct size was expressed as the ratio of the infarct zone to the area of risk.

Fig. 1. Effect of ischemia-reperfusion, precondition and cyclodextrin on the infarct size. The results are mean $\pm$ SEM of six animals per group. * $p < 0.05$ vs I/R, † $p < 0.05$ vs PC.

TUNEL Assay for assessment of Apoptotic Cell Death

Immunohistochemical detection of apoptotic cells was carried out by TUNEL assay using ApopTag *in situ* apoptosis detection kit (Intergen Company, Purchase, N.Y) [9]. Tissue sections were prepared from left ventricular tissue of the heart. Cells were counted at 100X magnification and at least 4 fields per sample. The sections were incubated again with mouse monoclonal antibody recognizing cardiac myosin heavy chain to specifically recognize apoptotic cardiomyocytes. The fluorescence staining was viewed with a confocal laser microscope. The number of apoptotic cells was counted and expressed as a percent of total myocytes population.

Transmission electron microscopy (TEM)

Small tissue samples (from, I/R, PC and cyclodextrin treated hearts), about 1 mm³, were fixed by immersion in 4% glutaraldehyde, postfixed for 1 hour in 1% OsO₄ in 0.1M cacodylate buffer, dehydrated and embedded in Epon 812 at 60°C for 48 hours. Routine 60 nm ultra thin sections were cut and mounted on coated grids, stained with 1% uranyl acetate and Reynolds's lead citrate. The transmission electron microscopy (TEM) examination was performed with Philips CM 12 at 60kV and digital images were recorded with Morada CCD, iTEM (Olympus Soft Imaging Solutions) [10, 11].

The number of caveolae/ μ m membrane length was counted on digital images using Olympus iTEM software on 25 random images at 15,000x magnification for each sample and data are expressed as mean $\pm$ SD.

Isolation of Caveolin-rich Membrane Fractions

The hearts were homogenized in sodium carbonate buffer containing protease inhibitor cocktail, pH 11.0, using a Polytron homogenizer [three 10s bursts] [Brinkman Instruments, Westbury, N.Y.]. The homogenate was sonicated [three 20s bursts], and adjusted to 45% sucrose by the addition of 2 ml of 90% sucrose prepared in MBS [25 mM Mes, pH 6.5, 0.15 M NaCl] and placed at the bottom of an ultracentrifuge tube as described previously [12]. A 35% discontinuous sucrose gradient was formed above [4 ml of 5% sucrose/4 ml of 35% sucrose – both in MBS containing 250 mM sodium carbonate] and centrifuged at 39,000 rpm for 16-20 h in an SW41 rotor [Beckman Instruments, Palo Alto, CA]. From the top of each gradient, 1 ml gradient fractions were collected to yield a total of 12 fractions as described elsewhere. Caveolin migrates mainly in fractions 5 and 6 of these sucrose density gradients [12].

Immunoprecipitation with Caveolin 1 and Caveolin 3

Only caveolin rich fractions (fraction 5 & 6) were used for immunoprecipitation. Immunoprecipitation was performed with Protein-A sepharose CL-B4 (Pharmacia Biotech Inc) using a polyclonal antibody against caveolin-1 or caveolin -3 [Santa Cruz Biotechnology, Santa Cruz, CA] Incubation conditions

Fig. 2. Effect of ischemia-reperfusion, precondition and cyclodextrin on the cardiomyocyte apoptosis. The results are mean $\pm$ SEM of six animals per group. * $p < 0.05$ vs I/R, † $p < 0.05$ vs PC.

were maintained as instructed by the supplier. Western blot analysis was then performed with antibodies against P38MAPK α , P38MAPK β , JNK, ERK, AKT, Phospho-AKT and Bcl-2 according to established Method [13].

Statistical Analysis

The values for number of apoptotic cardiomyocytes and infarct sizes as well as the functional parameters were all expressed as the Mean $\pm$ Standard Error of Mean (SEM) for at least six animals per group. The Western blot analyses were performed with at least three animals per group. The statistical analysis was performed by analysis of variance followed by Bonferroni's correction for any differences between the mean values of all groups. Differences between data were analyzed for significance by performing a Student's *t*-test. The results were considered significant if $p < 0.05$.

Table 1. Effect of ischemic PC and cyclodextrin on post ischemic ventricular performance. The results are mean $\pm$ SEM of six animals. * $p < 0.05$ vs I/R, $\dagger p < 0.05$ vs PC.

	Baseline	I/R	PC	PC + cyclodextrine
Heart rate (beats/min)	264 $\pm$ 6	259 $\pm$ 8	262 $\pm$ 9	239 $\pm$ 16
Aortic flow (ml/min)	36.4 $\pm$ 2	7.1 $\pm$ 1.5	16.8 $\pm$ 1.6*	6.8 $\pm$ 1.2 †
Coronary flow (ml/min)	22.9 $\pm$ 1.2	18.6 $\pm$ 1.6	21.1 $\pm$ 1.4	14.5 $\pm$ 1.4 †
Left ventricular developed pressure (mm Hg)	109 $\pm$ 4.1	52 $\pm$ 5.6	84 $\pm$ 4.4*	51 $\pm$ 5.3 †
Maximum first derivative of developed pressure (mm Hg/s)	2722 $\pm$ 51	599 $\pm$ 63	1478 $\pm$ 65*	689 $\pm$ 102 †

Fig. 3. Transmission electron microscopy of I/R, PC and cyclodextrin treated heart. Control (A): Cardiomyocyte (MYO) show a normal ultrastructure, arrays of mitochondria (M) with a dense matrix run parallel with myofibrils. Endothelial cells (E) have normal aspect also. L - capillary lumen. Scale bar: 5 μ m. Ischemia-reperfusion (B): Myocardium (MYO) showing ischemic lesions: increased mitochondria with lost cristae, matrix coagulation (*) and swelling (S), sarcoplasmic reticulum with dilatation (small arrows), myofibril disarray (F) and widened intercellular junctions (large arrows). Peripheral condensation of chromatin indicates the incipient phase of apoptosis (AP) in an endothelial cell (E). N - nerve fiber, ICLC - interstitial Cajal-like cell, P - pericyte. Scale bar - 2 μ m. Preconditioning (C): Normal ultrastructure of cardiomyocyte (MYO), endothelial cells and interstitial Cajal-like cell (ICLC). Mitochondria (M) showed a mild widening of the matrix compartment. Scale bar - 2 μ m. Cyclodextrin (D): Cardiomyocyte (MYO) with severe degeneration: mitochondria showing condensed cristae (small arrows) or swelling (S), intercellular junctions disorganization (large arrows), myofibril disarray and focal lysis (*). E - endothelial cells, FB - fibroblast, L - capillary lumen. Scale bar - 2 μ m.

Results

Cardioprotection by PC and abolition by Lipid raft disintegrator

As expected, ischemia reperfusion (I/R) caused significant tissue injury as reflected by increased amount of myocardial infarct size (Fig 1) and cardiomyocyte

apoptosis (Fig 2). Ischemic PC mediated by four cyclic episode of 5 min ischemia each followed by another period of 10 min reperfusion reduced tissue injury as evidenced by decreased amount of infarct size (Fig 1) and cardiomyocyte apoptosis (Fig 2). In concert, post-ischemic ventricular function was significantly higher in preconditioned heart compared to I/R hearts (Table 1).

Fig. 4. TEM micrographs show details of the cardiomyocyte plasma membrane under different experimental conditions: A - baseline control; B - Ischemia/Reperfusion; C - Preconditioning; D - Preconditioning and cyclodextrin. Caveolae (arrows) with normal ultrastructure could be seen along plasma membranes in panels A-C. In D plasma membranes show numerous disrupted caveolae (double arrows) and a single caveolae with normal structure (arrow). Note small vesicles (asterisks), about 100nm diameter, situated between plasma membrane and basal lamina (L) of the cardiomyocyte. Mitochondria (M) show swelling and disrupted cristae in D. ICLC - interstitial Cajal-like cellular process. Scale bar - 0.5 μ m.

Table 2. Number of caveolae/ μ m membrane length in cardiomyocyte.

	baseline (control)	I/R	PC	PC + cyclodextrin
number of caveolae / μ m membrane	0.32 caveolae/ μ m	0.10 caveolae/ μ m	0.17 caveolae/ μ m	0.04 caveolae/ μ m

LV developed pressure and aortic flow were reduced during post ischemic reperfusion in each group. However, the reduction in recovery was significantly less for PC group, which reached significance compared to the non-PC group at 120 min reperfusion (Table 1). Methyl- β -cyclodextrin, a lipid raft disintegrator at a dose of 0.2 mM, abolished all the cardioprotection offered by PC as evident from the increased infarct size, cardiomyocyte apoptosis and reduced post-ischemic ventricular function.

Transmission electron microscopy (TEM) images

TEM images of I/R and cyclodextrin treated heart showed severe degeneration, swelling of mitochondria, chromatin condensation and myofibril disarray. Whereas in PC heart the ultra structure of cardiomyocyte was normal like control heart, except that mitochondria showed slight swelling (Fig: 3). Images also showed that number of caveolae/ μ m was high in PC heart compared to those I/R and cyclodextrin treated heart (Fig 4; Table 2).

Differential association of MAP kinase with caveolin 1 vs. caveolin 3

Caveolin rich fractions (5th and 6th fractions of sucrose density gradient) of all three groups of hearts were immuno-precipitated with caveolin-1 and

caveolin-3. The examination of the existence of p38MAPK α/β in the caveolin immunoprecipitated sample by Western blot revealed that high amount of p38MAPK α bound with caveolin-1 and negligible interaction of caveolin-3 with p38MAPK α . In contrast, high amount of p38MAPK β bound with Caveolin-3 whereas its binding with caveolin-1 was negligible. JNK & ERK1/ 2 also bound with caveolin 1 whereas interaction with caveolin-3 was negligible.

In I/R heart, there was an increased association of p38MAPK β & ERK1/2 (anti-death signaling component) with caveolin-3 and caveolin -1, respectively, while there was a reduced association of p38MAPK α & JNK (death signaling component) with caveolin-1 indicating increased amount of death signaling components were available to I/R heart to generate a death signal. In contrast, in PC heart, there was a decreased association of anti-death signaling components (p38MAPK β , ERK 1/ 2) with caveolin-3 and caveolin-1, respectively, while there was an increased association of death signaling components (p38MAPK α , JNK) with caveolin-1 indicating reduced amount of death signaling components and increased amount of anti-death signaling components were available to the PC heart to generate a survival signal. When PC hearts were treated with Methyl- β -cyclodextrin, caveolin-

Fig. 5. Differential interaction of different MAPKs with caveolin-1 and caveolin-3 in I/R, PC and cyclodextrin heart.

MAP kinase interaction was negligible compared to PC (Fig 5).

Caspase-3, is a cysteine protease, has been identified a key mediator of apoptosis. Western blot revealed that caspase-3 interact with caveolin-1, but not with caveolin-3. In PC heart, caspase-caveolin interaction was high compared to the I/R heart. Whereas in cyclodextrin treated heart caveolin-caspase interaction was negligible.

In order to reconfirm our immunoprecipitation data, we tested all the twelve fractions of sucrose density gradient of PC and cyclodextrin treated heart. Generally, caveolin or caveolae is present in the 5th and 6th fraction of sucrose density gradient. In the PC heart, we found similar localization of caveolin. At the same time we found that p38MAPK β and ERK were present in non-caveolin region and p38MAPK α and JNK were present in caveolin-rich zone (fig 6). From these results it can be concluded that during PC, p38MAPK α and JNK (death signaling components) remain bound with caveolin making them unavailable. At the same time p38MAPK β and ERK (anti-death signaling components) remain in non-caveolin fractions making the heart more abundance to them. In case of cyclodextrin treated heart, caveolin was not limited only in 5th and 6th fraction; rather it was dispersed in wide (4th to 11th fractions) region. P38MAPK α/β , JNK, ERK all the signaling components were present mostly in all the fractions (Fig 6). It can be concluded that disruption of lipid raft by cyclodextrin, abolished the fine differentiation between non-caveolin region and caveolin and/or caveolae rich zone and simultaneously abolishing delicate control of lipid raft on the valuable signaling components.

Expression of different survival protein and apoptotic protein in PC and cyclodextrin treated heart

Bcl-2, Bcl-xl, phospho-AKT are known to transmit survival signal while Bax, JNK, p53 are known to transmit death signal. Expressions of these proteins were found in the cytosolic and crude fractions of the heart. PC heart showed high level of expression of Bcl-2, Bcl-xl and phospho-AKT. Similar to the I/R heart, cyclodextrin treated heart showed no expression of Bcl-2, Bcl-xl, phospho-AKT, rather showed high expression of JNK, p53 & BAX (fig 7).

Discussion

The present study demonstrated an important role of lipid raft in myocardial ischemia/reperfusion injury and ischemic PC. Earlier investigation from our laboratory showed that caveolin-MAP kinase interaction plays an important role in the development of survival signal in PC heart [5]. To study the role of lipid raft in PC, we used cholesterol depleting agent, methyl- β -cyclodextrin (M β CD), in an attempt to disintegration of lipid raft [14]. Lipid rafts are specialized membrane domain enriched with certain lipids, cholesterol and proteins. M β CD abolished the cardioprotection offered by PC as evident from the reduced post ischemic ventricular recovery, increased infarct size and increased number of apoptotic cardiomyocyte. Transmission Electron Microscopy revealed that M β CD disrupted normal ultra structure of caveolae. Cyclodextrin treated heart showed severe

Fig. 6. Distribution of different MAPKs in different fraction (sucrose density gradient) of preconditioned heart and cyclodextrin treated heart. In PC heart among all the 12 fraction of the heart tissue only 5th, 6th and 7th fractions are rich source of caveolin and /or lipid raft rich zone. But in cyclodextrin treated heart, where lipid rafts are disrupted, caveolin is dispersed from 4th to 11th fraction.

degeneration, swelling of mitochondria, chromatin condensation and myofibril disarray. Number of caveolae/ μm is also very low in cyclodextrin treated heart compared to the normal or PC myocardium. These results strongly indicate that myocardial preconditioning can not occur in the absence caveolae / lipid raft.

The MAP kinases play an essential role in mediating intracellular signal transduction during ischemic PC. MAP kinases are classified as: 1) Extracellular signal regulated kinase, ERK 1/ 2 (44/42 kDa), which are activated by growth hormone receptors via a Ras/ Raf pathway, 2) Stress activated kinase –Jun-NH2-terminal kinase 1/ 2 (JNK 1/ 2) and 3) p38MAPK α/β that are activated via MKK4/MKK7 and MKK3/MKK6 pathways, respectively, by cellular stress including reactive oxygen species, heat shock, inflammatory cytokines and ischemia. In response to extracellular stimulation, MAP kinase are rapidly activated and regulate cellular functions by inducing the phosphorylation of proteins, such as an oncogene product c-jun, S6 ribosomal protein kinase and MAP kinase activated protein kinase 2 [15, 16]. It appears that activation of stress-activated protein kinase is obligatory for myocardial adaptation to ischemia, but the activation is only transient, and the activities rapidly come down after subsequent ischemia and reperfusion.

In a previous study, we have shown that PC translocates p38MAPK from the nucleus to the cytosol prior to activation of MK2 and HSP27 phosphorylation [17]. To date, four members of the p38MAPK family have been identified: p38 α , p38 β , p38 γ and p38 δ [18].

Fig. 7. Effect of I/R, PC and cyclodextrin (cyclo) on different survival (Bcl-2, Bcl-xl and phospho-AKT) and death (JNK, p53 and bax) signal.

Although these isoforms share functional similarities, differences exist in their upstream and downstream kinases specificity, suggesting that they may have non-overlapping functions [19]. In general p38MAPK α is linked to the death signal while p38MAPK β is linked with the survival signal [20]. Mitogen-activated protein kinase 3 (MKK3) preferentially activates p38MAPK α by dual phosphorylation and increases cell death [21]. In case of heart, MK2 is activated specifically by p38MAPK α and

Fig. 8. Proposed model of the role of lipid raft in the ischemic preconditioning of the heart. In I/R heart, anti-death signaling components (p38MAPK β and ERK 1/2) remain bound (+) with caveolin, whereas there was reduced association (-) of death signaling components (p38MAPK α , JNK and caspase-3) with caveolin. These unbound death signaling components induces reperfusion injury in the heart by expressing (+) JNK, BAX and p53 in the myocardium. In PC heart, death signaling components remain bound (+) with caveolin, whereas there was reduced association (-) of anti-death signaling components with caveolin. These unbound anti-death/survival signaling components induced cardioprotection by expressing (+) AKT, Bcl-2 and Bcl-xL in the myocardium. When precondition was performed in presence of cyclodextrin, lipid raft disintegrator, there was no particular strong interaction of survival signaling components or death signaling components with caveolin. Due to the loss of fine control on the availability death and survival signals, heart can not generate survival signal (cardioprotection) in the PC heart in presence of lipid raft disintegrator, which was further confirmed by the expression (+) of JNK, BAX and p53 in myocardium of cyclodextrin treated heart. [I/R= ischemia reperfusion, PC= precondition].

p38MAPK β . We have shown that proapoptotic p38MAPK α binds with caveolin-1 and antiapoptotic p38MAPK β binds with caveolin-3. In I/R heart, more antiapoptotic p38MAPK β binds with caveolin-3 and less proapoptotic p38MAPK α binds with caveolin-1, rendering the heart more abundance to p38MAPK α thereby inducing death signal. More likely, in the PC heart, higher amount of proapoptotic p38MAPK α remains bound to caveolin-1 and less antiapoptotic p38MAPK α binds with caveolin-3, making the heart more abundance to p38MAPK β to induce a survival signal.

In an *in vivo* rat model, ischemia does not induce dual phosphorylation of ERK 1/2. However, PC increases ERK activation, subsequent to ischemia/reperfusion [22]. The cytosolic activation of ERK and the cardioprotection that is mediated by PC was attenuated by PD98059, ERK inhibitor. This indicates that cytosolic ERK activation is an important component of PC [23]. Using rat model of cerebral ischemia, PC appears to inhibit JNK activation, suggesting detrimental role JNK activation in the setting of PC [24]. Transgenic JNK knockout mice were found to be protected *in vivo* against myocardial ischemia-reperfusion injury. However, in transgenic mice with over-expressed MKK7, the upstream signaling components of JNK also paradoxically displayed resistant to myocardial ischemia-reperfusion injury [25].

Present investigation showed that, in I/R heart anti-death signaling component (p38MAPK β , ERK 1/2) bound with caveolin and binding of the death signaling component (p38MAPK α , JNK, caspase-3) with caveolin is less, exposing the heart to more abundance death signaling components thereby generating death signal. In contrast, in PC heart, binding of anti-death signaling component with caveolin is much less compared to the I/R heart exposing the heart to more abundance anti-death signaling component, generating a survival signal. It appears that differential translocation and/or interaction of MAPKs with caveolin or caveolae or lipid raft function as a switch for the conversion of I/R induced death signal into PC induced survival signal. In case of M β CD treated heart, where the lipid rafts or caveolae are disrupted, caveolin-MAP kinase interactions are very weak and no differential interaction can be observed between different MAP kinases and caveolin. To reconfirm our results, all 12 fractions (sucrose density gradient) of PC and cyclodextrin treated heart were tested. In PC heart we have found that death signaling components (p38MAPK α , JNK) remain in caveolin-rich zone, whereas anti-death signaling components (p38MAPK β , ERK 1/2) remains in non-caveolin rich zone. This observation further confirms the above mentioned hypothesis. In case of cyclodextrin treated heart, where

lipid rafts and/or caveolae are disrupted, caveolins (integral membrane protein of lipid raft) are scattered mostly in all fractions and at the same time all the signaling components (p38MAPK β , ERK 1/2, p38MAPK α , JNK) are scattered in all fractions. This observation confirmed that, due to disruption of lipid raft structure, there is no differentiation between caveolae/ caveolin rich zone and non-caveolin zone. As a result, caveolin, the integral membrane protein of the lipid raft, was scattered in all fractions. As lipid raft structure is disrupted, it can not block the death inducing signal and can not make anti-death signaling components predominantly available to the heart to induce survival signal or vice versa. It can be concluded that disruption of caveolae structure abolish the fine control of lipid raft on the availability of signaling components to induce particular signal. Due to the availability of both death and anti-death signaling components, any particular signaling components can not work predominantly and myocardium undergoes severe damage.

Recent investigations have suggested an emerging paradigm whereby stress-responsive intracellular signaling pathways involve transcription of survival gene Bcl-2 through PI-3-kinase pathway [26]. Akt is a critical regulator of PI3-kinase-mediated cell survival. [27]. Constitutive activation of Akt signaling is sufficient to block cell death induced by a variety of apoptotic stimuli [28]. Several down stream targets of Akt have been recognized as apoptosis regulatory molecules including Bcl-2-family

member BAD [29] procaspase-9, [27], cAMP response element-binding protein (CREB) [30], and transcription factors [31, 32]. Insulin like growth factor-1 (IGF-1) also regulates PI3-kinase and Akt [33], and induces over expression of Bcl-xL and down-regulation of Bax. Akt is activated by PC as a result of PI3-kinase activation leading to the stimulation of PKC and endothelial NO synthase (eNOS) [34]. Because of the suggested role of Akt in cell survival that directly antagonize mitochondrial-directed apoptosis, and because Akt is reported to be regulated by PC, we investigated if caveolin- p38MAPK β interaction mediated survival signal is transmitted through Akt. In this present investigation, as expected PC heart showed high level of expression of Bcl-2, Bcl-xl and phospho-AKT. Cyclodextrin treated heart showed no expression of Bcl-2, Bcl-xl, phospho-AKT, rather showed high expression of JNK, p53 & BAX, (Fig. 8).

In summary, it can be concluded that, PC was not possible to occur without the presence of lipid raft and disintegration of lipid raft abolished PC induced cardioprotection and also blocked the generation of survival signal via caveolin-MAP kinase interaction during preconditioning.

Acknowledgements

This study was supported by NIH HL 22559, HL 33889, HL 33889.

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