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Ischemic preconditioning enhances integrity of coronary endothelial tight junctions

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ABSTRACT

Ischemic preconditioning (IPC) is one of the most effective procedures known to protect hearts against ischemia/reperfusion (IR) injury. Tight junction (TJ) barriers occur between coronary endothelial cells. TJs provide barrier function to maintain the homeostasis of the inner environment of tissues. However, the effect of IPC on the structure and function of cardiac TJs remains unknown. We tested the hypothesis that myocardial IR injury ruptures the structure of TJs and impairs endothelial permeability whereas IPC preserves the structural and functional integrity of TJs in the blood–heart barrier. Langendorff hearts from C57BL/6J mice were prepared and perfused with Krebs–Henseleit buffer. Cardiac function, creatine kinase release, and myocardial edema were measured. Cardiac TJ function was evaluated by measuring Evans blue-conjugated albumin (EBA) content in the extravascular compartment of hearts. Expression and translocation of zonula occludens (ZO)-2 in IR and IPC hearts were detected with Western blot. A subset of hearts was processed for the observation of ultra-structure of cardiac TJs with transmission electron microscopy. There were clear TJs between coronary endothelial cells of mouse hearts. IR caused the collapse of TJs whereas IPC sustained the structure of TJs. IR increased extravascular EBA content in the heart and myocardial edema but decreased the expression of ZO-2 in the cytoskeleton. IPC maintained the structure of TJs. Cardiac EBA content and edema were reduced in IPC hearts. IPC enhanced the translocation of ZO-2 from cytosol to cytoskeleton. In conclusion, TJs occur in normal mouse heart. IPC preserves the integrity of TJ structure and function that are vulnerable to IR injury.

1. Introduction

Myocardial ischemic preconditioning (IPC) is defined as a short period of ischemia/reperfusion protects hearts against a subsequent prolonged period of ischemia/reperfusion (IR) injury [1]. Endogenous substances that coupled to Gi protein have been recognized as triggers, including adenosine, bradykinin, opioids, and sphingosine 1-phosphate. PKCε and PI_3kinase-Akt pathway are involved in downstream signal transduction [2]. Recently, it has been reported that PKCε can cause phosphorylation of Ser 369 at zonula occludens (ZO)-2, one of the tight junction proteins, and promote translocation of ZO-2 from nucleus to plasma membrane in kidney epithelial cells [3]. These observations suggest that PKCε may modulate tight junction structure and function. IPC may preserve the structural and functional integrity of tight junctions.

Tight junctions (TJs) are integral connections that occur between endothelial cells or epicardial cells. The fine structure of TJs was first described at the luminal side of the lateral membrane by using ultrathin-section electron microscopy [4]. TJs, a zipper-like structure, regulate the passage of ions and small molecules through paracellular pathways and are involved in the capillary permeability of numerous organs, such as: brain, retina, liver, lung, and kidney. It is well established that there is a blood–brain barrier resulting from the selectivity of the TJs between endothelial cells in brain blood vessels. Cerebral IPC can protect brain barrier integrity and reduce brain injury [5].

The capillary endothelial cells are situated between blood flow and cardiac myocytes and are essential for stabilizing the inner environment of myocardium. However, the endothelial TJs between blood and heart remain rarely studied. In particular, the role of IPC on TJ structure and function remains unknown. In this study we tested the hypotheses that myocardial IR disrupts coronary endothelial TJs and impairs the endothelial permeability of blood–heart barrier and that IPC maintains the structural and functional integrity of coronary endothelial TJs.

2. Materials and methods

2.1. Animals

All animal procedures adhered to the *Guide for the Care and Use of Laboratory Animals* provided by the Institute of Laboratory

Abbreviations: TJs, tight junctions; ZO-2, zonula occludens-2; EBA, Evans blue-conjugated albumin; IPC, ischemic preconditioning; IR, ischemia/reperfusion injury.

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Animal Resources, National Research Council (6th edition, The National Academies Press, Washington DC, 1996). All animal protocols were approved by the Institutional Animal Care and Use Committee of the South Dakota State University.

All C57BL/6J mice were purchase from Charles River Laboratories and housed in the Animal Research Wing of the South Dakota State University with free access to both food and water and received humane care.

2.2. Reagents

Evans blue dye, albumin, formaldehyde, anti-GAPDH antibody, and anti-flotillin-1 were purchased from Sigma–Aldrich (St. Louis, MO). Anti-ZO-1 antibody was purchased from Invitrogen (Carlsbad, CA). Anti-ZO-2 was purchased from Cell Signaling Tech (Beverly, MA). Anti-Claudin-5 antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Enhanced chemiluminescence was purchased from Amersham Bioscience (Piscataway, NJ). Protein assay kit, pre-stained m.w. standards, mini-format precast SDS-PAGE gel, nitrocellulose membrane were purchased from Bio-Rad (Hercules, CA).

2.3. Langendorff heart perfusion

Male C57BL/6J mice (22–25 g), were anesthetized with phenobarbital sodium (300 mg/kg, i.p.) and heparin (500 U/kg, i.p.). Hearts were rapidly excised, washed in ice-cold arresting solution (NaCl 120 mmol/L, KCl 30 mmol/L), and cannulated via the aorta on a 20 gauge stainless steel blunt needle. Hearts were perfused at 70 mm Hg on a modified Langendorff apparatus using Krebs–Henseleit solution (NaCl 118.5 mmol/L, $NaHCO_3$ 25.0 mmol/L, KCl 4.75 mmol/L, KH_2PO_4 1.18 mmol/L, $MgSO_4$ 1.19 mmol/L, D-glucose 11.0 mmol/L, and $CaCl_2$ 1.41 mmol/L) equipped with 95% O_2 and 5% CO_2at 37 °C as previously described in the literature [6]. Ventricular function was measured by a force displacement transducer (model T03, Grass) attached to the apex of the heart with a thin thread and metal hook. The resting tension was adjusted to 0.30 g [7]. Cardiac contractile force (Fc) and the maximal rate of development of contractile force ($\pm dFc/dt_{max}$), coronary flow, and heart rate were recorded with Biopac MP 100 data recording system (Goleta, CA).

2.4. Ischemic–reperfusion (IR) and ischemic preconditioning (IPC) protocols and Measurement of creatine kinase (CK) release

For IR experiments, the protocol consisted of a 20 min equilibration period, followed by 40 min of global ischemia and 40 min of reperfusion. For IPC [6], hearts were equilibrated for 13 min and then subjected to 2 min of global ischemia and 5 min of reperfusion, followed immediately by prolonged IR as described above. CK release in the coronary effluent was measured according to the manufacturer's instructions (Pointe Scientific, Canton, MI).

2.5. Electron microscope

A subset of cardiac tissues from control, IR, and IPC groups was fixed in 4% formaldehyde–1% glutaraldehyde. After post-fixing in 1% OsO4, the tissue was dehydrated in a series of graded acetone solutions and embedded in Spurr's resin. Thick sections were stained with toluidine blue and selected areas further trimmed and thin sections were prepared in a Reichert–Jung Ultracut E ultramicrotome, post stained with a saturated uranyl acetate solution, followed by lead citrate. Sections were examined with a JEOL JEM 1210 transmission electron microscope (Peabody, MA).

2.6. Determination of permeability of endothelial tight junctions and calculation of myocardial edema

At the end of reperfusion, Evans blue-conjugated albumin (EBA) in Krebs–Henseleit solution (0.2% Evans blue and 1% albumin) was perfused for 1 min at the same speed of coronary flow with Harvard pump, followed by washout with Krebs–Henseleit perfusion buffer for 5 min to remove the residues of EBA in coronary circulation. The wet weight of hearts was determined immediately. Hearts were reweighed after desiccation at 65° for 24 h. Dry hearts were homogenized with PBS buffer. Two volumes of formaldehyde were added to the homogenate. After incubation at 65 °C for 24 h, the homogenate was centrifuged at 5,000 × *g* for 30 min. The optical density of the supernatant was spectrophotometrically determined at both 620 and 740 nm, correcting for contaminating heme pigments by using the formula A_{620} (Evans blue) = $A_{620} - (1.426 \times A_{740} + 0.030)$ [8]. The extravasated EBA content in heart homogenate was calculated against a standard curve.

The percent myocardial edema was calculated as $100 \times [1 - (\text{dry weight/wet weight})]$ [9].

2.7. Cell cytoskeleton fraction preparation

At the end of reperfusion, mouse hearts were placed in liquid nitrogen and stored at −80 °C. The cytosol, membrane, and triton-insoluble fractions were isolated according to previously reported method with minor modifications [10]. In brief, hearts were minced and homogenized in 1.5 ml of TE buffer (10 mM Tris–HCI, pH 7.5; 1 mM EDTA) containing protease inhibitor cocktail (Sigma–Aldrich) with a glass poly-homogenizer. The tissue homogenate was centrifuged at 100*g* for 3 min. The supernatant was then centrifuged for 30 min at 100,000*g*. The cytosol fraction was in the supernatant and saved. The pellet was washed 3 times with TE buffer for 5 min each. The pellet was resuspended in TE buffer containing 1% Triton X-100 and mixed by using 1 ml syringe with 22 gauge needle on ice for 30 min. The mixture was centrifuged again at 100,000*g* for 30 min. The membrane fraction resided in the supernatant. The pellet was washed for 3 times with TE buffer containing 1% Triton X-100 for 5 min each. The pellet was resuspended in 0.5 ml of 1% Laemmli SDS sample buffer, then vortexed and boiled for 5 min, followed by centrifugation at 100,000*g* for 15 min. The cytoskeleton fraction was resided in the supernatant.

2.8. Western blotting

Protein concentration was determined by utilizing bovine serum albumin as a standard according to the method of Bradford (Bio-Rad Laboratories; Hercules, CA). Translocation of tight junction proteins (ZO-1, ZO-2, or Claudin-5) was conducted by utilizing standard Western blot as previously described [11]. Antibodies against ZO-1, ZO-2, or Claudin-5 were utilized to measure the expression of tight junction proteins in cytosolic, membrane, and cytoskeletal fractions. After incubation with corresponding secondary antibodies and washout, immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Amersham Bioscience, Piscataway, NJ) and quantified by densitometric analysis of digitized autoradiograms with NIH Image J software.

2.9. Histology

After 40 min of reperfusion with Krebs–Henseleit solution and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, × 10 min for fixation, hearts were removed from the perfusion apparatus and placed in 4% paraformaldehyde overnight at 4 °C. The heart

was cut into sections and dehydrated in graded acetone washes at 4 °C. Tissue sections were embedded in paraffin, and 5-μm-thick sections were cut. Standard hematoxylin and eosin (H&E) staining was performed and observed microscopically.

2.10. Statistic analysis

Data are mean ± SEM. The significance of the differences in mean values for hemodynamics, CK, edema, and EBA content between groups was evaluated by one-way ANOVA, followed by post hoc testing (Newman–Keuls). $P < 0.05$ was considered significant.

3. Results

3.1. Tight junctions occur between mouse coronary microvascular endothelial cells and are preserved in hearts subjected to ischemic preconditioning

To ascertain the presence of TJs in the coronary circulation of heart and explore the effects of IR or IPC on the structure of TJs, transmission electron microscopy study was performed (Fig. 1). Mice were divided into three groups: normal control (NC), ischemia/reperfusion injury (IR), and ischemic preconditioning (IPC). The results of ultrastructure study of mouse hearts indicated that there were distinct TJs between cardiac microvascular endothelial cells in both freshly prepared intact mouse hearts (*in vivo*) and isolated perfused mouse hearts (*ex vivo*). Myocardial IR injury caused disruption of TJs, and led to swollen mitochondria, and irregularly arrayed myofibrils. In contrast, the ultrastructure of TJs was preserved in hearts that are subjected to IPC.

3.2. Ischemic preconditioning decreases the permeability of coronary endothelial tight junctions

The protocols for IR and IPC were the same ones that we have reported previously with minor adjustment [6]. For this project, we performed the IR and IPC in a Langendorff mouse heart perfusion system. Cardiac contractile force (Fc) and CK release, an index reflecting the death of cardiac myocytes, were measured in both IR and IPC hearts. IPC improved cardiac function (75.2 ± 5.1% of preischemia baseline, *vs* 43.5 ± 9.7% in IR, $P < 0.05$) and an average of 2-fold reduction in the release of CK in the coronary effluent ($P < 0.05$). IR disrupted myofibril structure, induced vacuolization, and caused interstitial edema. IPC prevented these alternations (Fig. 2A).

To determine the permeability of coronary endothelial TJs, freshly prepared EBA was infused into isolated mouse hearts at the end of reperfusion for 1 min followed by washout for 5 min to remove the retained EBA. As shown in Fig. 2B, IR significantly increased extravascular EBA content in the heart tissue (21.44 ± 2.10 μg/ml in IR group, *vs* 9.97 ± 0.55 μg/ml in NC group, $P < 0.05$). Hearts subjected to ischemic preconditioning reduced EBA concentration in the cardiac extravascular tissue (11.87 ± 0.77 μg/ml in IPC group, $P < 0.05$ compared with IR group).

3.3. Ischemic preconditioning reduces myocardial edema

IR significantly increased the wet weight of hearts and myocardial edema (Fig. 3, $P < 0.05$ *vs* NC group). IPC reduced the wet weight of hearts and myocardial edema (Fig. 3, $P < 0.05$ *vs* IR group). There was no difference in dry weight of hearts among NC, IR, and IPC groups.

3.4. Ischemic preconditioning enhances translocation of the tight junction protein ZO-2

TJ protein ZO-2 plays a unique role in the organization and regulation of TJs. ZO-2 is one of the members of a family of membrane-associated guanylate kinase homologs. The subcellular localization of ZO-2 is highly sensitive to the state of cell–cell contact. To demonstrate the translocation of ZO-2, cytosol, membrane, and cytoskeleton fractions were isolated from mouse hearts. Anti-GAPDH was a marker of cytosol whereas anti-flotillin-1 was used as a marker of cytoskeletal fractions. Our results indicated that majority of ZO-2 is distributed between cytosol and triton-insoluble fractions (Fig. 4). IPC increased the translocation of ZO-2 from cytosol to triton-insoluble fractions, while IR reduced the amount of ZO-2 in the triton-insoluble fractions.

4. Discussion

The major finding of this study reveals that ischemia/reperfusion injury ruptures the structural and functional integrity of coronary endothelial tight junctions, coined blood–heart barrier [12], whereas ischemic preconditioning potently protects the integrity of cardiac tight junctions of the blood–heart barrier. These observations are based on the following evidence: (1) TJs were observed between the cardiac microvascular endothelial cells of normal mouse hearts. IR disrupted the structure of TJs. IPC maintained the structural integrity of TJs and protected cardiac function.

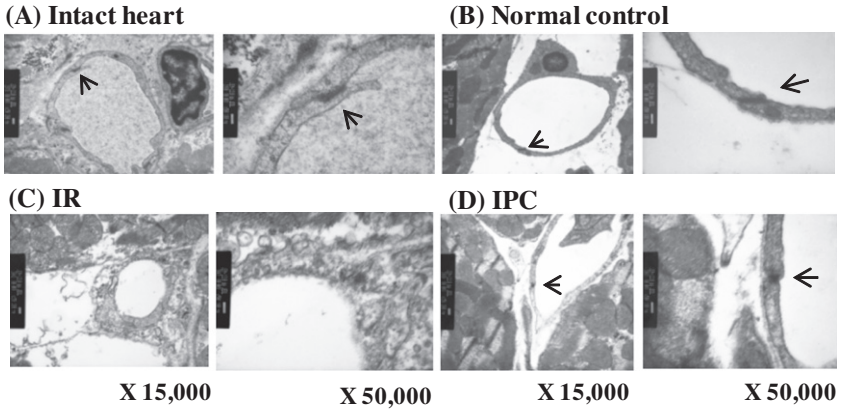

Fig. 1. Representative electronic microscopy images of tight junctions between the cardiac microvascular endothelial cells. (A). Cardiac sections were prepared directly from intactly anesthetized mice without isolated perfusion. (B). Cardiac sections were prepared from isolated hearts perfused with K–H buffer for 120 min. (C). Cardiac sections were prepared from isolate hearts subjected to IR injury. (D). Cardiac sections were prepared from isolated hearts subjected to IPC. Bar Ruler: 200 nm (15,000×); 100 nm (50,000×). Arrow: tight junction.

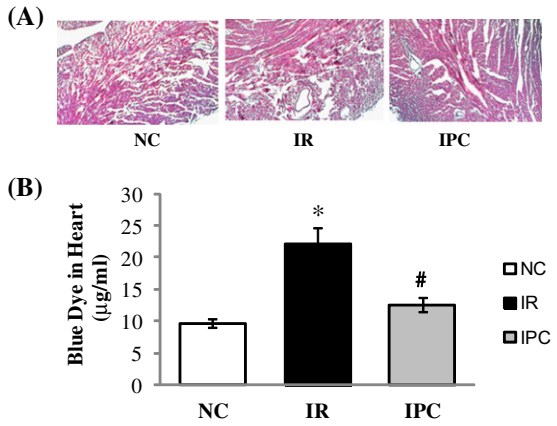

Fig. 2. Effects of IPC on structure and functional permeability of coronary endothelial tight junctions. (A). Representative hematoxylin and eosin (H&E) staining images in NC, IR, and IPC groups. Disarranged myofibril and interstitial vacuole were observed in hearts that were subjected IR injury. IR caused the extensively extravascular permeation of EBA. IPC reduced the staining of EBA. (B). Content of EBA in isolated perfused hearts. The quantifying method was described in Section 2. $n = 4$ per group. $^{*}P < 0.05$ *vs* normal control (NC) group. $^{\#}P < 0.05$ *vs* ischemia/reperfusion (IR) group.

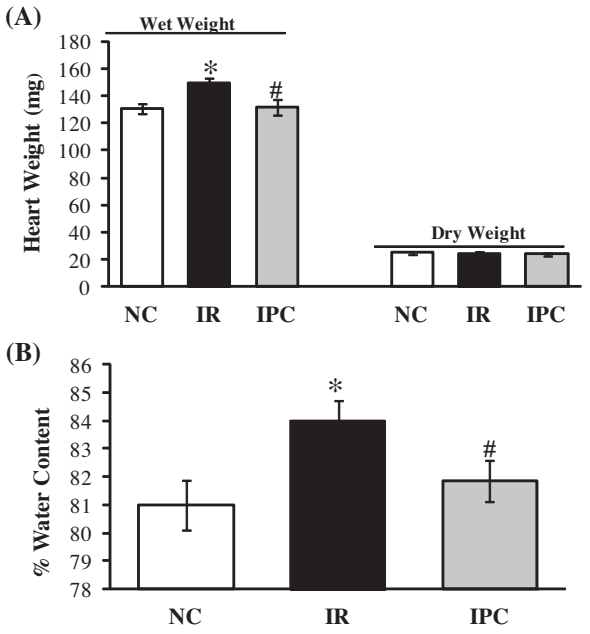

Fig. 3. Effects of IPC on myocardial edema formation. (A). After IR, wet weight of hearts increased significantly. IPC reduced the wet weight of hearts. There was no difference of dry weight among NC, IR, and IPC groups. (B). Water content within IR hearts was increased whereas IPC decreased water content. $n = 4$ per group. $^{*}P < 0.05$ *vs* normal control (NC) group. $^{\#}P < 0.05$ *vs* ischemia/reperfusion (IR) group.

(2) IR increased the permeability of cardiac barriers and caused intramyocardial edema. IR caused the dysfunction of cardiac TJs and cell death. IPC attenuated the functional impairment of TJs and tissue edema. (3) The content of TJ protein ZO-2 in triton-insoluble fractions was decreased in hearts that were subjected to IR. IPC increased the translocation of ZO-2 from cytosol to cytoskeleton.

4.1. Tight junctions in hearts and effects of ischemia/reperfusion injury or ischemic preconditioning on TJs

There are four types of intercellular junctions in mammalian cells: adherens junctions, desmosomes, gap junctions, and tight

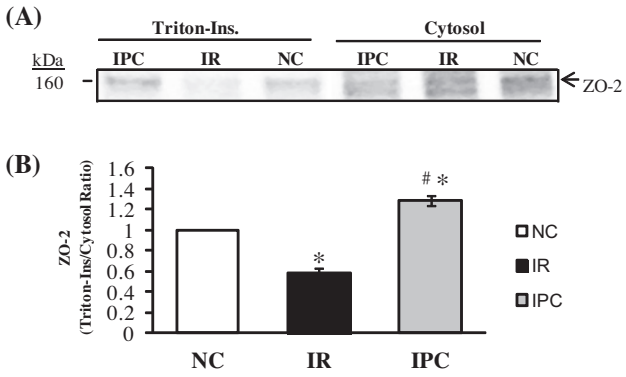

Fig. 4. Translocation of tight junction protein ZO-2 in cytosol, membrane, and triton-insoluble fractions of hearts from IR or IPC group. (A). Representative images of Western blotting incubated with anti-ZO-2 primary antibody. (B). Translocation ratio of ZO-2 from cytosol to triton-insoluble fractions. $^{*}P < 0.05$ *vs* normal control (NC) group. $n = 3$–6 per group. $^{\#}P < 0.05$ *vs* ischemia/reperfusion (IR) group.

junctions. These junctions were originally identified and defined by electron microscopy. It has been recognized that specific types of integral membrane proteins are concentrated in these junctions. C adherins are the major proteins in adherens junctions. A similar adherens junction in cardiomyocytes is named fascia adherens. Desmogleins and desmocollins are the major components of desmosomes. The integral membrane proteins in gap junctions are connexins. Gap junctions are particularly important in cardiac muscle cells. Tight junctions are elements of the endothelial and epithelial junctional complex. The intercellular barrier is formed by rows of the transmembrane proteins occludin and claudins, which are bound on the cytoplasmic surface to ZO-1 and ZO-2. On ultrathin-section electron microscopy, TJs appear as a series of discrete sites of apparent fusion, involving the outer leaflet of the plasma membrane of adjacent endothelial or epithelial cells. Bundgaard reported the TJ structure in endothelium of rat heart capillaries by utilizing serial-section electron microscopy and noted that paracellular pathway in capillary endothelium is permeable not only to small solutes but also to certain small macromolecules less than 4 nm molecular diameter based on the distance between adjacent cells [13]. Many factors govern the permeability of TJs, including lipid molecules around capillaries, net electric charge, and TJ protein affinity, among others. Endothelial junctions of skeletal muscle capillaries are impermeable to tracers larger than microperoxidase (molecular weight 1.9 kDa, 2 nm). In the present study, clear TJ structures were observed in normal hearts. IR impaired the structure of TJs, while IPC maintained the integral structure of TJs.

4.2. Permeability of coronary endothelial tight junctions

Evans blue dye is widely used for quantitative evaluation of blood–brain barrier permeability [14]. The molecular weight of Evans blue is 960.81 Da. Evans blue has high affinity with serum albumin (66.5 kDa). Virtually, no trace of Evans blue is detected in normal brain after intravenous injection. Inflammation and ischemic injury can accelerate the entry of Evans blue into brain through ruptured BBB. Evans blue dye-conjugated albumin (EBA) has been used to assess mouse lung capillary leakage [15]. Disruption of lung endothelial barrier can increase extravascular level of EBA and pulmonary edema [16]. EBA has seldom been utilized for assessment of cardiac capillary permeability. Sutherland et al. indicated that injection of EBA into the femoral veins of anaesthetized rats did not show any significant leakage of EBA into hearts from the vasculature. In contrast to the *in vivo* study, EBA was detected

extensively in the extravascular compartment of hearts [17]. This discrepancy might be related to the less optimal heart perfusion system *ex vivo*. In the present study, a certain amount of EBA was found in extravascular tissue of normal perfused hearts. IR increased the permeation of EBA into extravascular cardiac tissue and produced myocardial edema. IPC significantly attenuated the content of EBA in extravascular tissue and reduced myocardial edema.

4.3. Scaffolding proteins of tight junctions–ZO-2

Like other intercellular junctions, TJs are protein complexes composed of integral membrane proteins, cytoplasmic plaque proteins, and cytoskeletal proteins. Claudins and occludin are transmembrane proteins at the TJs. Through their cytoplasmic domains, they bind to a vast array of cytoplasmic proteins, such as: ZO-1/-2/-3 and cytoskeletal proteins such as actin filaments. Deficiency of ZO-1 is embryonic lethal with massive apoptosis and defect in vascular development [18]. Similarly, it was found that ZO-2 knockout mice were also embryonic lethal with significant apoptosis and enhanced permeability [19]. These studies demonstrate that TJs are vital for cell viability. ZO-1 and -2, two members of the membrane-associated guanylate kinase homologue family of proteins, are cytosolic scaffolding proteins that are critical determinants of barrier formation [20]. Unlike ZO-1, ZO-2 is uniquely restricted to the microvascular endothelial cells of heart [21]. ZO-2 is a target of phosphorylation by PKC [22]. Bryostatin-1, a specific novel PKC activator for PKC-ε, and δ, enhances barrier function and recruitment of ZO-2 into the tight junction complex [23]. PKC-ε activation increases the translocation of new synthesized ZO-2 from nucleus to plasma membrane whereas ZO-2 can also reach the plasma membrane directly from the cytoplasm [3]. It has been shown that overexpression of activated PKC-ε protects heart from IR injury [24], and IPC is abolished in PKC-ε null mice [25]. The present study indicates that IR reduces the distribution of ZO-2 in the TJ fractions. IPC increases the translocation of ZO-2 from cytosol to TJ fractions, which may relate to the enhancement of cardiac endothelial TJ function.

4.4. Coronary endothelial tight junctions: a key interconnection for the survival of cardiac myocytes?

Interplay between cardiac endothelial cells and cardiac myocytes may represent a novel therapeutic target for heart disease [12]. It has been reported that capillary-like networks formed by endothelial cells promotes marked cardiomyocyte reorganization and less apoptosis [26]. The role of TJs in this dynamic process is unclear. We hypothesize that TJs between endothelial cells play a crucial role in the intercellular network and homeostasis of inner environment of cardiac tissue. Disruption of TJs may increase the accumulation of exotic macromolecules released from endothelial cells into cardiac tissue and reduce the survival of cardiac myocytes. Augmentation of endothelial TJ number or function in the heart will minimize the content of "exogenous" molecules and increase the survival of cardiomyocytes. We are testing this hypothesis in a primary cardiac microvascular cell culture model system. In summary, our present studies reveal that myocardial IR ruptures the structure of TJs and impairs the endothelial barrier function whereas IPC preserves the structural integrity of TJs and cardiac endothelial barrier function. The signaling pathway involved in this process requires further exploration.

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