PROTEIN KINASE C DELTA (δ) PEPTIDE ACTIVATOR EXERTS ANTI-INFLAMMATION AND CARDIO-PROTECTIVE EFFECTS

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Abstract—Ischemia followed by reperfusion (I/R) causes additional cell injury. Reperfusion injury is initiated by vascular endothelial dysfunction and/or oxidative stress, which is further augmented by leukocyte (i.e., polymorphonuclear leukocyte [PMN]) activation and recruitment. Protein kinase C (PKC) is a key signaling molecule mediating reperfusion injury. The role of PKC delta (δ) isoform in myocardial I/R injury is still being debated. In this study, we tested the effects of PKC δ peptide activator (PKC δ+) on inflammation and PMN-induced postreperfused cardiac function. We found that PKC δ+ significantly reduced phorbol-12-myristate-13-acetate (PMA, 15 nM)-induced superoxide (SO) release in isolated rat PMNs. Furthermore, PKC δ+ (5 and 10 μM, both n = 5) dose-dependently decreased N^G-nitro-L-arginine methyl ester (L-NAME)-induced leukocyte-endothelial interactions in rat mesenteric microcirculation *in vivo* by intravital microscopy. Lastly, we tested the effects of PKC δ+ on I/R+PMNs-induced postreperfused cardiac dysfunction in isolated perfused rat hearts. We found that PKC δ+ (10 μM, n = 6) significantly attenuated PMN-induced cardiac dysfunction as compared with control I/R+PMNs hearts (n = 10) in left ventricular developed pressure (LVDP), end diastolic pressure (EDP), and the maximal rate of LVDP ($+dP/dt_{max}$; all $p < 0.05$). We also found that PKC δ+ treated postreperfused heart tissue showed significantly lower leukocyte vascular adherence and tissue infiltration. These results suggest that PKC δ+ attenuated PMN-induced post I/R cardiac contractile and diastolic dysfunction, possibly by inhibiting leukocyte-endothelial interactions and attenuating PMN SO release.

Keywords—Ischemia/reperfusion injury; PKC delta isoform; Superoxide; Leukocyte-endothelial interactions; Cardiac function

1. Introduction

Reperfusion of ischemic myocardium can elicit pathologic consequences, such as cardiac arrhythmias, prolonged left ventricular dysfunction, endothelial damage, and myocardial cell injury (Yellon and Hausenloy 2007). Reperfusion injury is characterized by endothelial dysfunction (i.e., decrease in endothelium-derived nitric oxide [NO]), enhanced polymorphonuclear leukocyte (PMN) /endothelial interactions, and PMN extravasation to release reactive oxygen species (ROS) and proteolytic enzymes to induce the injury of vascular endothelium and cardiomyocytes (Lucchesi, Werns et al. 1989, Tsao and Lefer 1990, Shandelya, Kuppusamy et al. 1993, Lefer and Lefer 1996, Duilio, Ambrosio et al. 2001).

Protein kinase C (PKC), a critical signal transduction enzyme, plays an important role in myocardium ischemia/reperfusion (I/R; Young, Balin et al. 2005, Budas, Churchill et al. 2007). Studies show that PKC activation is enhanced during acute myocardial ischemia, particularly during the early reperfusion period (Strasser, Braun-Dullaeus et al. 1992, Churchill and Szweda 2005). Moreover, PKC is an important mediator for PMN activation, such as integrin-dependent PMN adhesion, chemotaxis, and superoxide (SO) release; therefore, inhibition of PKC activity can protect the heart from the PMN-induced reperfusion injury when administrated during reperfusion (Laudanna, Mochly-Rosen et al. 1998, Young, Ikeda et al. 2001, Young, Balin et al. 2005). Several PKC isoforms, such as PKC α, PKC βII, PKC δ, PKC ε, PKC ζ, have been shown to express in vascular endothelium and PMN (Majumdar, Rossi et al. 1991, Young, Balin et al. 2005). Moreover, recent evidence suggests that different PKC isoforms exert different regulation on NO and SO release from endothelium and PMN activation, respectively (Young, Balin et al. 2005, Teng, Kay et al. 2008, Perkins, Pershad et al. 2012).

Moreover, considerable research has suggested that PKC δ is a key enzyme involved in myocardial I/R injury, although the role of PKC δ activation to mitigate MI/R injury is controversial. Among several PKC isoforms, only PKC δ activation and translocation to the plasma membrane have been observed in both transient ischemia and α1-adrenergic preconditioning in isolated rat hearts (Mitchell, Meng et al. 1995). Conversely, ischemic preconditioning can exaggerate myocardial I/R injury in PKC δ null mice (Mayr, Metzler et al. 2004). Furthermore, application of JTV519 prior to ischemia or during early reperfusion can protect the heart from I/R injury also through activating PKC δ (Inagaki, Kihara et al. 2000). By contrast, Chen et al. found that PKC δ peptide activator (PKC δ+) given prior to ischemia increases cardiac damage induced by simulated ischemia in isolated adult myocytes and in an intact heart (Chen, Hahn et al. 2001). Moreover, administration of PKC δ peptide inhibitor during reperfusion improves postreperfusion cardiac function (Inagaki, Hahn et al. 2003). Therefore, further research is needed to clearly elucidate the role of PKC δ in myocardial I/R.

This study utilized PKC δ+ to test the role

of PKC δ on SO release *in vitro* and leukocyte-endothelial interactions *in vivo.* Then the effects of the PKC δ+ on myocardial I/R injury was also tested in a PMN-induced heart I/R injury model. We found that PKC δ+ reduces PMN SO release and leukocyte-endothelial interactions. Furthermore, administration of PKC δ+ significantly improved postreperfused cardiac function accompanied with significantly less leukocyte vascular adherence and infiltration in postreperfused cardiac tissue.

2. Methods

The Institutional Animal Care and Use Committee (IACUC) of Philadelphia College of Osteopathic Medicine approved all animal protocols performed in this study.

2.1 Isolation of PMNs

Sprague-Dawley rats (350-400 g) were used to produce PMNs for SO release and isolated heart experiments. Rats were anesthetized with 3% isoflurane and injected intraperitoneally (i.p.) with 16 ml of 0.5% glycogen (Sigma Chemical Co., St. Louis, MO) dissolved in phosphate buffered saline (PBS). After 16-18 hours, PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (Omiyi, Brue et al. 2005, Phillipson, Peterman et al. 2005). The peritoneal lavage fluid was centrifuged at 250 x g for 20 minute (min) at 4 ºC. The PMNs were then washed in 15 ml of PBS and centrifuged at 250 x g for 10 min at 4 ºC. Thereafter, the PMNs were resuspended in 2.5 ml of PBS and stored at 4 ºC for later experiments. The PMN preparations were > 90% pure and > 95% viable, according to microscopic analysis and exclusion of 0.3% trypan blue, respectively.

2.2 Measurement of SO radical release from rat PMNs

The SO anion release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech, El Cajon, CA) by the reduction of ferricytochrome C (Young, Ikeda et al. 2001). The PMNs (5×10^6) were resuspended in 450 μl PBS and mixed with ferricytochrome C (100 μM, Sigma Chemical Co., St. Louis, MO). PKC δ+ (0.5-20 μM) or a PKC δ inhibitor, rottelin (20 μM), was added to the PMN/ferricytochrome C suspension and mildly vortexed. The total volume of the suspension was 900 μl and was incubated for 15 min at 37 ºC in spectrophotometric cells. Control samples did not contain PKC δ+. The PMNs were stimulated with 15 nM phorbol 12-myristate-13-acetate (PMA, Sigma Chemical Co., St. Louis, MO) in a final reaction volume of 1.0 ml. Positive control samples were given superoxide dismutase (SOD; 10 μg/ml) just prior to addition of PMA. Absorbance at 550 nm was measured every 30 second (sec) up to 360 sec (peak response) and the change (Δ) in absorbance from PMNs was determined from time zero (Phillipson, Peterman et al. 2005, Young, Balin et al. 2005).

2.3 Measurement of rat mesenteric leukocyte-endothelial interactions by intravital microscopy

Leukocyte-endothelial interactions were recorded as previously described (Chen, Rueter et al. 2010). Briefly, male Sprague-

Dawley rats (weighing 275-325 g) were anesthetized using 60 mg/kg of sodium pentobarbital i.p., and the left carotid artery was cannulated for monitoring of mean arteriolar blood pressure (MABP). After abdominal laparotomy, a loop of ileal mesentery was exteriorized and placed in a temperature controlled Plexiglas chamber (37 °C) for adequate superfusion of the test solutions. The mesentery was placed over a Plexiglas pedestal in the observation chamber for visualization under a Nikon Eclipse microscope (Nikon Co., Japan). The microcirculation was recorded with Image Pro, MDA (Media Cybernetics, Bethesda, MD) and leuckocyte-endothelial interactions were analyzed offline.

The rats were allowed to stabilize for 30 min with superfusion of Krebs buffer after surgery. After stabilization, a nonbranched postcapillary venule was chosen for observation. A baseline recording was made to establish basal values for leukocyte rolling, adherence, and transmigration. The mesentery then was superfused with the experimental test solutions for 120 min. Test solutions included Krebs buffer alone, 50 μM L-NAME alone, and 50 μM L-NAME in the presence of PKC δ+ (5 or 10 μM). All the drugs were dissolved into the Krebs buffer. The Krebs buffer is composed of the following (in mmol/l): 17 dextrose, 120 NaCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 EDTA, 5.9 KCl, and 1.2 $MgCl_2$. The solution was aerated with 95% N_2-5% CO_2 equilibrated at a pH of 7.3 to 7.4 at 37 °C. Two min video recordings were made at baseline, and then 30, 60, 90, and 120 min after superfusion of the test solutions for quantification of leukocyte rolling, adherence, and transmigration as previously described (Chen, Rueter et al. 2010).

2.4 PMN-Induced Myocardial I/R Injury
2.4.1 Isolatin of Plasma

Blood was collected from the aorta in citrate phosphate buffer (Sigma Chemical Co., St. Louis, MO) over a period of 1 min just before isolation of the rat heart. The blood was centrifuged at 10,000 x g for 10 min. Then the plasma decanted, and 5 ml of plasma collected from a single rat was used for the same rat heart experiment.

2.4.2 PMN-Induced Myocardial I/R Injury Model

The isolated rat Langendorff heart was prepared as previously described (Omiyi, Brue et al. 2005, Perkins, Pershad et al. 2012). The isolated heart was perfused with a modified Krebs buffer with 95% O_2-5% CO_2 at 37 ºC by a constant pressure of 80 mmHg. The two side arms in the perfusion line proximal to the heart inflow cannula allowed PMNs (200 X 10^6) and plasma with/without PKC δ+ (1, 5, 10 μM) to be directly infused into the coronary inflow line. Left ventricular developed pressure (LVDP, defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure [EDP]) and the maximal rate of LVDP ($+dP/dt_{max}$) were monitored using a pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX), which was positioned in the left ventricular cavity. Coronary flow was monitored by a flowmeter (T106, Transonic Systems, Inc., Ithaca, NY) in the perfusion line. Coronary flow, LVDP, and $+dP/dt_{max}$ were recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO) in conjunction with a computer (Gateway).

Figure 1 illustrates a schematic diagram of

the protocol for I/R experiments in the isolated perfused rat heart. After 15 min of stabilization of the heart to obtain the baseline cardiac function, the flow of the Krebs buffer was reduced to zero for 20 min to induce global ischemia. Throughout the entire ischemic period, the heart remained immersed in a water-jacketed reservoir containing 37 ºC Krebs buffer, and the temperature was maintained by a circulating water bath (ThermoHaake P5, Fisher Scientific, Pittsburgh, PA). At reperfusion of Krebs buffer, some hearts were infused with PMNs and/or different concentrations of PKC δ+ dissolved in plasma for the first 5 min of reperfusion. LVDP, $+dP/dt_{max}$, and coronary flow were measured and recorded every 5 min during the whole protocol. After each experiment, the left ventricle was isolated, fixed in 4% paraformaldehyde, and stored at 4 °C for subsequent histological analysis.

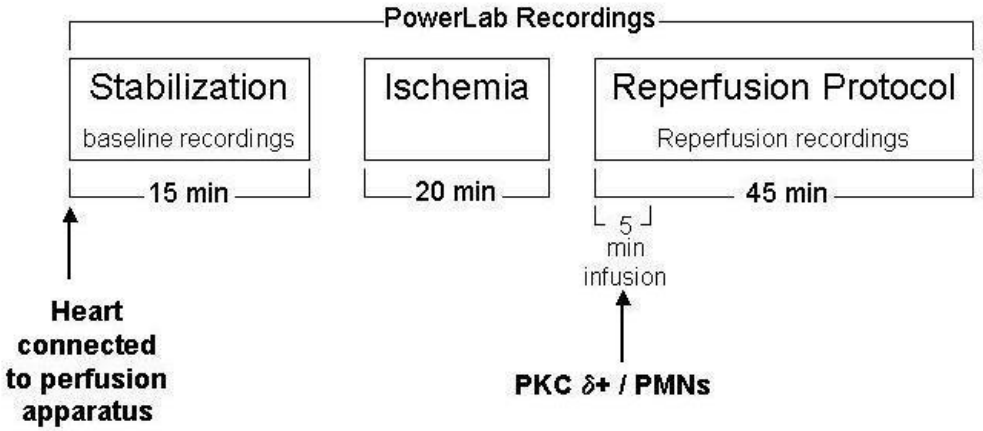

Figure 1. The diagram for PMN-induced myocardial I/R experiments.

2.4.3 Groups of isolated perfused hearts

Eight experimental groups were included: (a) Sham I/R hearts (n=6) were not subjected to ischemia and were not perfused with PMNs, but received plasma at 35 min perfusion (i.e., the same time that I/R hearts received plasma). This sham group is used to show that cardiac function will remain unchanged throughout the 80 min protocol. Moreover, previous studies showed that sham I/R hearts given PMNs exhibited no changes of cardiac function from initial control values (Phillipson, Peterman et al. 2005). (b) I/R hearts (n=6) were reperfused with plasma without PMNs at a rate of 1 ml/min for the first 5 min of reperfusion. These hearts represent a control group to show that 20 min of ischemia followed by 45 min of reperfusion does not result in sustained cardiac contractile dysfunction. (c and d) In some sham and I/R hearts, 10 μM PKC δ+ (Genemed Synthesis, Inc., San Francisco, CA) were dissolved in

plasma and infused at a rate of 1ml/min for 5 min after 35 min of perfusion (sham) or at the beginning of reperfusion (I/R) (both n=6). (e) The control I/R+PMN heart group (n=10) was given the 200 x 10^6 PMNs (resuspended in 5 ml of Krebs buffer) plus 5 ml of plasma for 5 min at a rate of 1 ml/min at the beginning of reperfusion. This group shows sustained cardiac contractile dysfunction throughout reperfusion. (f, g, and h) The different concentrations of PKC δ+ (1, 5, 10 μM) were dissolved in plasma and reperfused with the PMNs at the beginning of reperfusion to test the effect of PKC δ+ on PMN-induced reperfusion cardiac dysfunction (n=6 for all three groups).

2.4.4 Determination of PMNs vascular adherence and infiltration into the cardiac tissue

Three rat hearts from each of the 8 experimental groups were used for histological analysis. The hearts chosen represented those in which the values in cardiac function were closest to the group mean for all control and treatment groups. After hematoxyline&eosin (H&E) staining, ten similar areas of each rat heart, ranging from the endocardium throughout the myocardium to the epicardium of the left ventricle, were counted for PMN vascular adherence and infiltration and expressed as PMNs/mm^2 area of cardiac tissue.

The selective PKC δ+ (Myr-MRAAEDPM, M.W. = 1,130, Genemed Synthesis) is derived from receptors for activated C kinase (RACK)-like PKC δ sequence. It can enhance PKC δ binding to RACK and translocation to membrane to act with its cellular substrates (Chen, Hahn et al. 2001). On the other hand, this PKC δ+ is myristolated (fatty acid moiety) to allow for rapid cell permeability (within 10 sec) (Omiyi, Brue et al. 2005, Phillipson, Peterman et al. 2005). L-NAME, SOD, and rottlerin were purchased from Sigma (St. Louis, MI). Other chemicals used in making Krebs buffer were obtained from Fisher Scientific Co. (Pittsburgh, PA).

2.5 Statistical Analysis

All data in the text and figures are presented as means ± SEM. The data were analyzed by ANOVA using post hoc analysis with the Bonferroni/Dunn test. Probability values of <0.05 were considered to be statistically significant.

3 Results

3.1 PKC δ+ exerted inhibition of SO release in isolated rat PMNs

We tested the effect of PKC δ+ on SO release from PMA-stimulated rat PMNs. As seen in figure 2, control SO release (i.e., change in absorbance) induced by 15 nM PMA from rat PMNs was 0.47 ± 0.03. Comparing with control (PMA only), only 5 μM and 10 μM PKC δ+ significantly inhibited SO release by 32 ± 11 % (both $p < 0.05$). By contrast, PKC δ inhibitor, rottlerin (20 μM), significantly increased PMA-stimulated SO release by 79 ± 13 % ($p < 0.01$). SOD (10 μg/ml), was used as a negative control in the SO assays, and scavenged SO release from PMA-stimulated rat PMNs by 94 ± 4 % ($p < 0.01$).

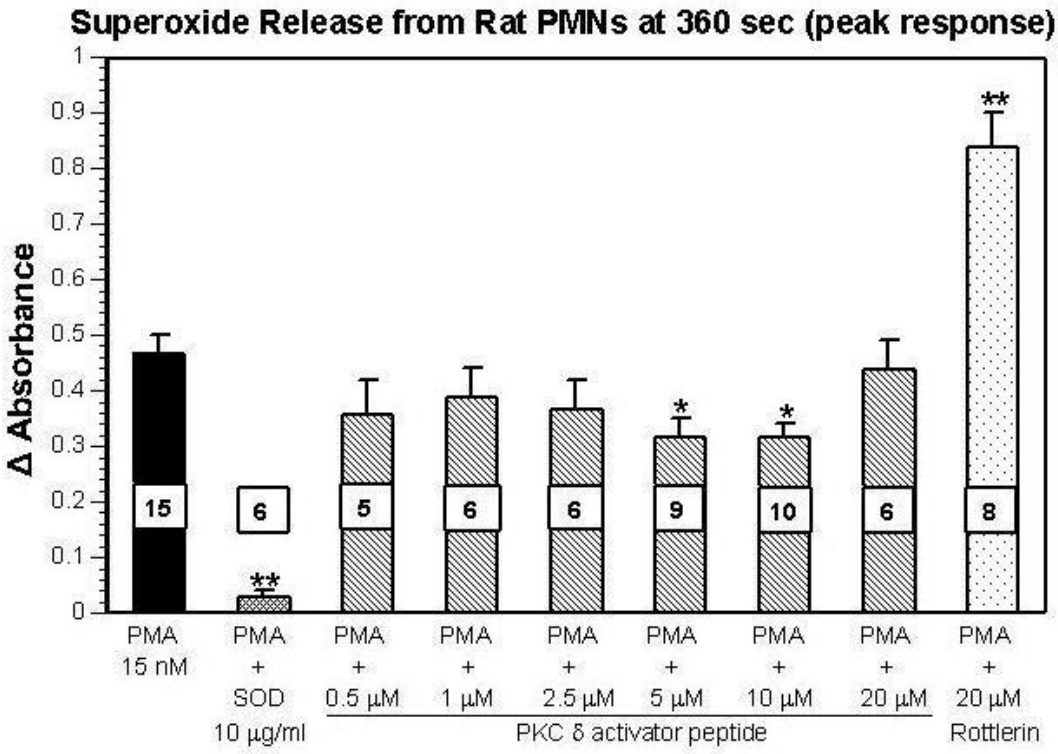

Figure 2. The effects of PKC δ+ and PKC δ inhibitor on SO release from isolated PMNs. PKC δ+ (5 and 10 μM) significantly reduced PMA-induced PMNs SO release, whereas a PKC δ inhibitor, rottlerin, significantly increased SO release. * $p < 0.05$, ** $p < 0.01$ vs PMA.

3.2 PKC δ+ exhibited anti-inflammatory effects

We further investigated the effect of PKC □+ on leukocyte-endothelial interactions in rat mesenteric post-capillary venules via intravital microscopy. We found that there was no significant difference in the basal leukocyte-endothelial interactions, including leukocyte rolling, adherence and transmigration among different experimental groups. Moreover, superfusion of Krebs buffer did not significantly change leukocyte-endothelial interactions during the 120 min observation period (see figure 3). The leukocyte-endothelial interactions at 2 hours in Krebs group were 16 ± 6 cells/min, 2 ± 1 cells/100 μm, and 1 ± 1 cells/20 X 100 μm^2 for rolling, adherence and transmigration, respectively. By contrast, 50 μM L-NAME induced a significant increase in leukocyte-endothelial interactions from 30-60 min, and this increase lasted for the rest of the observation period (see figure 3). At 2 hours of superfusion, leukocyte rolling, adherence, and transmigration in L-NAME group were 82 ± 11 cells/min, 22 ± 5 cells/100 μm, and 21 ± 4 cells/20 X 100 μm^2, respectively, which were significantly higher compared to Krebs group (all $p < 0.01$, figure3). These data suggest that

reduced NO production by using NO synthase inhibitor (i.e., L-NAME) can induce increased leukocyte-endothelial interactions in rat mesenteric postcapillary venules. Conversely, PKC □+ dose-dependently reduced L-NAME induced leukocyte-endothelial interactions. After 2 hours of superfusion, 5 μM PKC □+ reduced L-NAME-induced leukocyte rolling, adherence, and transmigration to 41 ± 8 cells/min, 12 ± 2 cells/100 μm, and 7 ± 2 cells/20 X 100 μm^2 respectively. A higher dose of PKC □+ (i.e., 10 μM) exerted more inhibition on L-NAME induced leukocyte rolling (11 ± 1 cells/min), vascular adherence (2 ± 1 cells/100 μm), and tissue infiltration (2 ± 1 cells/20 X 100 μm^2; all $p < 0.01$ vs L-NAME).

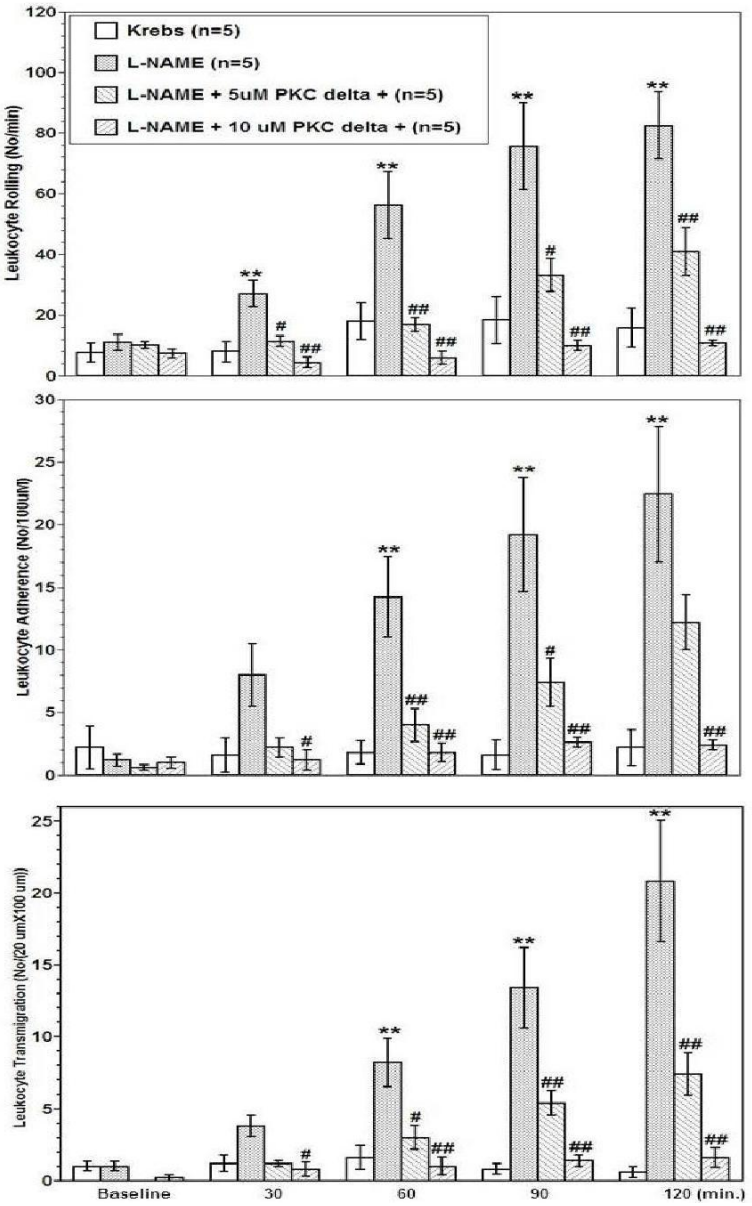

Figure 3. The inhibitory effects of PKC δ activator on L-NAME induced leukocyte rolling (top panel), adherence (middle panel), and transmigration (bottom panel). ** $p < 0.01$ vs Krebs control; # $p < 0.05$, ## $p < 0.01$ vs L-NAME group.

3.3 PKC δ+ exhibited cardio-protection in isolated rat heart

Figure 4 showed the initial and final values for LVDP and +dP/dt$_{max}$ in the different experimental groups. Initial baselines of LVDP and +dP/dt$_{max}$ were similar for all groups in this study. As shown, the sham I/R (n=6) remained near 100% of initial baseline values for LVDP and +dP/dt$_{max}$. I/R (n=6) hearts recovered to 82 ± 4% and 74 ± 7% of initial LVDP and +dP/dt$_{max}$, respectively, by the end of the 45 min postreperfusion. By contrast, final LVDP and +dP/dt$_{max}$ at 45 min postreperfusion was significantly decreased compared to initial baseline in the I/R+PMNs group (both $p < 0.01$). These hearts only recovered to 49 ± 8% and 45 ± 8% of initial baseline values for LVDP and +dP/dt$_{max}$, respectively. The I/R hearts without PMNs did not result in prolonged severe cardiac contractile dysfunction comparing with I/R+PMNs group, indicating that global ischemia for only 20 min could not cause sustained contractile dysfunction in this model of I/R. In contrast, PKC δ+ dose-dependently attenuated cardiac contractile dysfunction induced by I/R+PMNs. LVDP in 1, 5, 10 μM PKC δ+ treated hearts was improved to 61 ± 5%, 80 ± 9%, and 82 ± 3% of initial values, respectively. Similarly, +dP/dt$_{max}$ at 45 min postreperfusion were 54 ± 4%, 67 ± 8%, and 79 ± 5% of initial values for 1, 5, 10 μM PKC δ+ - treated I/R+PMNs hearts, respectively. Comparing with I/R+PMNs hearts, only 10 μM PKC δ+ significantly restored both LVDP and +dP/dt$_{max}$ (both $p < 0.01$, figure 4).

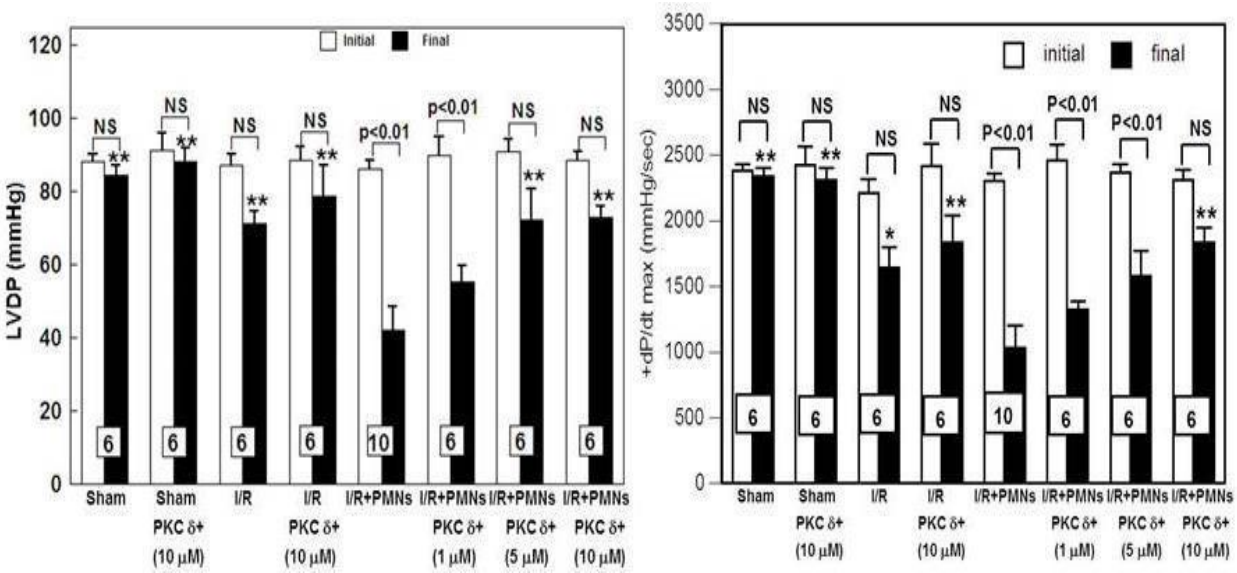

Figure 4. The comparison of initial and final LVDP (left panel) and +dP/dt$_{max}$ (right panel) among the eight experimental groups. I/R+PMNs hearts showed significantly compromised LVDP and +dP/dt$_{max}$ compared to its initial baselines. By contrast, PKC δ+ dose-dependently improved LVDP and +dP/dt$_{max}$ compared to those in I/R+PMNs hearts. ** $p < 0.01$ vs final value in I/R+PMN heart.

To determine whether the PKC δ+ produced direct inotropic effects on cardiac contractile function, nonischemic sham I/R hearts (n=6) were perfused with PKC δ+ (10 μM). We did

not find any significant changes in LVDP or $+dP/dt_{max}$ during the 80 min protocol (figure 4); which demonstrated that the PKC δ+ did not exert any direct effect on cardiac contractile functioning.

Additionally, I/R hearts treated with PKC δ+ (10 μM) recovered similarly to untreated I/R hearts (figure 4), further indicating that PKC δ+ exerted no direct effect on cardiac contractile function, even in the setting of I/R without PMN infusion.

The time course of cardiac contractile function (i.e., LVDP) and diastolic function (i.e., EDP) data from the sham I/R, I/R, I/R+PMNs and I/R+PMNs+ PKC δ+ (10 μM) groups are illustrated in Figure 5. As shown, the sham I/R (n=6) remained near 100% of initial LVDP for the entire perfusion period. I/R (n=6) hearts experienced a depression of 39 ± 4% in LVDP during the first 5 minutes of reperfusion, but eventually recovered to 82 ± 4% of initial baseline values by the end of the 45-minute postreperfusion. However, the I/R+PMNs (n=10) hearts experienced sustained cardiac contractile dysfunction, recovering to only 49 ± 8% of initial baseline values by the end of reperfusion. In contrast, the I/R+PMNs+ PKC δ+ (10 μM) hearts (n=6) significantly recovered to 52 ± 11% of initial baseline values at 10 min postreperfusion by comparing with that of I/R+PMNs group (19 ± 5%, $p < 0.01$). LVDP of these hearts significantly recovered better throughout the rest postreperfusion and reached to 82 ± 3% of initial baseline at end of reperfusion. Moreover, the LVDP time courses from 10-45 min of postreperfusion between I/R group and I/R+PMNs+ PKC δ+ (10 μM) group were almost overlaid, which indicated that PKC δ+ (10 μM) almost fully prevented the deleterious influence of PMNs on cardiac contractile function from a very early time point of reperfusion.

PKC δ+ (10 μM) also significantly improved postreperfused diastolic function as illustrated in figure 5. As shown, the sham I/R hearts (n=6) maintained the normal and low EDP (7 ± 2 mmHg) through the whole 80 min experimental procedure. EDP in I/R hearts (n=6) increased to 36 ± 5 mmHg at 5 min of reperfusion, and gradually decreased to 28 ± 4 mmHg at 45 min of reperfusion. By contrast, I/R+PMNs hearts sustained the high EDP through the whole 45 min reperfusion, from 57 ± 6 mmHg (5min) to 46 ± 6 mmHg (45 min). On the contrary, PKC δ+ (10 μM) treatment given for the first 5 min of reperfusion significantly reversed the severe high EDP induced by I/R+PMNs from 10 min of reperfusion (31 ± 10 mmHg, $p < 0.01$) to the end of reperfusion (19 ± 7 mmHg, $p < 0.01$). On the other hand, it was noticeable that the EDP time course in I/R+PMNs+ PKC δ+ (10 μM) hearts showed a better recovery trend than that of I/R hearts (figure 5).

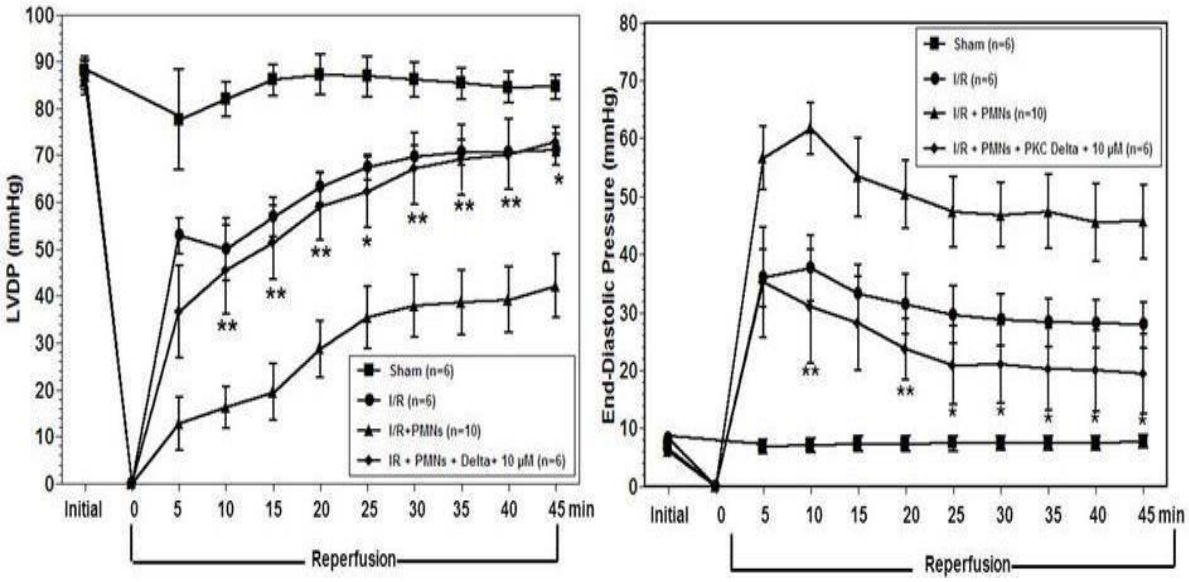

Figure 5. The comparison of time course of LVDP (left panel) and EDP (right panel) among the sham, I/R, I/R+PMNs, and I/R+PMNs+ PKC δ+ (10 μM). I/R+PMNs hearts showed compromised LVDP and EDP. By contrast, PKC δ+ (10 μM) significantly improved LVDP and EDP from 10 min of reperfusion and maintained for the rest of reperfusion compared to those in I/R+PMNs hearts. (* $p < 0.05$; ** $p < 0.01$ illustrates significance between I/R+PMNs hearts and I/R+PMNs+ PKC δ+ 10 μM).

3.4 PKC δ+ dose-dependently reduced adhered and transmigrated PMNs in postreperfused heart tissue

In this I/R model, cardiac contractile dysfunction was correlated with an increase in the number of PMNs infiltrating the myocardial tissue within the 45 min reperfusion period. Upon reperfusion, a significant number of PMNs transmigrated into the myocardium, increasing from 25 ± 2 PMNs/mm^2 in sham I/R hearts to 158 ± 12 PMNs/mm^2 in I/R+PMNs hearts. Application of PKC δ+ dose-dependently attenuated the number of infiltrated PMNs in reperfused left ventricle, showing 63 ± 3% (1 μM), 77 ± 4% (5 μM) and 83 ± 2% (10 μM) reduction compared to that in I/R+PMNs hearts, respectively (all $p < 0.01$, figure 6). Furthermore, we found that PKC δ+ dose-dependently reduced the number of adherent PMNs to the vascular endothelium, in comparison to untreated I/R+PMNs hearts. The 5 μM and 10 μM PKC δ+ treated I/R+PMNs hearts experienced a 64 ± 9% and 82 ± 9% reduction in the number of adherent PMNs, respectively (both $p < 0.01$, figure 6).

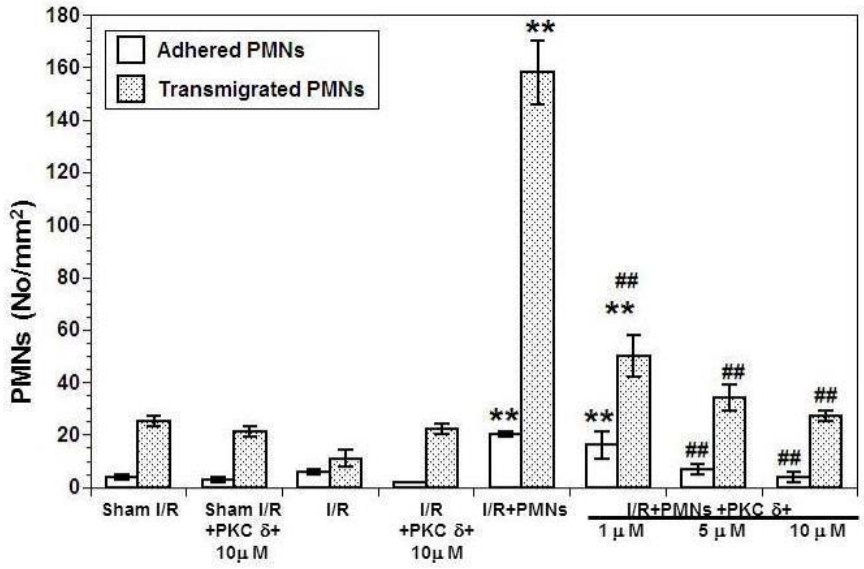

Figure 6. Histological assessments of adhered and transmigrated PMNs in postreperfused heart tissue among eight experimental groups. I/R+PMNs hearts exhibited significant increase of adhered and transmigrated PMNs. By contrast, PKC δ+ treated I/R+PMNs hearts showed a dose-dependent reduction in adhered and transmigrated PMNs. ** $p < 0.01$ from sham I/R; ## $p < 0.01$ from I/R+PMNs.

4. Discussion

4.1 Summary of Major Findings

This study shows: 1) PKC δ+ significantly attenuated SO release from isolated rat PMNs; Moreover, 2) PKC δ+ exhibited anti-inflammatory effects by reducing L-NAME induced leukocyte-endothelial interactions; 3) PKC δ+ dose-dependently improved the postreperfusion cardiac function (i.e., LVDP, EDP, and + dP/dt_{max}), especially 10 μM PKC δ+ significantly enhanced the postreperfusion cardiac function from 10 min of reperfusion and maintained for the rest of reperfusion compared to those of I/R+PMN hearts; 4) I/R+PMN+PKC δ+ treated hearts showed significant attenuation of intravascular PMN adherence and transmigration in postreperfused heart tissue in a dose-dependent manner.

4.2 Role of PKC δ in inflammation

PKC activation mediates MI/R injury by regulating various events, in particular, leukocyte-mediated inflammation following reperfusion. During MI/R, leukocytes are activated by ROS, pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-8, and complement fragment C5a (Arumugam, Shiels et al. 2004, Vinten-Johansen 2004). Activated PMNs can cause endothelial and myocardium dysfunction by releasing cytotoxic substances, such as SO, when they are adherent to vascular endothelium and

infiltrate into the myocardium. Moreover, activated PMNs contribute to reperfusion no-reflow phenomenon by its plugging effects and tight junction loss at the vascular endothelial barrier. Therefore, increased PMNs infiltration into heart tissue directly correlates with larger infarct size and compromised cardiac function (Omiyi, Brue et al. 2005, Phillipson, Peterman et al. 2005, Perkins, Pershad et al. 2012). We have shown that inhibition of PKC by a broad-spectrum inhibitor, Gö 6983, completely reduced SO production from PMA-stimulated PMNs. Moreover, Gö 6983 significantly improved postreperfused cardiac function accompanied with reduced PMN vascular adherence and tissue infiltration in a PMN-induced MI/R injury model (Peterman, Taormina et al. 2004).

It is well-known that PKC includes classical (α, βI, βII, γ), novel (ε, δ, θ, η), and atypical (ζ, λ/ι) isozymes. These isoforms have different cellular locations and may have different roles in myocardial I/R injury (Young, Balin et al. 2005, Churchill, Budas et al. 2008). Five isoforms-PKC α, PKC βI, PKC βII, PKC δ, and PKC ζ, are expressed on PMNs (Korchak, Rossi et al. 1998). PMA can activate all of 5 PKC isoforms and cause NADPH oxidase activation resulting in SO production. Inhibition of PKC βII and PKC ζ using selective PKC βII and PKC ζ peptide inhibitors can reduce PMA-induced SO production by 50% and 40%, respectively (Omiyi, Brue et al. 2005, Phillipson, Peterman et al. 2005, Young, Balin et al. 2005). In this study, we found that a selective PKC δ activator peptide inhibits PMA-stimulated SO release by 32 ± 11 %. By contrast, a PKC δ inhibitor, rottlerin, increased PMA-stimulated SO release by 79 ± 13 %. Our data suggest that PKC δ negatively regulates NADPH oxidase activity in isolated PMNs.

We further confirm the anti-inflammatory effects of PKC δ+ by demonstrating the significant reduction in L-NAME induced leukocyte-endothelial interactions in mesenteric microcirculation when PKC δ+ was applied with L-NAME together. L-NAME is a nonselective NO synthase inhibitor, and it simulates vascular endothelial dysfunction which is characterized by decreased endothelial-derived NO. Dysfunctional vascular endothelium in turn induces leukocyte-endothelial interactions, including increased leukocyte rolling, vascular adherence and tissue infiltration. We found that PKC δ+ dose-dependently reduced L-NAME induced inflammatory responses. This anti-inflammatory effect of PKC δ+ may be partially related to the reduction of SO production from activated PMNs.

Additionally, PKC δ activation may desensitize TNF-α receptor during the inflammation process. TNF-α is one of key pro-inflammatory cytokines promoting leukocyte adherence and infiltration. Kilpatrick LE et al. showed that both PMA and TNF-α can cause serine phosphorylation of TNF-α receptor, which further desensitizes PMN degranulation and reduces release of proinflammatory mediators. In particular, PKC δ is the only PKC isoform initiating this negative feedback loop (Kilpatrick, Song et al. 2000). Therefore, using PKC δ+ can attenuate leukocyte adherence/transmigration and decrease the respiratory burst of SO from activated

neutrophils by phosphorylating TNF-α receptor. Furthermore, cyclooxygenase (COX) may also be another substrate of PKC δ (Kwak, Park et al. 2010). Activation of PKC δ can enhance COX activity and further reduce the release of leukotrienes (Mao, Tsu et al. 2004), which can slow down the process of inflammation.

4.3 Mechanisms related to the cardioportection of PKC δ+ in I/R injury

Many studies indicate that inhibition of leukocyte-endothelial interactions and reduction of leukocyte SO release can mitigate I/R injury. Our study fully supports this idea by showing PKC δ+ significantly improved postreperfused cardiac function in a PMN-induced myocardial I/R injury model. In this *ex vivo* MI/R model, sham I/R hearts perfused with PMNs does not result in cardiac contractile dysfunction (Peterman, Taormina et al. 2004). Moreover, the I/R group which had 20 min of global ischemia following 45 min reperfusion only showed transient and reversible postreperfused cardiac dysfunction. However, in this setting of I/R, reperfusion of PMNs can induce a significant decrease in LVDP and $+dP/dt_{max}$ during the entire 45 min postreperfusion. Therefore, this PMNs+I/R injury heart model emphasizes the role of PMNs in exacerbation of I/R injury. Although PMN infiltration into postreperfused heart tissue requires a longer time for *in vivo* myocardial I/R model, the time course of endothelium dysfunction and PMN/endothelial interaction is similar in the *ex vivo* and *in vivo* MI/R model.

In this study, 10 μM PKC δ+ treated I/R+PMNs hearts had maximal restoration of postreperfusion systolic and diastolic cardiac functions, showing no significant difference between initial and final (45-min postreperfusion) LVDP, EDP, and $+dP/dt_{max}$, values respectively. Moreover, all cardiac function indexes in this group of hearts were significantly higher than those in I/R+PMN hearts. This group of hearts also exhibited identical or better recovery in LVDP and EDP postreperfusion time course relative to I/R control. Additionally, PKC δ+ (10 μM) did not elicit cardiodepressant or cardiotonic effects on normal and I/R cardiac contractile function. Hearts treated with 5 μM PKC δ+ also had significant recovery in LVDP, but not $+dP/dt_{max}$. Comparably, 1 μM PKC δ+ provided minimal cardioprotection, the postreperfusion cardiac contractile function was not significantly different from that of I/R+PMN hearts. Furthermore, leukocyte vascular adherence and tissue infiltration also showed dose-dependent reduction in PKC δ+ treated postreperfused tissue. It is noticeable that the dose-dependent cardioprotective effects of PKC δ+ is very closely related to the dose-dependent effects of PKC δ+ on L-NAME induced leukocyte-endothelial interactions and on PMN-SO release. Therefore, the cardioprotective effects of PKC δ+ is possibly mediated by inhibiting postreperfused inflammation and SO release from PMNs.

Our data are consistent with the study by Inagaki et al. showing that JTV519 exerted cardioprotection specifically by translocation of PKC δ to the cell membrane (Inagaki, Kihara et al. 2000). However, Chen et al. found that PKC δ+ (i.e., 1 μM) given for 10 min before

ischemia caused increased cardiac damage in isolated rat myocytes (Chen, Hahn et al. 2001). The divergence of their and our studies is possibly due to different time and dose of applying PKC δ+ (1 vs. 10 μM), as well as different myocardial I/R injury model. PKC δ+ may exert cardioprotection by other possible mechanisms. Activation of PKC δ mediates ischemia preconditioning (Zhao, Renner et al. 1998). In PKC δ knockout mice, ischemia preconditioning can exacerbate the heart damage (Mayr, Metzler et al. 2004). Studies show that ischemic preconditioning activates PKC δ, then PKC δ translocates to the mitochondria to open mitochondrial ATP-dependent potassium channels to increase the tolerance of myocytes to prolonged ischemia. It has been indicated that mitochondrial ATP-dependent potassium channel transient opening is the final common path for ischemic preconditioning (Wang, Hirai et al. 1999). Recently, researchers also disclose the existence of ischemic postconditioning, which also enhances the ischemia tolerance of the heart after introducing a few transient I/R cycles at the beginning of reperfusion (Zhao and Vinten-Johansen 2006). Furthermore, ischemia postconditioning may share the same signaling path as ischemia preconditioning (Hausenloy and Yellon 2009, Zhang, Zhao et al. 2011). In this study, we applied PKC δ+ only for 5 min when reperfusion started. The cardioprotection of PKC δ+ may suggest that PKC δ activation may simulate ischemia postconditioning by transient opening of mitochondrial ATP-dependent potassium channels (Wang, Hirai et al. 1999). However, Churchill EN et al. found that ischemia for 30 min followed by reperfusion for 15 min stimulated PKC δ translocation to the mitochondria within the first 5 min of reperfusion and remained there for 60 min (Churchill and Szweda 2005). The prolonged translocation of PKC δ induces SO production from mitochondria and cell apoptosis. It has been suggested that prolonged opening of ATP-dependent potassium channel can collapse the mitochondria membrane potential and result in mitochondria dysfunction (Pomerantz, Robinson et al. 2000). It is still unclear about the role of PKC δ in cell apoptosis. Yoshida showed PKC δ activation induces DNA damage and cell apoptosis (Yoshida 2007). However, Liao suggests that rottlerin, a PKC δ inhibitor, can induce mitochondrial membrane depolarization and initiate cell apoptosis (Liao, Hung et al. 2005).

PKC δ also translocates to the cell membrane, intercalated disc, and nucleus after ischemia preconditioning. The translocation may further regulate other rate-limiting enzymes and gene transcription to protect the heart (Nishizuka 1992, Mitchell, Meng et al. 1995). Additionally, PKC δ is involved in cardiomyocyte metabolism. PKC δ null cardiomyocytes shift the energy usage from glucose to lipid metabolism, which predispose myocytes to be vulnerable to ischemia and result in more heart tissue damage after ischemia preconditioning (Mayr, Chung et al. 2004, Mayr, Metzler et al. 2004). It will be great interest for us to further investigate the potential substrates of PKC δ activation in I/R+PMNs hearts in the future.

4.4 Conclusion

In summary, this study strongly suggests that PKC δ activator exerted anti-

inflammatory effects and provided cardio-protective effects against myocardial I/R injury when given at the beginning of reperfusion. It may be a potential strategy to mitigate the damage caused by I/R.

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