Naunyn-Schmiedeberg's Arch Pharmacol (2008) 378:1–15
DOI 10.1007/s00210-008-0288-5

ORIGINAL ARTICLE

Mechanisms related to the cardioprotective effects of protein kinase C epsilon (PKC ε) peptide activator or inhibitor in rat ischemia/reperfusion injury

Jane Chun-wen Teng · Helen Kay · Qian Chen · Jovan S. Adams · Christopher Grilli · Giuseppe Guglielmello · Christopher Zambrano · Samuel Krass · Adrian Bell · Lindon H. Young

Received: 18 February 2008 / Accepted: 22 March 2008 / Published online: 22 May 2008
© Springer-Verlag 2008

Abstract The role of protein kinase C epsilon (PKC ε) in polymorphonuclear leukocyte (PMN)-induced myocardial ischemia/reperfusion (MI/R) injury and novel-related mechanisms, such as regulation of vascular endothelium nitric oxide (NO) and hydrogen peroxide (H_2O_2) release from blood vessels, have not been previously evaluated. A cell-permeable PKC ε peptide activator (1–10 μM) significantly increased endothelial NO release from non-ischemic rat aortic segments ($p<0.01$). By contrast, PKC ε peptide inhibitor (1–10 μM) dose-dependently decreased NO release ($p<0.01$). Then, these corresponding doses of PKC ε activator or inhibitor were examined in MI/R. The PKC ε inhibitor (5 μM given during reperfusion, $n=6$) significantly attenuated PMN-induced postreperfused cardiac contractile dysfunction and PMN adherence/infiltration (both $p<0.01$), and expression of intracellular adhesion molecule-1 (ICAM-1; $p<0.05$). By contrast, only PKC ε activator pretreated hearts (5 μM PKC ε activator given before ischemia (PT), $n=6$), not PKC ε activator given during reperfusion (5 μM, $n=6$) exerted significant cardioprotection ($p<0.01$). Moreover, the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester, did not block the cardioprotection of PKC ε inhibitor, whereas it completely abolished the cardioprotective effects of PKC ε activator PT. In addition, PKC ε inhibitor (0.4 mg/kg) significantly decreased H_2O_2 release during reperfusion in a femoral I/R model ($p<0.01$). Therefore, the cardioprotection of PKC ε inhibitor maybe related to attenuating ICAM-1 expression and H_2O_2 release during reperfusion. By contrast, the cardioprotective effects of PKC ε activator PT may be mediated by enhancing vascular endothelial NO release before ischemia.

Keywords Endothelial dysfunction · Nitric oxide · Neutrophils · Superoxide radicals · Hydrogen peroxide · Left ventricular developed pressure

Introduction

The benefit of reperfusion for patients who suffer myocardial ischemia is largely limited by reperfusion-induced cardiac contractile dysfunction and myocardial cell injury (Lucchesi and Mullane 1986; Forman et al. 1989). Reperfusion injury is characterized by an increase in oxygen-derived free radicals that damage cell membrane permeability leading to intracellular calcium overload and cardiac hypercontracture (Yellon and Hausenloy 2007). A principle reactive oxygen species is superoxide anion (SO), which is produced by several sources that include polymorphonuclear leukocyte (PMN) and endothelial NADPH oxidase, incomplete oxidative phosphorylation in mitochondria, and endo-

Jane Chun-wen Teng and Helen Kay contributed the same efforts for this manuscript.

J. C.-w. Teng · H. Kay · C. Zambrano · S. Krass
Biomedical Sciences,
Philadelphia College of Osteopathic Medicine,
Philadelphia, PA, USA

Q. Chen · J. S. Adams · C. Grilli · G. Guglielmello · A. Bell
Philadelphia College of Osteopathic Medicine,
Philadelphia, PA, USA

L. H. Young (✉)
Department of Pathology, Microbiology and Immunology,
Philadelphia College of Osteopathic Medicine,
4170 City Avenue,
Philadelphia, PA 19131-1694, USA
e-mail: LindonYo@pcom.edu

thelial nitric oxide (NO) synthase (eNOS) when essential eNOS cofactor, tetrahydrobiopterin (BH_4), is oxidized to dihydrobiopterin (BH_2). SO is further converted to hydrogen peroxide (H_2O_2) by superoxide dismutase, then H_2O_2 is converted to water by catalase. However, these endogenous oxidative stress defense mechanisms are overwhelmed during reperfusion. SO also attenuates the bioavailability of NO through the formation of peroxynitrite anion (Vasquez-Vivar et al. 2002). An abrupt decrease in endothelium-derived NO occurs within 5 min of reperfusion and results in endothelial dysfunction (Lefer and Lefer 1996). Endothelial dysfunction promotes the upregulation of endothelial adhesion molecules [e.g., intracellular adhesion molecule-1 (ICAM-1)] to facilitate PMN adherence and infiltration (Tsao et al. 1990; Tsao and Lefer 1990; Lefer and Lefer 1996). After 30 min, the transmigrated PMNs release cytotoxic substances such as SO radicals to directly injure the myocardium that contributes to cardiac contractile dysfunction (Tsao et al. 1990; Hansen 1995).

Protein kinase C (PKC) is an important enzyme involved in myocardial ischemia/reperfusion (MI/R). PKC regulates eNOS in both man and rats (Rubanyi et al. 1989; Young et al. 2005) and exerts positive or negative regulation of NO release with respect to its different isoforms (Hirata et al. 1995; Li et al. 1998; Young et al. 2005). Previous studies have shown that PKC beta II (βII), PKC delta (δ), and PKC zeta (ζ) inhibition increases NO release from non-ischemic rat aortic segments (Omiyi et al. 2005; Phillipson et al. 2005; Young et al. 2006). By contrast, PKC epsilon (ε) activation leads to eNOS phosphorylation and increased expression (Li et al. 1998; Zhang et al. 2005). Moreover, different PKC isoforms also regulate ICAM-1 expression under different biological conditions. For instance, PKC β inhibition attenuates ICAM-1 expression in the kidney of diabetic rats and improves renal function (Wu et al. 2006). Conversely, activation of PKC ζ by tumor necrosis factor-alpha increases PMN adhesion to human pulmonary artery endothelial cells through phosphorylation of ICAM-1 (Javaid et al. 2003). In addition, upregulation of PKC epsilon (PKC ε) accompanying with enhanced ICAM-1 expression are observed in salt-sensitive hypertensive rats (Kobayashi et al. 2005). In PMNs, PKC activation increases SO release via phosphorylation of cytosolic factor $p47^{phox}$ that is required for NADPH oxidase activation (Park and Babior 1997).

It has been well documented that PKC activation is an adaptive response in preconditioning, whereas PKC inhibition is cardioprotective during the reperfusion phase after acute ischemia in both ex vivo and in vivo experiments (Lasley et al. 1997). However, the specific mechanisms mediating the dual roles of PKC have not been well understood in I/R injury. For example, PKC ε activation is cardioprotective in reperfusion injury only when given/activated before prolonged ischemia (i.e., 20 min ischemia) but not when given/activated during early reperfusion (Inagaki et al. 2003). The effect of PKC ε inhibition has not been evaluated in postreperfused cardiac function. Due to the absence of PKC ε expression in rat/man PMNs, the vascular endothelial cell may be the principle target related to the cardioprotection of PKC ε in MI/R injury. Moreover, broad-spectrum PKC inhibition with staurosporine and related analogs decreases H_2O_2 release from endothelial cells (Zulueta et al. 1995). However, the role of PKC ε inhibition mediating this response has not been previously characterized.

Therefore, in this study, the selective PKC ε inhibitor peptide (N-Myr-EAVSLKPT, MW=1054, Genemed Synthesis) and PKC ε activator peptide (N-Myr-HDAPIGYD, MW=1097, Genemed Synthesis) are used to attenuate or promote the interaction of PKC ε with its membrane substrates such as eNOS (Li et al. 1998) through receptor for activated C kinase (RACK-1). The selective PKC ε inhibitor or activator peptide has been demonstrated in rat cardiac myocytes to be specific for PKC ε in the range of 0.1–10 μM (Chen et al. 2001; Johnson et al. 1996a; Johnson et al. 1996b). Firstly, we tested the effects of PKC ε inhibitor or PKC ε activator in nonischemic aortic segments to determine the effective dose range that would increase or decrease NO release. Secondly, we tested the effects of PKC ε inhibitor or PKC ε activator in isolated MI/R rat hearts using the determined effective dose. Thirdly, we evaluated total PMN content in postreperfused hearts. Then N^G-nitro-L-arginine methyl ester (L-NAME) was used to test if the cardioprotection and decreased PMN adherence/infiltration provided by PKC ε inhibitor when given during reperfusion (RP) or PKC ε activator when given before ischemia (PT) could be blocked. We further tested the effect of PKC ε inhibitor on oxidative stress during reperfusion by directly measuring H_2O_2 release from femoral veins subjected to I/R in anesthetized rats.

Materials and methods

Isolated rat heart preparation

The Institutional Animal Care and Use Committee of the Philadelphia College of Osteopathic Medicine approved all animal protocols performed in this study. As previously described, hearts were rapidly excised from male Sprague–Dawley rats (275–325 g, Ace Animals, Boyertown, PA, USA) after injecting pentobarbital sodium 60 (mg/kg) and sodium heparin (1,000 U) intraperitoneally (i.p.). Then, the heart was subjected to retrograde perfusion with a modified Krebs' buffer (in mmol/l: 17.0 dextrose, 120.0 NaCl, 25.0 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 ethylenediamine-

tetraacetic acid, 5.9 KCl, and 1.2 $MgCl_2$) maintained at 37°C, pH of 7.3–7.4 and aerated with 95% O_2–5% CO_2. Three side arms in the perfusion line proximal to the heart inflow cannula allowed infusing PKC ε inhibitor or PKC ε activator/plasma, PMNs, and L-NAME. Coronary flow was monitored by a flowmeter (T106, Transonic Systems, Ithaca, NY, USA). Left ventricular developed pressure [LVDP, defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure (LVEDP)] and the maximal rate of LVDP ($+dP/dt_{max}$) were monitored using a pressure transducer (SPR-524, Millar Instruments, Houston, TX, USA) which was positioned in the left ventricular cavity and recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO, USA; Peterman et al. 2004).

The isolated rat heart was cannulated via the aorta onto a perfusion needle and immersed in a water-jacketed reservoir that contained 160 ml of Krebs' buffer maintained at 37°C. The preload volume came from Krebs' buffer that filled the left ventricle upon insertion of the pressure transducer catheter in the base of the left side of the heart. We also used animals in the same weight range for all study groups, and therefore, the preload should be similar among all study groups as in previous studies (Peterman et al. 2004; Omiyi et al. 2005; Phillipson et al. 2005). The initial baseline left ventricular end-diastolic pressure was between 4 and 8 mmHg for all hearts in each study group.

Groups of isolated perfused hearts

Table 1 indicated the 16 groups (control and treatment conditions) of isolated perfused rat hearts used in the study. Three types of control groups were used in the study based on the well-established PMN-induced MI/R model (Lefer et al. 1997). (1) Sham hearts were not subjected to ischemia and were not perfused with PMNs, but they were perfused with 5 ml of plasma (1 ml/min) at 35 min into perfusion (i.e., the same time point that I/R hearts would be given 5 ml of plasma). This group was employed to show that cardiac function (i.e., LVDP and $+dP/dt_{max}$) could be maintained throughout the 80-min protocol. (2) I/R hearts were subjected to 20 min of ischemia/45 min of reperfusion and were perfused with 5 ml of plasma (1 ml/min) in the absence of PMNs during the first 5 min of reperfusion. This group was employed to show that these hearts would recover to near baseline values by the end of 45 min of reperfusion. Therefore, 20 min ischemia followed by 45 min reperfusion would stun the heart but was a form of reversible cell injury. (3) I/R + PMN hearts were subjected to 20 min of ischemia and were reperfused with PMNs (200×10^6, resuspended in 5 mL Krebs' buffer) and 5 ml of plasma (1 ml/min) during the first 5 min of reperfusion. This group was employed to show that 20 min of ischemia followed by 45 min reperfusion in the presence of PMNs resulted in a sustained cardiac contractile dysfunction throughout the 45 min reperfusion period compared to initial baseline values. To show that PKC ε activator or PKC ε inhibitor did not exert a cardiotonic or cardiodepressant effect in Sham or I/R settings, PKC ε activator (5 μM) or PKC ε inhibitor (5 μM) was dissolved in plasma and perfused into hearts. In some I/R + PMN hearts, different doses of PKC ε activator or inhibitor were infused either just before ischemia (1 and 5 μM PKC ε activator, PT) or at the beginning of reperfusion (2.5, 5 or 10 μM PKC ε activator, 1 or 5 μM PKC ε inhibitor, RP) to test whether PKC ε activator or PKC ε inhibitor might elicit cardioprotective effects. Furthermore, a NO synthase inhibitor, L-NAME (50 μM), was used throughout the 45 min reperfusion in some PKC ε activator or PKC ε inhibitor-treated I/R + PMN hearts to determine if a NO mechanism was involved in the cardioprotective effect. The dose of L-NAME (50 μM) used in this study did not significantly affect LVDP in Sham hearts (Young et al. 2000).

Isolation of plasma and PMNs

As previously described, 5 ml plasma was collected from the aorta just before isolation of the heart and used for infusion for all cardiac perfusion groups (Peterman et al. 2004). PMNs were prepared after peritoneal lavage from Sprague–Dawley rats (350–400 g, Ace Animals, Boyertown,

Table 1 Groups of rat hearts and treatment received at the beginning of reperfusion (RP) or prior to ischemia (PT)

Group	Sham	I/R	I/R + PMN	I/R + PMN + L-NAME
Control (no drug)	$n=6$	$n=7$	$n=11$	
PKC ε activator (2.5 μM, RP)			$n=4$	
PKC ε activator (5 μM, RP)			$n=6$	
PKC ε activator (10 μM, RP)			$n=5$	
PKC ε activator (1 μM, PT)			$n=5$	
PKC ε activator (5 μM, PT)	$n=6$	$n=6$	$n=6$	$n=6$
PKC ε inhibitor (1 μM, RP)			$n=6$	
PKC ε inhibitor (5μM, RP)	$n=6$	$n=6$	$n=6$	$n=7$

PA, USA) receiving 0.5% glycogen (i.p.; Sigma Chemical, St. Louis, MO, USA; Omiyi et al. 2005).

Determination of PMN vascular adherence and infiltration into the cardiac tissue

Three rat hearts that were closest to the group mean for the cardiac function studies were embedded in plastic and sectioned into 2.5 μm serial sections. Then, sections were stained with hematoxylin and eosin by previously established methods (Omiyi et al. 2005). Under light microscopy, ten areas of each rat heart from the left ventricle were counted for PMN vascular adherence and infiltration into the heart tissue and expressed as adhered and total PMNs/mm^2.

Western blot analysis for expression of ICAM-1

Right ventricular tissue from isolated heart experiments was homogenized and separated into cytosol and membrane fractions. Then, equal amounts (50 μg) of protein from each experimental group and pre-stained molecular weight marker were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was probed with a specific antibody against ICAM-1 [1:400, mouse monoclonal 1A29, Endogen, Woburn, MA, USA (Scalia et al. 2000)]. ICAM-1 was located between 80 and 110 kD. Protein levels were quantified by analyzing densitometry by using Image J. The membranes were stained with Ponceau S and probed with β-actin (1:3,000, mouse monoclonal JLA 20, Calbiochem, San Diego, CA, USA) to ensure that approximately equal amounts of protein were loaded in each lane.

Measurement of NO release from rat aortic segments

As previously published (Omiyi et al. 2005), the NO release from non-ischemic aortic segment was measured using a calibrated NO meter (Iso-NO; World Precision Instruments (WPI), Sarasota, FL, USA; Guo et al. 1996). Basal rat aortic endothelial NO release was determined by the difference of reading between a well containing only Krebs'–Henseleit buffer solution (in mmol/l: 10.0 dextrose, 119.0 NaCl, 12.5 $NaHCO_3$, 2.5 $CaCl_2$, 4.8 KCl, 1.2 KH_2PO_4, and 1.2 $MgSO_4$) and a well containing aortic tissue. After basal NO measurement, the effect of acetylcholine (Ach, 5 μM, positive control) and PKC ε activator (1–10 μM) or PKC ε inhibitor (1–10 μM) were determined. The respective drug effects were reassessed 30 min later after adding L-NAME (400 μM). All measurements were reported in picomoles per second per milligram of aortic tissue. Between 16 and 41 trials were performed for each group.

Measurement of SO radical release from rat PMNs

The SO anion release from PMNs in the presence of PKC ε activator, PKC ε inhibitor, or PKC ζ peptide inhibitor (Genemed Synthesis, San Francisco, CA, USA) was measured spectrophotometrically (model 260 Gilford, Nova Biotech, El Cajon, CA, USA) when stimulated with phorbal myristate acetate (PMA) as previously described (Ohlstein and Nichols 1989; Phillipson et al. 2005). Superoxide dismutase (SOD, 10 ug/ml) served as positive control and the change in absorbance (Δ) in SO anion release was determined from time 0 to 360 s.

Measurement of H_2O_2 release from rat femoral veins during reperfusion

We recently developed a novel technique to measure H_2O_2 release from femoral veins in rats. One was subjected to I/R by clamping the femoral artery/vein for 20 min followed by 45 min reperfusion (similar to the I/R time course in our isolated MI/R model; Kuntscher et al. 2002), and the other is a non-ischemic sham control. Rats were anesthetized with sodium pentobarbital (i.p., 60 mg/kg for induction and 28 mg/kg for maintenance). The H_2O_2 microsensors (100 μm, WPI) connected to a free radical analyzer (Apollo 4000, WPI) were inserted into a catheter and placed inside each femoral vein. PKC ε inhibitor (0.4 mg/kg diluted in saline, which corresponds to about 5 μM in the blood concentration) and saline (for nondrug control group) were applied through tail vein injection at the beginning of reperfusion. The changes in H_2O_2 release during reperfusion (in pA) were expressed as relative changes to baseline (initial). Thereafter, the values were converted to the concentration of H_2O_2 (μM) after correction to the calibration curve of H_2O_2 microsensors.

Statistical analysis

All data in the text and figures were presented as means± SEM. The data were analyzed by ANOVA using post hoc analysis with the Bonferroni/Dunn test. Student *t* test was used to analyze differences between sham and I/R limbs with or without PKC ε inhibitor. Probability values of <0.05 were considered to be statistically significant.

Results

Regulation of PKC ε on endothelial NO release

NO release from rat non-ischemic aortic endothelium was measured to determine the role of PKC ε in the regulation of eNOS. Moreover, the dose–responses of PKC ε activator

and PKC ε inhibitor on NO release also provided the optimal dose ranges for testing their effects in cardiac function experiments. As shown in Fig. 1, PKC ε activator dose-dependently (1–10 μM) increased NO release up to 0.33±0.02 ($p<0.01$) pmol NO/s/mg tissue. By contrast, PKC ε inhibitor dose-dependently (1–10 μM) decreased NO release down to 0.01±0.02 pmol NO/s/mg tissue ($p<0.01$) from basal values. Ach (5 μM) was used as a positive control and increased NO release to 0.30±0.02 pmol NO/s/mg tissue ($p<0.01$). The eNOS inhibitor, L-NAME (400 μM), served as negative control and significantly blocked NO release from aortic endothelium.

Cardioprotection of PKC ε on PMN-induced MI/R injury

Different doses of PKC ε activator or inhibitor were applied individually to the isolated perfused hearts to determine their effects on postreperfused cardiac function. Figure 2a,b showed the time course of cardiac contractile function (i.e., LVDP) and diastolic function (i.e., LVEDP) for the I/R + PMN, I/R + PMN + PKC ε activator (5 μM PT) and I/R + PMN + PKC ε inhibitor (5 μM RP) groups. The hearts in the I/R + PMN group exhibited sustained cardiac contractile and diastolic dysfunction, 56±5% of initial LVDP and 46±5 mmHg in LVEDP, respectively, at 45-min reperfusion. Compared to I/R + PMN hearts, PKC ε inhibitor (5 μM, RP) significantly increased final LVDP to 99±6% of initial baseline and significantly decreased final LVEDP to 16±2 mmHg (both $p<0.01$). By contrast, PKC ε activator (5 μM, PT) provided significant recovery in LVDP, not in LVEDP, compared to I/R + PMN hearts. The hearts in the Sham group maintained LVDP and LVEDP throughout the entire duration of the perfusion period. Hearts in the I/R group experienced a depression in LVDP and an increase in LVEDP during the initial stages of reperfusion, but by the end of reperfusion they had recovered to initial baseline values (data not shown). This data suggested that both PKC ε inhibitor (RP) and activator (PT) provided significant restoration in postreperfused cardiac contractile function, while only PKC inhibitor (RP) showed significant recovery in cardiac diastolic function.

Figure 3 and Table 2 showed the initial and final values for LVDP and $+dP/dT_{max}$ from different experimental groups. There was no significant difference between the initial baseline values of all the groups studied. There was also no significant difference between the initial and final values of LVDP and $+dP/dt_{max}$ in Sham and I/R hearts, and Sham or I/R with PKC ε activator (5 μM) or inhibitor (5 μM; data not shown). However, I/R + PMN hearts only recovered to 56±5% of initial LVDP and 48±5% of initial $+dP/dt_{max}$ at 45 min postreperfusion; both were significantly lower than initial baseline (Fig. 3 and Table 2, both $p<0.01$). This result suggested that PMNs were principally responsible for the sustained cardiac contractile dysfunction in this model of I/R. PKC ε activator (5 μM) applied during reperfusion did not significantly improve the postreperfusion cardiac function (75±7% and 71±8% of initial LVDP and $+dP/dt_{max}$, respectively, Fig. 3 and Table 2). Moreover, 2.5 and 10 μM PKC ε activator-treated hearts were more compromised compared to 5 μM PKC ε activator applica-

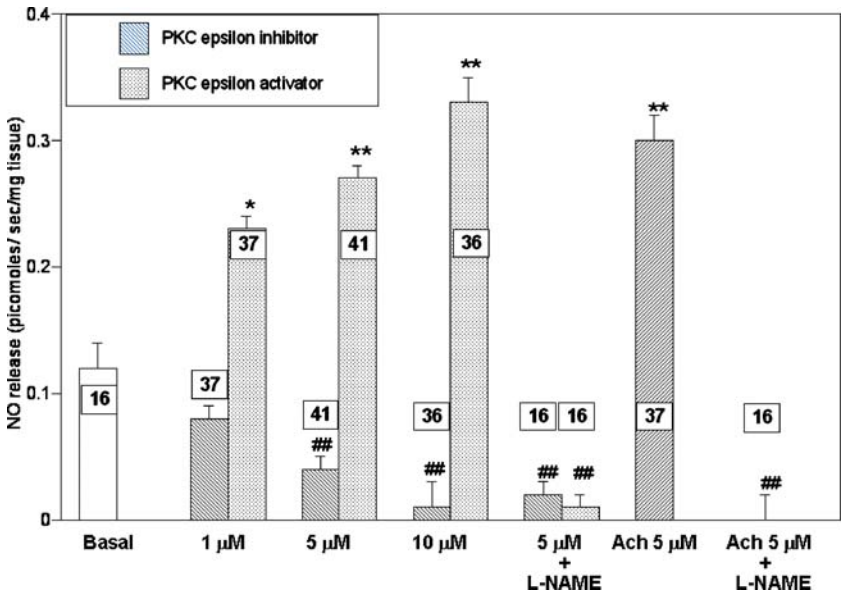

Fig. 1 Measurement of NO release from rat aortic segments. Endothelial NO release was significantly increased from basal NO release in PKC ε activator-treated segments (1–10 μM) and acetylcholine treatment (Ach, 5 μM). By contrast, endothelial NO release was significantly decreased from basal NO release in PKC ε inhibitor-treated segments (5 and10 μM). NO release was significantly reduced in both groups given 400 μM L-NAME. All values are expressed as means±SEM; numbers of rat aortic segments are at the *bottom of the bars*. *$p<0.05$, **$p<0.01$, $^{\#\#}p<0.01$ from basal values

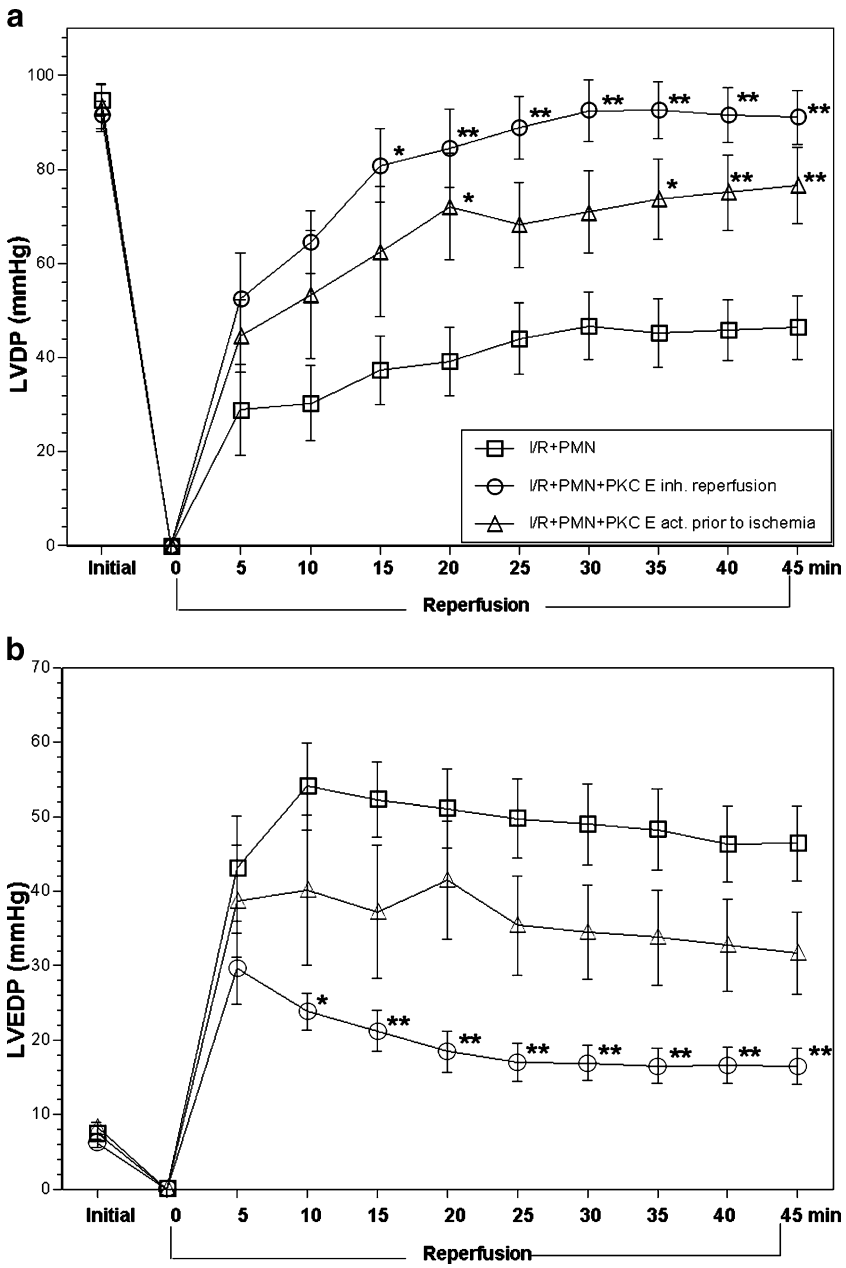

Fig. 2 Time course of cardiac function parameters from I/R + PMN, I/R + PMN + PKC epsilon peptide activator [PKC ε activator (*act.*), 5 μM, before ischemia] and I/R + PMN + PKC epsilon peptide inhibitor [PKC ε inhibitor (*inh.*), 5 μM, reperfusion] rat hearts. **a** Left ventricular developed pressure (LVDP) data are expressed in mmHg and shown at initial (baseline) and reperfusion from 0 to 45 min after 20 min ischemia. The I/R + PMN group (n=11) exhibited a significant and sustained reduction in LVDP compared to I/R + PMN + PKC ε activator (n=6) and I/R + PMN + PKC ε inhibitor (n=6) groups. **b** Left ventricular end diastolic pressure (LVEDP) data are expressed in mmHg and shown at initial (baseline) and reperfusion from 0 to 45 min after 20 min ischemia. The I/R + PMN group (n=11) exhibited a significant and sustained elevation in LVEDP compared I/R + PMN + PKC ε inhibitor (n=6) groups. All values are expressed as mean± SEM. [*p<0.05, **p<0.01 from I/R + PMN values]

tion (data not shown). By contrast, 5 μM PKC ε activator PT I/R + PMN hearts significantly recovered to 90±10% and 89±9% for LVDP (p<0.05) and +dP/dt_{max} (p<0.01), respectively, at 45 min of reperfusion, compared to I/R + PMN hearts (Figs. 2a, 3 and Table 2).

However, I/R + PMN + PKC ε inhibitor (RP)-treated hearts exhibited significant dose-dependent restoration of postreperfusion cardiac contractile function to 99±6% and 87±5% of initial LVDP and +dP/dt_{max}, respectively, at 45 min postreperfusion (both p<0.01).

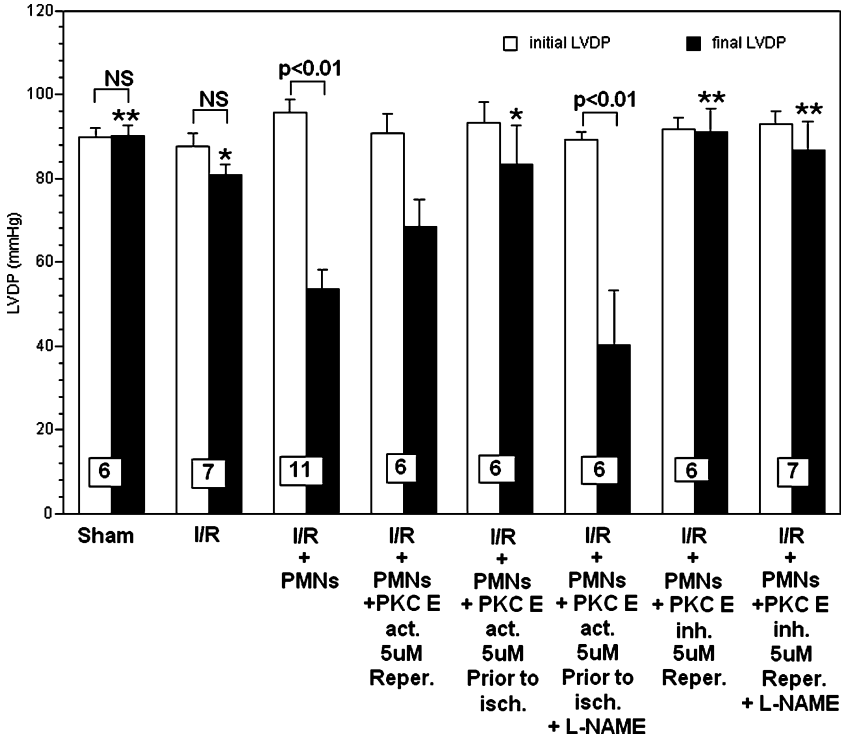

Fig. 3 Initial and final LVDP expressed in mmHg from isolated perfused rat hearts before ischemia (*I*; initial) and after 45 min reperfusion (*R*; final). Hearts were perfused in the presence or absence of PMNs. PMNs induced a significant decrease in LVDP, which was attenuated by the PKC ε activator [*act*.; 5 μM, before ischemia (*isch*.)] and PKC ε inhibitor [*inh*.; 5 μM, reperfusion (Reper.)]. Moreover, L-NAME (50 μM) blocked the cardioprotective effect of PKC ε activator pretreatment, not the cardioprotective effect of PKC ε inhibitor. All values are expressed as mean±SEM. Numbers of hearts are at the *bottom of the bars* (*$p<0.05$, **$p<0.01$ from final I/R + PMN values; *NS* Not significant compared to initial LVDP values)

Effects of L-NAME on cardioprotective effects of PKC ε activator or PKC ε inhibitor

L-NAME (50 μM) was used to determine if a NO mechanism was involved in the cardioprotection of PKC ε activator or inhibitor. Previous studies have shown that L-NAME given by itself during reperfusion was not associated with cardioprotection (Shi et al. 2004). In the presence of L-NAME, the cardioprotective effects of PKC ε activator, not inhibitor, were totally blocked by L-NAME (Fig. 3 and Table 2). This result indicated that a NO mechanism might be related to the cardioprotection of PKC ε activator PT, but might not mediate the beneficial effects of PKC ε inhibitor.

Table 2 Initial and final + dP/dt_{max} expressed in mmHg/s in isolated perfused rat hearts before ischemia (I) and after reperfusion (R)

Group	Initial	Final
Sham	2,329±61.9	2,479±70.9*
I/R alone	2,277±87.1	2,037±64.4*
I/R + PMNs	2,450±96.9	1,188±117.3**
I/R + PMNs + PKC ε activator (5 μM, RP)	2,483±156.9	1,772±199.1
I/R + PMNs + PKC ε activator (5 μM, PT)	2,307±137.7	2,045.±197.8*
I/R + PMNs + PKC ε activator (5 μM, PT) + L-NAME (50 μM)	2,182±115.4	830±239.5**
I/R + PMNs + PKC ε inhibitor (5 μM, RP)	2,545±105.9	2,212±117.7*
I/R + PMNs + PKC ε inhibitor (5 μM, RP)+L-NAME (50 μM)	2,340±90.7	2,109±201.0*

Hearts were perfused in the presence or absence of PMNs. PMNs induced a significant decrease in +dP/dt_{max}, which was significantly attenuated by PKC ε inhibitor (5 μM) given during reperfusion (RP) or PKC ε activator (5 μM) given before ischemia (PT). The cardioprotective effects of PKC ε activator (5 μM, PT) were blocked by L-NAME, but not in PKC ε inhibitor (5 μM, RP)-treated hearts. All values are expressed as mean±SEM.
*$p<0.01$ from final I/R + PMNs
**$p<0.01$ from initial +dP/dt_{max}

Effects of PKC ε activator or PKC ε inhibitor on postreperfused PMNs adherence/infiltration

The infiltration of PMNs into the myocardium within the 45-min reperfusion period was closely correlated with the cardiac injury associated with I/R in this model. The representative PMN coronary vascular adherence and transmigration from I/R + PMN, I/R + PMN + PKC ε activator (5 μM, PT) and I/R + PMN + PKC ε inhibitor (5 μM, RP) groups were shown by light microscopy picture under ×20 and ×40 magnification (Fig. 4). The I/R + PMN + PKC ε activator (5 μM, PT) and I/R + PMN + PKC ε inhibitor (5 μM, RP) hearts displayed a considerable reduction in PMN vascular adherence and tissue infiltration compared to the I/R + PMN hearts. The total and adhered intravascular PMNs from different experimental groups were listed in Table 3. Sham and I/R hearts exhibited very few vascular adhered and transmigrated PMNs, which represented only resident PMNs. However, I/R + PMN hearts exhibited a significantly increased total intravascular and infiltrated PMNs, and vascular adhered PMNs. By contrast, PKC ε activator (5 μM) PT, not RP, and PKC ε inhibitor RP-treated hearts exhibited significant decrease in total PMNs and vascular adhered PMNs compared to I/R + PMN hearts (all $p<0.05$). L-NAME completely blocked the attenuation of adhered/infiltrated PMNs by 5 μM PKC ε activator PT, but not in 5 μM PKC ε inhibitor RP.

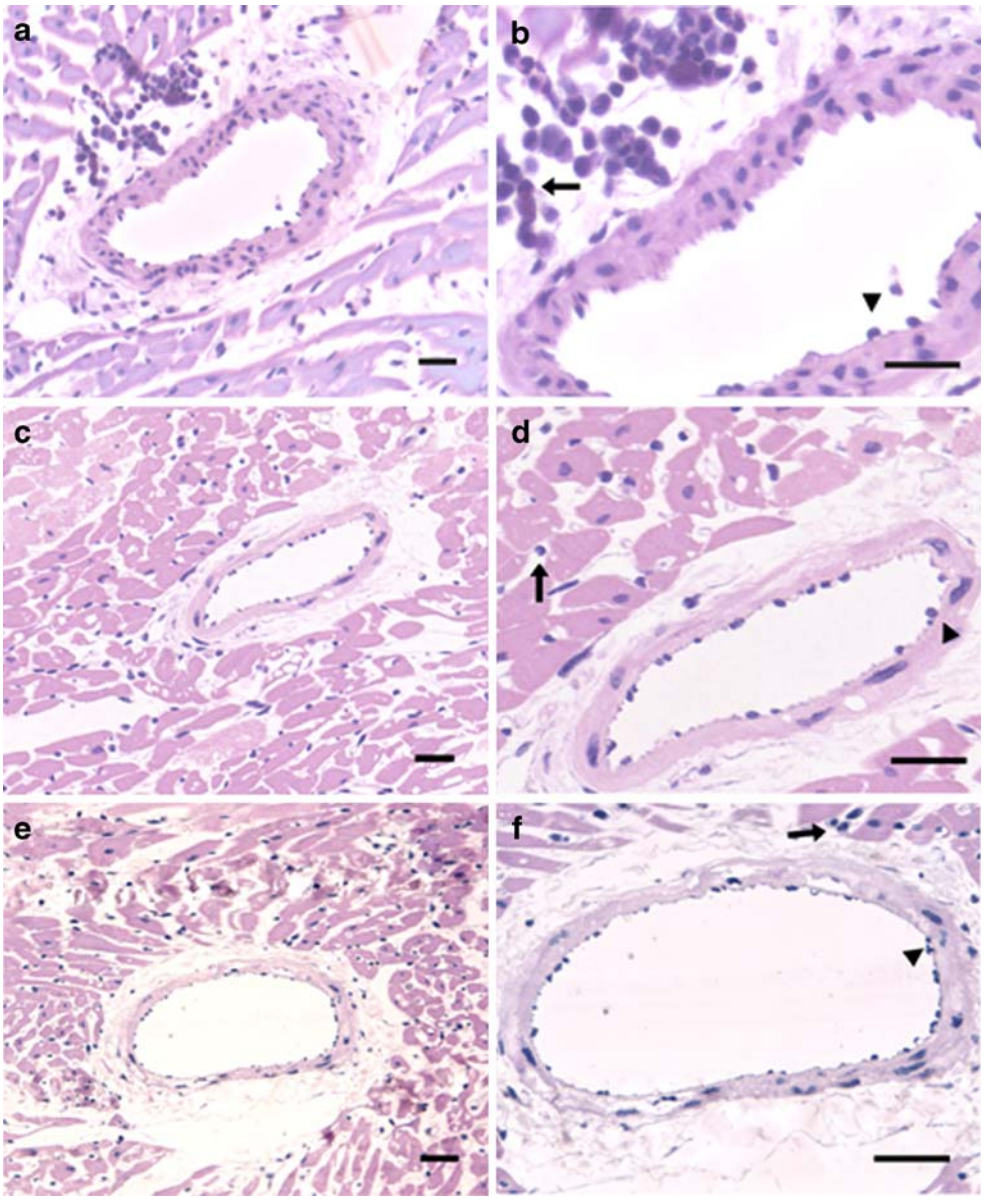

Fig. 4 Representative light microscopy photograph of hematoxylin and eosin-stained rat heart tissue from I/R + PMN, I/R + PMN + PKC ε activator (5 μM, before ischemia) and I/R + PMN + PKC ε inhibitor (5 μM, reperfusion) groups. **a**–**b**, I/R + PMN hearts illustrated enhanced PMN coronary vascular adherence and tissue infiltration (**a**: ×20; **b**: ×40). **c**–**d** I/R + PMN + PKC ε inhibitor (5 μM, reperfusion)-treated hearts exhibited a reduction in PMN coronary vascular adherence and tissue infiltration compared with I/R + PMN (**c** ×20; **d**: ×40). **e**–**f** I/R + PMN + PKC ε activator (5 μM, before ischemia)-treated hearts showed a reduction in PMN coronary vascular adherence and tissue infiltration compared with I/R + PMN (**c** ×20; **d** ×40). *Arrows* indicate infiltrated PMN, and *arrowheads* indicate PMN adherence to coronary vasculature. *Scale bar*, 20 μm

Table 3 Histological assessment of PMNs in isolated perfused rat heart samples

Group	Total Intravascular and Infiltrated	Adhered
Sham	19.6±1.4**	1.4±0.70***
I/R alone	37.1±2.52**	4.2±2.10***
I/R + PMNs	230.3±13.95	58.1±8.07
I/R + PMNs + PKC ε activator (5 μM, RP)	200.2±19.60	32.2±5.05*
I/R + PMNs + PKC ε activator (5 μM, PT)	115.5±14.75**	22.4±4.9***
I/R + PMNs + PKC ε activator (5 μM, PT) + L-NAME (50 μM)	240.1±10.59**	54.6±7.57
I/R + PMNs + PKC ε inhibitor (5 μM, RP)	121.80±5.28**	26.6±3.90*
I/R + PMNs + PKC ε inhibitor (5 μM, RP) + L-NAME (50 μM)	127.4±12.1**	40.6±3.7

Total intravascular/infiltrated PMNs and adhered PMNs were counted up to three sections in ten areas (mm^2) per heart. Three hearts were selected from each of the cardiac function experimental groups. All values are mean numbers of PMNs per squared millimeter of heart area±SEM.
*$p<0.05$ from adhered I/R + PMN values.
**$p<0.01$ from total intravascular and infiltrated I/R + PMN values
***$p<0.01$ from adhered I/R + PMN values

Effects of PKC ε activator or PKC ε inhibitor on ICAM-1 expression

The representative ICAM-1 blot and summary data ($n=3$) from PKC ε inhibitor-treated heart tissue were shown in Fig. 5a and b, respectively. There was very low expression of ICAM-1 in the membrane fraction of postreperfused heart tissue from Sham, Sham + PKC ε inhibitor (5 μM), I/R, or I/R + PKC ε inhibitor (5 μM) groups. By contrast, ICAM-1 expression in the membrane fraction of I/R + PMN hearts was significantly enhanced to 201±39% compared to Sham ($p<0.05$). The PKC ε inhibitor dose-dependently decreased ICAM-1 expression up to 47±5% (5 μM) compared to I/R + PMN ($p<0.05$). There was no detectable expression of ICAM-1 in the cytosol fraction of I/R + PMN + PKC ε inhibitor heart tissue (Fig. 5a). Moreover, PKC ε activator PT also decreased ICAM-1 expression compared to I/R + PMN, but the effect was not significant (data not shown). Therefore, the decreased expression of ICAM-1 might be a mechanism related to the cardioprotective effects of PKC ε inhibitor.

Effects of PKC ε activator or PKC ε inhibitor on PMN SO release

We also tested the effects of PKC ε activator or inhibitor on PMA-stimulated PMN SO release. As expected, neither PKC ε activator nor inhibitor had any effect on PMN SO release, as this PKC isoform was not expressed in rat PMNs (Dang et al. 2001).

Effects of PKC ε inhibitor on H_2O_2 release from rat femoral veins during reperfusion

The femoral vein subjected to I/R in anesthetized rats exhibited a significant increase in H_2O_2 release during reperfusion compared to the sham vein (Fig. 6a). This data supported the concept that oxidative stress was increased during reperfusion. Moreover, the H_2O_2 release difference between I/R and sham femoral veins was significantly decreased at most time points throughout the whole 45-min reperfusion by applying PKC ε inhibitor (0.4 mg/kg, i.v.) at the start of reperfusion compared to saline control ($p<0.05$, Fig. 6b). This finding indicated that PKC ε inhibitor used at the beginning of reperfusion decreased the oxidative stress during reperfusion and might be a mechanism related to the observed cardioprotective effects.

Discussion

Summary of major findings

The major findings of this study were as follows: (1) PKC ε positively regulated NO release from vascular endothelium in rat non-ischemic aortic segments, that is, PKC ε activator significantly increased, whereas PKC ε inhibitor significantly decreased NO release in a dose-dependent manner. (2) I/R + PMN + PKC ε activator (5 μM, PT) and I/R + PMN + PKC ε inhibitor (5 μM, RP)-treated rat hearts exerted significant restoration in postreperfused cardiac function (i.e., LVDP and $+dP/dt_{max}$) associated with significant attenuation of intravascular PMN adherence/infiltration in postreperfused myocardium. Especially, PKC ε inhibitor restored postreperfused cardiac contractile function by 99% with significant recovery in postreperfused diastolic pressure. (3) The cardioprotective effects of PKC ε activator, not PKC ε inhibitor were blocked by L-NAME. (4) PKC ε inhibitor (5 μM, RP), not PKC ε activator (5 μM, PT)-treated rat hearts exhibited significantly lower ICAM-1 expression compared to I/R + PMN hearts. (5) The increased oxidative stress (i.e., H_2O_2) during reperfusion was significantly decreased by PKC ε inhibitor (0.4 mg/kg, RP) in femoral I/R veins in vivo. Therefore, this study

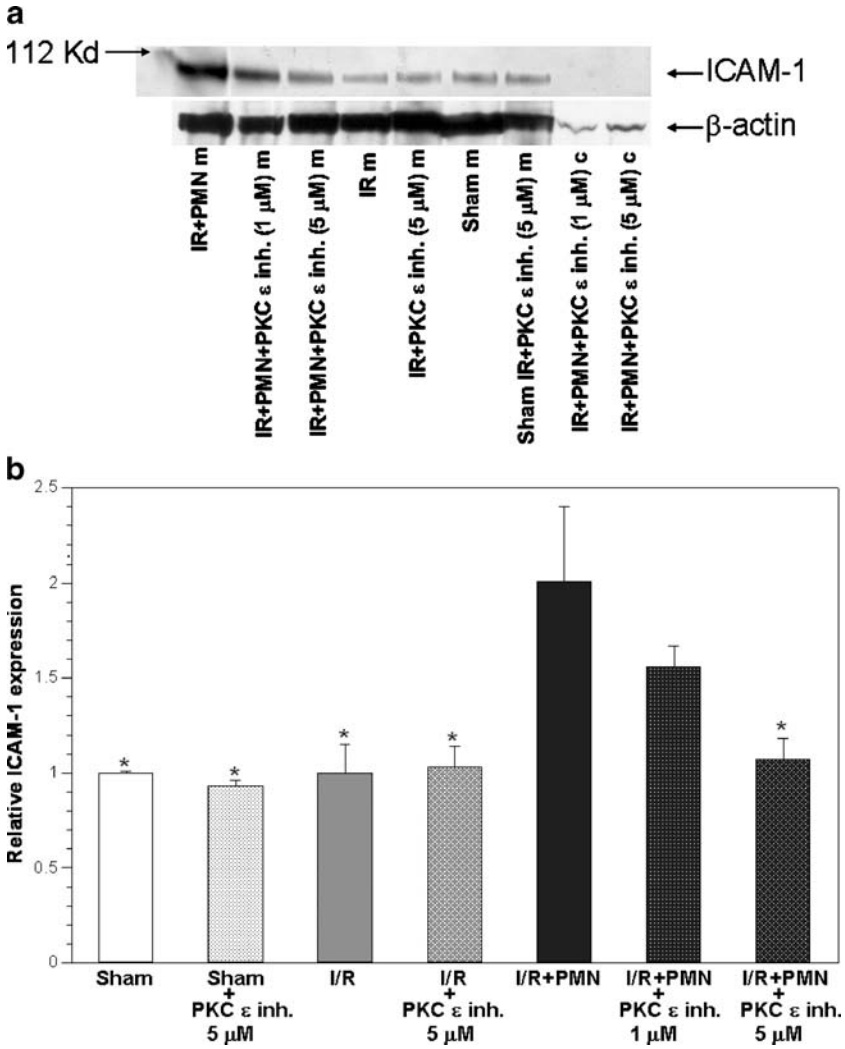

Fig. 5 The expression of ICAM-1 from different experiment groups. **a** A representative Western blot photograph of ICAM-1 and β-actin expression in tissue lysates from isolated perfused rat hearts with different treatments. PKC ε inhibitor (*inh*.; 5 μM, reperfusion)-treated hearts exhibited attenuated ICAM-1 expression compared to control I/R + PMN hearts in the membrane fraction (*m* Membrane fraction, *c* cytosol fraction). The similar densities of β-actin among the membrane fractions from different groups indicate the equal amount of protein loading. **b** A summary of relative ICAM-1 expression in membrane fractions from isolated perfused rat hearts with different treatments. PMNs induced a significant increase in ICAM-1 expression compared to non-PMN perfused hearts ($p<0.05$). The increase was significantly attenuated by PKC ε inhibitor ($p<0.05$); *$p<0.05$ from I/R + PMN values

supported that PKC ε activator (PT) or PKC ε inhibitor (RP) could be an effective treatment to protect the reperfused heart from PMN-induced I/R injury.

Effects of PKC ε activator or PKC ε inhibitor on cardiac function after I/R

This study established the dual role of PKC ε in MI/R injury, and the data was consistent with previous studies suggesting that PKC activation mediated preconditioning, whereas PKC inhibition was involved in the cardioprotection during the reperfusion phase after prolonged acute ischemia (Lasley et al. 1997). We found that both PKC ε inhibitor (RP) and activator (PT) provided significant restoration in postreperfused LVDP and $+dP/dt_{max}$. By contrast, PKC ε inhibitor (5 μM, RP), not PKC ε activator (5 μM, PT), showed significantly lower LVEDP compared to I/R + PMN hearts.

Our results are consistent with the study of Inagaki et al. showing that PKC ε activator PT significantly increased LVDP in a MI/R model without perfusing PMNs (Inagaki et al. 2003). However, Zatta et al. showed that PKC ε inhibitor RP had no effect on myocardium infarct size induced by local 25 min ischemia/3-h reperfusion in vivo (Zatta et al. 2006). It was noted that their PKC ε inhibitor might be ninefold higher (i.e., 3.8 mg/kg) compared to the current study (i.e., 5 μM or 0.4 mg/kg), which might compromise the selectivity or induce a direct effect on cardiac function. The concentration of PKC ε inhibitor or activator used in our study did not to exert a direct effect on

cardiac function (i.e., Sham + PKC ε inhibitor and I/R + PKC ε inhibitor).

Mechanisms underlying cardioprotection by PKC ε activator pretreatment

It is well known that endothelial dysfunction, which is characterized by decreased eNOS-derived NO within 2.5 or 5 min of reperfusion, is an important initiative step to induce post-I/R injury (Tsao et al. 1990; Tsao and Lefer 1990). In this regard, using a NO donor or L-arginine to maintain endothelial function can quench SO, down-regulate adhesion molecules, and inhibit PMN aggregation and infiltration into the reperfused myocardium (Weyrich et al. 1995; Pabla et al. 1996). In this study, we found that a NO mechanism possibly mediated the cardioprotection of PKC ε activator PT based on: (1) PKC ε activator significantly increased NO release from non-ischemic rat aortic segments, which was consistent with activation of PKC ε directly phosphorylating eNOS to enhance eNOS

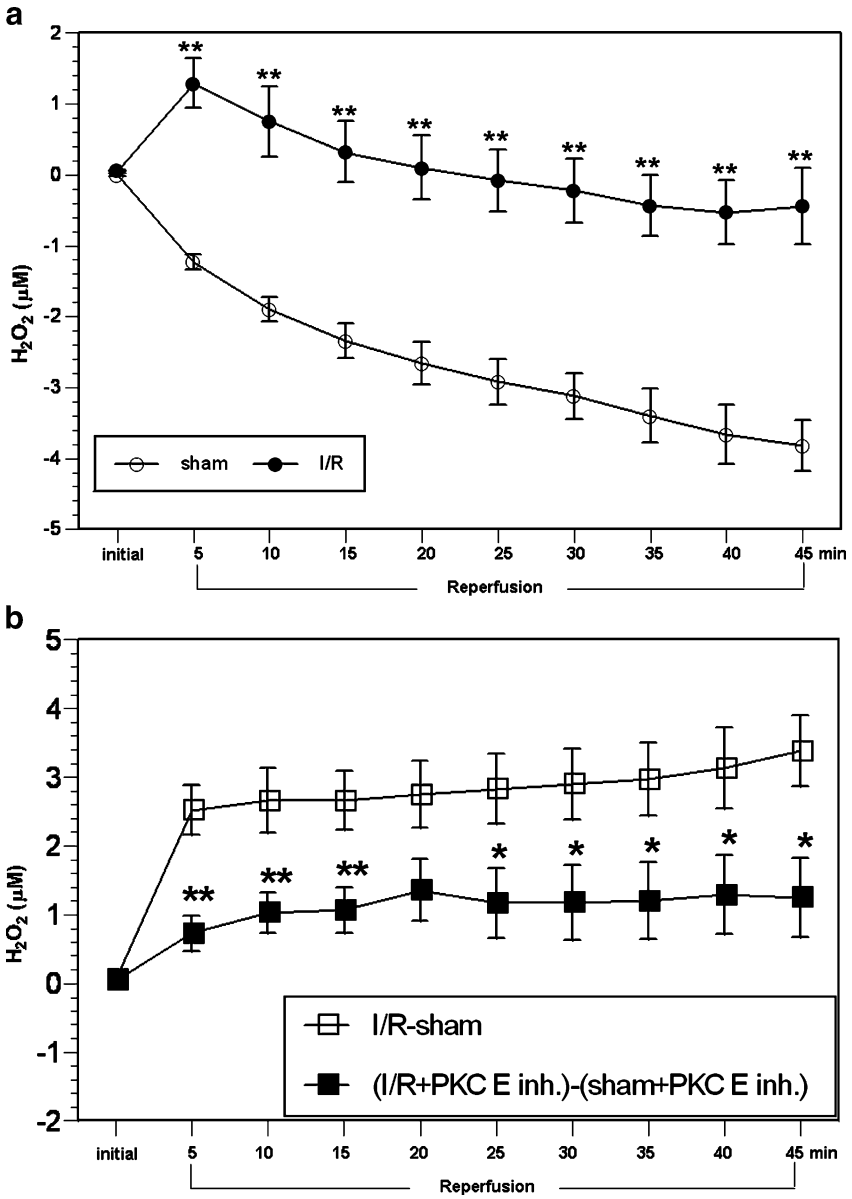

Fig. 6 Measurement of H_2O_2 (μM) release from rat femoral veins during reperfusion in the saline control and PKC ε inhibitor (*inh.*)-treated groups (0.4 mg/kg). **a** H_2O_2 (μM) releases from rat Sham and I/R femoral veins during reperfusion in the control group. Anesthetized rats were given saline I.V. at the beginning of reperfusion (n=5). There was a significant increase in H_2O_2 release from I/R veins compared to sham veins during reperfusion (**p<0.01). **b** The relative difference in H_2O_2 release between I/R and Sham femoral veins in the control group (n=5) and PKC ε inhibitor-treated group during reperfusion (n=6). PKC ε inhibitor treatment significantly decreased H_2O_2 release at most time points throughout reperfusion compared to saline control. *p<0.05, **p<0.01 from the difference between Sham and I/R femoral veins in control group

activity (Li et al. 1998; Zhang et al. 2005); (2) The significant restoration of postreperfusion cardiac function caused by PKC ε activator PT was completely abolished using L-NAME; (3) The significant attenuation of adhered and infiltrated PMNs in PKC ε activator PT postreperfused heart tissue were also blocked by L-NAME.

In addition, the significant decrease of total intravascular and infiltrated PMNs also contributed to the cardioprotection of PKC ε activator PT. At 45 min reperfusion, the majority of PMNs (~75% of 230 PMNs/mm^2) in control I/R + PMN hearts had transmigrated from the coronary vasculature into the myocardium and was principally responsible for inducing MI/R injury. Therefore, PMNs were less likely to form microemboli in the coronary vasculature. Moreover, PMN adherence at 45 min reperfusion might not be a sensitive indicator to reflect cell injury in MI/R because only ~25% adhered PMNs were observed at this time. By contrast, PKC ε activator PT dose-dependently (1 and 5 μM) significantly attenuated total intravascular and infiltrated PMNs (i.e., 159 and 116 PMNs/mm^2 respectively), which correlated with dose-dependent improvement in LVDP at 45 min postreperfusion (i.e., 70% and 90%, respectively).

Moreover, inhibition of L-type Ca^{2+} channels and activation of mitochondrial KATP channels due to PKC ε activation on cardiomyocytes might be related to postreperfused cardioprotection during I/R (Hu et al. 2000; Ohnuma et al. 2002). However, due to the brief drug application, the endothelial cells and PMNs were the principle cell types involved in our PMN-induced MI/R injury model (Omiyi et al. 2005; Phillipson et al. 2005). Firstly, PKC ε activator exerted direct effects on vascular endothelium (i.e., increase NO release), and PKC ε activator would first diffuse into coronary endothelial cells before entering into the myocardium. Secondly, PKC ε activator (5 μM) did not by itself influence cardiac contractile function (i.e., Sham + PKC ε activator hearts), suggesting that any diffusion of PKC ε activator into the myocardium would not elicit significant effects. Thirdly, the locus of PKC ε activator cardioprotection was in the coronary vascular endothelium, as PKC ε is not expressed in PMNs. Therefore, it was not likely that regulation of myocardial Ca^{2+} channels or mitochondrial KATP channels would be the principle mechanisms mediating PKC ε activator cardioprotection.

Mechanisms underlying cardioprotection by PKC ε inhibitor application during early reperfusion

Significantly lower PMN adherence/infiltration was also observed in PKC ε inhibitor treated hearts dose-dependently, which correlated to its cardioprotective effects. The data supported the concept that PMNs significantly contributed to MI/R injury (Hansen 1995).

ICAM-1 is expressed constitutively only at low levels on vascular endothelial cells and promotes PMN binding to the coronary endothelium (Rothlein et al. 1986). In both in vivo and in vitro models, ICAM-1 expression could be increased in response to cytokines and oxygen radicals (Kukielka et al. 1995; Fan et al. 2002). ICAM-1 expression could be increased within 1 h in vitro or ex vivo (Entman et al. 1991); (Olanders et al. 2002), whereas, in vivo expression was increased 2.5 to 3 h after MI/R (Weyrich et al. 1995; Fan et al. 2002). Inhibition of ICAM-1 could significantly decrease MI/R-induced myocardial necrosis, PMN infiltration, and endothelial dysfunction (Ma et al. 1992; Hartman et al. 1995; Zhao et al. 1997; Soriano et al. 1996). Similarly, ICAM-1 expression was significantly increased in I/R + PMN hearts compared to Sham hearts (Fig. 5). This effect might be attributed to the large amount of PMN infusion during reperfusion that might release increased amounts of oxygen radicals resulting in ICAM-1 translocation from cytosol to cell membrane. By contrast, PKC ε inhibitor (RP) dose-dependently decreased ICAM-1 expression, which might be a possible mechanism involved in the cardioprotective effects of PKC ε inhibitor in this PMN-induced MI/R model.

We also found out that postreperfusion cardiac function in I/R + PMN + PKC ε inhibitor-treated hearts was still significantly restored in the presence of L-NAME during reperfusion (Fig. 3). This data indicated that NO might not mediate the cardioprotection of PKC ε inhibitor.

Furthermore, the effect of PKC ε inhibitor on H_2O_2 release during reperfusion was directly measured from

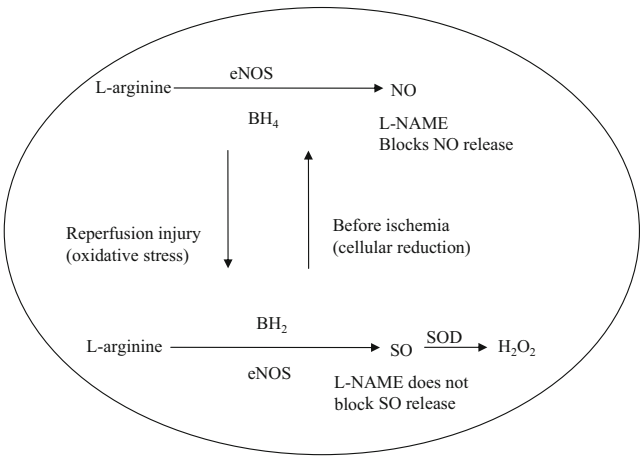

Fig. 7 Schematic diagram of the role of tetrahydrobiopterin (BH_4)- and dihydrobiopterin (BH_2)-regulating nitric oxide (NO) and superoxide (SO) production from endothelial nitric oxide synthase (eNOS) and the effect of N^G-nitro-L-arginine methyl ester (L-NAME) on blocking NO release from an endothelial cell. SO is further converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD)

femoral veins in vivo, one subjected to similar I/R time periods as in our MI/R model and another one served as sham. We found that femoral I/R veins showed significantly higher H_2O_2 release throughout the reperfusion compared to sham veins. Moreover, PKC ε inhibitor significantly decreased H_2O_2 release compared to I/R saline control group without any drug treatment. H_2O_2 is produced from SOD via SO; therefore, this data suggested that PKC ε inhibitor decreased oxidative stress during reperfusion, which might be a mechanism related to its cardioprotective effects.

Role of eNOS in effects of PKC ε activator or PKC ε inhibitor application during early reperfusion

Our results about the regulation of PKC ε on NO release from non-ischemic endothelium of rat aortic segments were consistent with other studies showing that PKC ε positively regulated eNOS function (Li et al. 1998; Zhang et al. 2005). However, PKC ε activator (RP) did not significantly restore cardiac function. By contrast, PKC ε inhibitor (RP) did significantly improve postreperfused cardiac function. The opposite connection of PKC ε activator and PKC ε inhibitor on NO release suggested that vascular endothelium might change its physiological function during reperfusion. eNOS catalyzes L-arginine to NO in vascular endothelium; however, eNOS can switch its product profile from NO to SO (eNOS uncoupling) with the change of ratio between the reduction state of the essential co-factor BH_4 to the oxidized state of BH_2 metabolites regardless of L-arginine concentrations (Stroes et al. 1998; Vasquez-Vivar et al. 2002). Moreover, L-NAME blocks NO release by binding to the heme domain site on NOS, but does not block SO release that is generated by the oxygenase domain site when BH_2 is the cofactor (Fig. 7).

Some studies indicate that MI/R can alter the availability or production of BH_4, which contributes to endothelium-dependent vasodilation dysfunction during MI/R (Tiefenbacher et al. 1996). By contrast, exogenous application of different BH_4 metabolites attenuates endothelial dysfunction by increasing the synthesis and/or stability of NO in vessels of patients with hypercholesterolemia and atherosclerosis, and animals after MI/R (Tiefenbacher et al. 1996; Stroes et al. 1997). In this regard, inhibition of eNOS (i.e., PKC ε inhibitor) during reperfusion may reduce SO release from eNOS to prevent endothelial and heart dysfunction. This speculation is consistent with our experiments showing that PKC ε inhibitor significantly decreased H_2O_2 release during reperfusion, and its cardioprotective effects could not be blocked by L-NAME (Fig. 3 and 7). To further elucidate the product profile of eNOS during reperfusion in vivo, the effects of BH_4 and BH_2 on H_2O_2 release from femoral I/R veins and MI/R injury will be determined.

Significance of findings

In summary, this study further supported that PKC ε activator (PT) and PKC ε inhibitor (RP) restored post-reperfused cardiac function and attenuated PMN adherence/infiltration in the isolated perfused rat heart. The cardio-protection provided by PKC ε activator was mainly mediated by the increase of endothelial-derived NO release. By contrast, The PKC ε inhibitor effects on postreperfusion cardiac function might be correlated with reduced SO release from coronary vascular endothelium by suppressing eNOS uncoupling and decreased ICAM-1 expression during reperfusion.

Acknowledgments Richard J Brue (M.S., D.O. student at Philadelphia College of Osteopathic Medicine) assisted in tissue sectioning rat hearts. Laura Triplett (M.S. student at Philadelphia College of Osteopathic Medicine) assisted in Western blot technique. Christine Hammond (Philadelphia College of Osteopathic Medicine) assisted in the light microscopy photography of the hematoxylin and eosin-stained isolated rat perfused heart tissue sections.

This study was supported by the National Heart, Lung, and Blood Institute Grant 1R 15HL-76235-01. This study was also supported in part by the Philadelphia College of Osteopathic Medicine, Department of Pathology, Microbiology, and Immunology, and the Center for the Study of Chronic Disorders of Aging at Philadelphia College of Osteopathic Medicine (4170 City Avenue, Philadelphia, PA 19131, PA).

References

Chen L, Hahn H, Wu G, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo Y, Bolli R, Dorn GW 2nd, Mochly-Rosen D (2001) Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. Proc Natl Acad Sci USA 98:11114–11119

Dang PM, Fontayne A, Hakim J, El Benna J, Perianin A (2001) Protein kinase C zeta phosphorylates a subset of selective sites of the NADPH oxidase component p47phox and participates in formyl peptide-mediated neutrophil respiratory burst. J Immunol 166:1206–1213

Entman ML, Michael L, Rossen RD, Dreyer WJ, Anderson DC, Taylor AA, Smith CW (1991) Inflammation in the course of early myocardial ischemia. Faseb J 5:2529–2537

Fan H, Sun B, Gu Q, Lafond-Walker A, Cao S, Becker LC (2002) Oxygen radicals trigger activation of NF-kappaB and AP-1 and upregulation of ICAM-1 in reperfused canine heart. Am J Physiol Heart Circ Physiol 282:H1778–H1786

Forman MB, Puett DW, Virmani R (1989) Endothelial and myocardial injury during ischemia and reperfusion: pathogenesis and therapeutic implications. J Am Coll Cardiol 13:450–459

Guo JP, Murohara T, Buerke M, Scalia R, Lefer AM (1996) Direct measurement of nitric oxide release from vascular endothelial cells. J Appl Physiol 81:774–779

Hansen PR (1995) Role of neutrophils in myocardial ischemia and reperfusion. Circulation 91:1872–1885

Hartman JC, Anderson DC, Wiltse AL, Lane CL, Rosenbloom CL, Manning AM, Humphrey WR, Wall TM, Shebuski RJ (1995) Protection of ischemic/reperfused canine myocardium by

CL18/6, a monoclonal antibody to adhesion molecule ICAM-1. Cardiovasc Res 30:47–54

Hirata K, Kuroda R, Sakoda T, Katayama M, Inoue N, Suematsu M, Kawashima S, Yokoyama M (1995) Inhibition of endothelial nitric oxide synthase activity by protein kinase C. Hypertension 25:180–185

Hu K, Mochly-Rosen D, Boutjdir M (2000) Evidence for functional role of epsilonPKC isozyme in the regulation of cardiac Ca(2+) channels. Am J Physiol Heart Circ Physiol 279:H2658–2664

Inagaki K, Hahn HS, Dorn GW 2nd, Mochly-Rosen D (2003) Additive protection of the ischemic heart ex vivo by combined treatment with delta-protein kinase C inhibitor and epsilon-protein kinase C activator. Circulation 108:869–875

Javaid K, Rahman A, Anwar KN, Frey RS, Minshall RD, Malik AB (2003) Tumor necrosis factor-alpha induces early-onset endothelial adhesivity by protein kinase Czeta-dependent activation of intercellular adhesion molecule-1. Circ Res 92:1089–1097

Johnson JA, Gray MO, Chen CH, Mochly-Rosen D (1996a) A protein kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. J Biol Chem 271:24962–24966

Johnson JA, Gray MO, Karliner JS, Chen CH, Mochly-Rosen D (1996b) An improved permeabilization protocol for the introduction of peptides into cardiac myocytes. Application to protein kinase C research. Circ Res 79:1086–1099

Kobayashi N, Hara K, Tojo A, Onozato ML, Honda T, Yoshida K, Mita S, Nakano S, Tsubokou Y, Matsuoka H (2005) Eplerenone shows renoprotective effect by reducing LOX-1-mediated adhesion molecule, PKCepsilon-MAPK-p90RSK, and Rho-kinase pathway. Hypertension 45:538–544

Kukielka GL, Smith CW, Manning AM, Youker KA, Michael LH, Entman ML (1995) Induction of interleukin-6 synthesis in the myocardium. Potential role in postreperfusion inflammatory injury. Circulation 92:1866–1875

Kuntscher MV, Schirmbeck EU, Menke H, Klar E, Gebhard MM, Germann G (2002) Ischemic preconditioning by brief extremity ischemia before flap ischemia in a rat model. Plast Reconstr Surg 109:2398–2404

Lasley RD, Noble MA, Mentzer RM Jr (1997) Effects of protein kinase C inhibitors in in situ and isolated ischemic rabbit myocardium. J Mol Cell Cardiol 29:3345–3356

Lefer AM, Lefer DJ (1996) The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia–reperfusion. Cardiovasc Res 32:743–751

Lefer DJ, Scalia R, Campbell B, Nossuli T, Hayward R, Salamon M, Grayson J, Lefer AM (1997) Peroxynitrite inhibits leukocyte-endothelial cell interactions and protects against ischemia–reperfusion injury in rats. J Clin Invest 99:684–691

Li H, Oehrlein SA, Wallerath T, Ihrig-Biedert I, Wohlfart P, Ulshofer T, Jessen T, Herget T, Forstermann U, Kleinert H (1998) Activation of protein kinase C alpha and/or epsilon enhances transcription of the human endothelial nitric oxide synthase gene. Mol Pharmacol 53:630–637

Lucchesi BR, Mullane KM (1986) Leukocytes and ischemia-induced myocardial injury. Annu Rev Pharmacol Toxicol 26:201–224

Ma XL, Lefer DJ, Lefer AM, Rothlein R (1992) Coronary endothelial and cardiac protective effects of a monoclonal antibody to intercellular adhesion molecule-1 in myocardial ischemia and reperfusion. Circulation 86:937–946

Ohlstein EH, Nichols AJ (1989) Rabbit polymorphonuclear neutrophils elicit endothelium-dependent contraction in vascular smooth muscle. Circ Res 65:917–924

Ohnuma Y, Miura T, Miki T, Tanno M, Kuno A, Tsuchida A, Shimamoto K (2002) Opening of mitochondrial K(ATP) channel occurs downstream of PKC-epsilon activation in the mechanism of preconditioning. Am J Physiol Heart Circ Physiol 283:H440–447

Olanders K, Sun Z, Borjesson A, Dib M, Andersson E, Lasson A, Ohlsson T, Andersson R (2002) The effect of intestinal ischemia and reperfusion injury on ICAM-1 expression, endothelial barrier function, neutrophil tissue influx, and protease inhibitor levels in rats. Shock 18:86–92

Omiyi D, Brue RJ, Taormina P 2nd, Harvey M, Atkinson N, Young LH (2005) Protein kinase C betaII peptide inhibitor exerts cardioprotective effects in rat cardiac ischemia/reperfusion injury. J Pharmacol Exp Ther 314:542–551

Pabla R, Buda AJ, Flynn DM, Blesse SA, Shin AM, Curtis MJ, Lefer DJ (1996) Nitric oxide attenuates neutrophil-mediated myocardial contractile dysfunction after ischemia and reperfusion. Circ Res 78:65–72

Park JW, Babior BM (1997) Activation of the leukocyte NADPH oxidase subunit p47phox by protein kinase C. A phosphorylation-dependent change in the conformation of the C-terminal end of p47phox. Biochemistry 36:7474–7480

Peterman EE, Taormina P 2nd, Harvey M, Young LH (2004) Go 6983 exerts cardioprotective effects in myocardial ischemia/reperfusion. J Cardiovasc Pharmacol 43:645–656

Phillipson A, Peterman EE, Taormina P Jr, Harvey M, Brue RJ, Atkinson N, Omiyi D, Chukwu U, Young LH (2005) Protein kinase C-zeta inhibition exerts cardioprotective effects in ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 289:H898–907

Rothlein R, Dustin ML, Marlin SD, Springer TA (1986) A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. J Immunol 137:1270–1274

Rubanyi GM, Desiderio D, Luisi A, Johns A, Sybertz EJ (1989) Phorbol dibutyrate inhibits release and action of endothelium-derived relaxing factor(s) in canine blood vessels. J Pharmacol Exp Ther 249:858–863

Scalia R, Coyle KM, Levine BJ, Booth G, Lefer AM (2000) C-peptide inhibits leukocyte-endothelium interaction in the microcirculation during acute endothelial dysfunction. Faseb J 14:2357–2364

Shi Y, Rafiee P, Su J, Pritchard KA Jr, Tweddell JS, Baker JE (2004) Acute cardioprotective effects of erythropoietin in infant rabbits are mediated by activation of protein kinases and potassium channels. Basic Res Cardiol 99:173–182

Soriano SG, Lipton SA, Wang YF, Xiao M, Springer TA, Gutierrez-Ramos JC, Hickey PR (1996) Intercellular adhesion molecule-1-deficient mice are less susceptible to cerebral ischemia-reperfusion injury. Ann Neurol 39:618–624

Stroes E, Kastelein J, Cosentino F, Erkelens W, Wever R, Koomans H, Luscher T, Rabelink T (1997) Tetrahydrobiopterin restores endothelial function in hypercholesterolemia. J Clin Invest 99:41–46

Stroes E, Hijmering M, van Zandvoort M, Wever R, Rabelink TJ, van Faassen EE (1998) Origin of superoxide production by endothelial nitric oxide synthase. FEBS Lett 438:161–164

Tiefenbacher CP, Chilian WM, Mitchell M, DeFily DV (1996) Restoration of endothelium-dependent vasodilation after reperfusion injury by tetrahydrobiopterin. Circulation 94:1423–1429

Tsao PS, Lefer AM (1990) Time course and mechanism of endothelial dysfunction in isolated ischemic- and hypoxic-perfused rat hearts. Am J Physiol 259:H1660–1666

Tsao PS, Aoki N, Lefer DJ, Johnson G 3rd, Lefer AM (1990) Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation 82:1402–1412

Vasquez-Vivar J, Martasek P, Whitsett J, Joseph J, Kalyanaraman B (2002) The ratio between tetrahydrobiopterin and oxidized tetrahydrobiopterin analogues controls superoxide release from endothelial nitric oxide synthase: an EPR spin trapping study. Biochem J 362:733–739

Weyrich AS, Buerke M, Albertine KH, Lefer AM (1995) Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of the ischemic feline myocardium. J Leukoc Biol 57:45–55

Wu Y, Wu G, Qi X, Lin H, Qian H, Shen J, Lin S (2006) Protein kinase C beta inhibitor LY333531 attenuates intercellular adhesion molecule-1 and monocyte chemotactic protein-1 expression in the kidney in diabetic rats. J Pharmacol Sci 101:335–343

Yellon DM, Hausenloy DJ (2007) Myocardial reperfusion injury. N Engl J Med 357:1121–1135

Young LH, Ikeda Y, Scalia R, Lefer AM (2000) C-peptide exerts cardioprotective effects in myocardial ischemia-reperfusion. Am J Physiol Heart Circ Physiol 279:H1453–1459

Young LH, Balin BJ, Weis MT (2005) Go 6983: a fast acting protein kinase C inhibitor that attenuates myocardial ischemia/reperfusion injury. Cardiovasc Drug Rev 23:255–272

Young LH, Phillipson A, Omiyi D, Atkinson N, Jivani M, Adams J, Peterman EE, Taormina P, 2nd, Brue RJ, Harvey M (2006) Protein kinase C isoform (PKC) peptide activator/inhibitors exert cardioprotective effects in polymorphonuclear leukocyte (PMN)-induced ischemia/reperfusion (I/R) injury. In: Blondelle SE (ed) Understanding biology using peptides. 19th American Peptide symposium: 457–458

Zatta AJ, Kin H, Lee G, Wang N, Jiang R, Lust R, Reeves JG, Mykytenko J, Guyton RA, Zhao ZQ, Vinten-Johansen J (2006) Infarct-sparing effect of myocardial postconditioning is dependent on protein kinase C signalling. Cardiovasc Res 70:315–324

Zhang J, Baines CP, Zong C, Cardwell EM, Wang G, Vondriska TM, Ping P (2005) Functional proteomic analysis of a three-tier PKCepsilon-Akt-eNOS signaling module in cardiac protection. Am J Physiol Heart Circ Physiol 288:H954–961

Zhao ZQ, Lefer DJ, Sato H, Hart KK, Jefforda PR, Vinten-Johansen J (1997) Monoclonal antibody to ICAM-1 preserves postischemic blood flow and reduces infarct size after ischemia–reperfusion in rabbit. J Leukoc Biol 62:292–300

Zulueta JJ, Yu FS, Hertig IA, Thannickal VJ, Hassoun PM (1995) Release of hydrogen peroxide in response to hypoxia-reoxygenation: role of an NADPH oxidase-like enzyme in endothelial cell plasma membrane. Am J Respir Cell Mol Biol 12:41–49