Protein kinase C-ζ inhibition exerts cardioprotective effects in ischemia-reperfusion injury

Aisha Phillipson, Ellen E. Peterman, Philip Taormina, Jr., Margaret Harvey, Richard J. Brue, Norrell Atkinson, Didi Omiyi, Uchenna Chukwu, and Lindon H. Young

Department of Pathology, Microbiology, and Immunology, Philadelphia College of Osteopathic Medicine, Philadelphia, Pennsylvania

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Protein kinase C-ζ inhibition exerts cardioprotective effects in ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 289: H898–H907, 2005. First published March 25, 2005; doi:10.1152/ajpheart.00883.2003.—Ischemia followed by reperfusion (I/R) in the presence of polymorphonuclear leukocytes (PMNs) results in marked cardiac contractile dysfunction. A cell-permeable PKC-ζ peptide inhibitor was used to test the hypothesis that PKC-ζ inhibition could attenuate PMN-induced cardiac contractile dysfunction by suppression of superoxide production from PMNs and increase nitric oxide (NO) release from vascular endothelium. The effects of the PKC-ζ peptide inhibitor were examined in isolated ischemic (20 min) and reperfused (45 min) rat hearts reperfused with PMNs. The PKC-ζ inhibitor (2.5 or 5 μM, n = 6) significantly attenuated PMN-induced cardiac dysfunction compared with I/R hearts (n = 6) receiving PMNs alone in left ventricular developed pressure (LVPD) and the maximal rate of LVPD (+dP/dt max) cardiac function indexes (P < 0.01), and these cardioprotective effects were blocked by the NO synthase inhibitor, Nω-nitro-ω-arginine methyl ester (50 μM). Furthermore, the PKC-ζ inhibitor significantly increased endothelial NO release 47 ± 2% (2.5 μM, P < 0.05) and 54 ± 5% (5 μM, P < 0.01) over basal values from the rat aorta and significantly inhibited superoxide release from phorbol-12-myristate-13-acetate-stimulated rat PMNs by 33 ± 12% (2.5 μM) and 40 ± 8% (5 μM) (P < 0.01). The PKC-ζ inhibitor significantly attenuated PMN infiltration into the myocardium by 46–48 ± 4% (P < 0.01) at 2.5 and 5 μM, respectively. In conclusion, these results suggest that the PKC-ζ peptide inhibitor attenuates PMN-induced post-I/R cardiac contractile dysfunction by increasing endothelial NO release and by inhibiting superoxide release from PMNs thereby attenuating PMN infiltration into I/R myocardium.

PMN-endothelium interaction, which occurs by 10 to 20 min postreperfusion, and subsequent PMN infiltration into the myocardium is observed by 30 min postreperfusion (20, 21, 35, 39). The time course of events are similar in ex vivo and in vivo myocardial ischemia-reperfusion (I/R) models within the first 30 min of reperfusion with respect to endothelial dysfunction and PMN-endothelium interactions (26, 35–37). However, the in vivo model requires a longer reperfusion period (i.e., 270 min) to accumulate PMNs in the reperfused myocardium and induce myocardial injury (i.e., infarct size) (26, 35) compared with the ex vivo I/R model, in which PMNs are infused at the beginning of reperfusion and readily accumulate into the myocardium between 30 and 45 min of reperfusion to induce myocardial injury (i.e., sustained cardiac contractile dysfunction) (5, 29, 41–43).

Chemotactic substances released from reperfused tissue and plasma factors activate PMNs that augment PMN release of cytotoxic substances (i.e., superoxide anion) and contribute to cardiac contractile dysfunction after I/R (24, 25, 35, 37). Superoxide combines with NO to produce peroxynitrite anion, thus reducing the bioavailability of NO, and promotes endothelial dysfunction and PMN infiltration after myocardial I/R (6, 13, 24, 31, 37, 38). Likewise, compounds that increase endothelium-derived NO release quench superoxide anion from PMNs and reperfused tissues and inhibit PMN infiltration and preserve cardiac function after I/R (21, 41). These findings suggest that compounds that enhance endothelial NO release or inhibit endothelium–PMN superoxide release may exert cardioprotective effects in I/R injury (29).

Protein kinase C (PKC) is an important cytosolic enzyme involved in the signaling pathway of PMNs and endothelial cells. In PMNs, PKC activation increases superoxide release via phosphorylation of the cytosolic factor p47phox, which is required for NADPH oxidase activation (1, 7, 22, 40). In endothelial cells, PKC activation inhibits endothelial NO synthase (eNOS) activity, resulting in decreased endothelial NO release (14, 30), and augments superoxide and hydrogen peroxide production via NADPH oxidase (44). Consequently, inhibition of PKC leads to enhanced NO bioavailability in endothelial cells by these two mechanisms (41, 42).

PKC exists in different isoforms that are subdivided into three biochemical classes of function (9). The classical isoforms are Ca2+ dependent and activated by diacylglycerol (DAG); the novel isoforms are Ca2+ independent but are activated by DAG; and, finally, the atypical isoforms are Ca2+ and DAG independent (9, 11). Of particular interest is the...
atypical isoform PKC-ζ, which can be stimulated by phospholipids and augments superoxide production in PMNs and endothelial cells and PMN chemotaxis (7, 8, 10, 19). However, the role of PKC-ζ in myocardial I/R and endothelial NO release has not been previously characterized.

In this study, the hypothesis tested was that PKC-ζ inhibition attenuates PMN-induced cardiac contractile dysfunction and PMN vascular adherence and infiltration in postperfused hearts. In addition, the hypothesis predicted that PKC-ζ inhibition attenuates PMN superoxide release and augments endothelial NO release. The selective PKC-ζ peptide inhibitor used in these assays was myristoylated (fatty acid moiety) to allow for rapid cell permeability (within 10 s) and was 13 amino acids in length (N-myristoyl-SIYRRGARRWRKL, mol wt = 1,928, Genemed Synthesis). The peptide sequence corresponds to positions 113–125 of the pseudosubstrate domain on PKC-ζ, thereby inhibiting PKC-ζ from interacting with other cellular substrates (i.e., NADPH oxidase) (7, 19). Dang et al. (7) observed an IC₅₀ equal to ~10 μM with respect to inhibition of PMN superoxide release.

METHODS

Isolated Rat Heart Preparation

The Institutional Animal Care and Use Committee of Philadelphia College of Osteopathic Medicine approved all animal protocols performed in this study. Male Sprague-Dawley rats (275–325 g, Ace Animals; Boyertown, PA) were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally. Heparin sodium (1,000 units) was also administered intraperitoneally. The hearts were rapidly excised, the ascending aorta were cannulated, and retrograde perfusion of the heart was initiated with a modified Krebs buffer maintained at 37°C at a constant pressure of 80 mmHg. The Krebs buffer had the following composition (in mmol/l): 113 NaCl, 25 NaHCO₃, 2.5 CaCl₂, 0.5 EDTA, 5.9 KCl, and 1.2 MgCl₂. The perfusate was aerated with 95% O₂-5% CO₂ and equilibrated at a pH of 7.3–7.4. The two side arms in the perfusion line were monitored using a pressure transducer (SPR-524, Millar Instruments; Houston, TX), which was positioned in the left ventricular cavity. Hearts were immersed in a water-jacketed reservoir containing 160 ml Krebs buffer maintained at 37°C. Coronary flow, LVDP, and +dP/dt were recorded using a Powerlab Station acquisition system (ADInstruments; Grand Junction, CO) in conjunction with a computer (GateWay).

LVDP, +dP/dt, and coronary flow were measured every 5 min for 15 min to equilibrate the hearts and obtain a baseline measurement. LVDP was defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure.

After 15 min, the flow of the Krebs buffer was reduced to zero for 20 min to induce global ischemia. At reperfusion, hearts were infused for 5 min with 200 × 10⁶ PMNs resuspended in 5 ml Krebs buffer plus 5 ml plasma at a rate of 1 ml/min. In some experiments, the PKC-ζ peptide inhibitor (Genemed Synthesis) was added to plasma at a final concentration of 1, 2.5, or 5 μM. Sham I/R hearts were not subjected to ischemia and were not perfused with PMNs. We (29) recently showed that sham I/R hearts given PMNs exhibited no changes from initial control values.

Groups of Isolated Perfused Hearts

The following groups of isolated perfused rat hearts were used.

Group 1. Sham I/R hearts were not subjected to ischemia and were not perfused with PMNs but were perfused with 5 ml plasma (1 ml/min) at 35 min into perfusion (the same time point that I/R hearts would be given 5 ml plasma, 15 min of baseline recordings plus 20 min ischemia). These hearts represented a control group to determine whether the isolated rat heart could maintain LVDP and +dP/dt throughout the 80-min protocol (n = 6).

Group 2. Sham I/R + PKC-ζ peptide inhibitor (5 μM) hearts were not subjected to ischemia and were not perfused with PMNs. These hearts were administered the PKC-ζ peptide inhibitor (5 μM, dissolved in plasma from a 5 mM stock in H₂O) 35 min into perfusion. This group was employed to determine whether the PKC-ζ peptide inhibitor causes a cardiotoxic or cardiodepressant effect (n = 6).

Group 3. I/R hearts were subjected to 20 min of ischemia and were perfused with 5 ml plasma (1 ml/min) during the first 5 min of reperfusion but were not perfused with PMNs. These hearts represented a control group to determine whether 20 min of ischemia followed by reperfusion stuns the heart but whether LVDP and +dP/dt would recover to baseline values (initial) by the end of the 45-min reperfusion period (n = 6).

Group 4. I/R + PKC-ζ peptide inhibitor (5 μM, dissolved in plasma) hearts were subjected to 20 min of ischemia and were not perfused with PMNs. These hearts are perfused with 5 ml plasma + PKC-ζ inhibitor during the first 5 min of reperfusion. This group was employed to determine whether the PKC-ζ peptide inhibitor causes a cardiodepressant effect in the setting of I/R without PMNs (n = 6).

Group 5. I/R + PMN hearts were subjected to 20 min of ischemia and were perfused with 5 ml plasma (1 ml/min) and PMNs (resuspended in 5 ml Krebs buffer) during the first 5 min of reperfusion. These hearts represented a control group to determine whether 20 min of ischemia followed by 45 min reperfusion in the presence of PMNs (200 × 10⁶) results in a sustained cardiac contractile dysfunction throughout the 45-min reperfusion period compared with initial baseline values (5, 29, 41–43) (n = 6).

Group 6. I/R + PMN + PKC-ζ peptide inhibitor (1 μM) hearts were subjected to 20 min of ischemia and were perfused with 1 μM PKC-ζ peptide inhibitor (dissolved in plasma) and PMNs (200 × 10⁶) during the first 5 min of reperfusion. These hearts represented a group to determine the effect of PKC-ζ inhibition in attenuating PMN-induced cardiac contractile dysfunction (n = 6).

Group 7. I/R + PMN + PKC-ζ peptide inhibitor (2.5 μM) hearts were subjected to 20 min of ischemia and were perfused with 2.5 μM PKC-ζ peptide inhibitor (dissolved in plasma) and PMNs (200 × 10⁶) during the first 5 min of reperfusion. These hearts represented a group to determine the effect of PKC-ζ inhibition in attenuating PMN-induced cardiac contractile dysfunction (n = 6).

Group 8. I/R + PMN + PKC-ζ peptide inhibitor (5 μM) hearts were subjected to 20 min of ischemia and were perfused with 5 μM PKC-ζ peptide inhibitor (dissolved in plasma) and PMNs (200 × 10⁶) during the first 5 min of reperfusion. These hearts represented a group to determine the effect of PKC-ζ inhibition at a higher concentration of the PKC-ζ peptide inhibitor in attenuating PMN-induced cardiac contractile dysfunction (n = 6).

Group 9. I/R + PMN + PKC-ζ peptide inhibitor (5 μM) + N⁴-nitro-l-arginine methyl ester (l-NAME; 50 μM) hearts were subjected to 20 min of ischemia and were perfused with 5 μM PKC-ζ peptide inhibitor (dissolved in 5 ml plasma) and 50 μM l-NAME (dissolved in Krebs buffer from 50 mM stock in H₂O) during the first 5 min of reperfusion. l-NAME (50 μM) was continually infused into the heart throughout the 45-min reperfusion period. These hearts represented a group to determine whether the cardioprotective effect of PKC-ζ peptide inhibition can be blocked with a NO synthase inhibitor (l-NAME) (n = 5).

Data were recorded every 5 min for 45 min postreperfusion. After each experiment, the left ventricle was isolated, fixed in 4% paraformaldehyde, and stored at 4°C for later histological analysis.
Isolation of Plasma

The plasma used for infusion with the PMNs was isolated from the same rat from which the heart was isolated from for each cardiac perfusion experiment to more closely simulate the conditions in vivo. Blood was collected from the aorta in citrate phosphate buffer (Sigma Chemical; St. Louis, MO) over a period of 1 min just before isolation of the rat heart. The blood was centrifuged at 10,000 g for 10 min at 4°C. The plasma was then decanted and used for infusion in each heart. Five milliliters of plasma collected from a single rat were used for each perfused heart.

Isolation of PMNs

Male Sprague-Dawley rats (350–400 g, Ace Animals), used as PMN donors, were anesthetized with ethyl ether and given a 16 ml ip injection of 0.5% glycerogen (Sigma Chemical) dissolved in PBS (42). Rats were reanesthetized with ethyl ether 16–18 h later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (42). The peritoneal lavage fluid was centrifuged at 250 g for 20 min at 4°C. The PMNs were then washed in 20 ml PBS and centrifuged at 250 g for 10 min at 4°C. Thereafter, the PMNs were resuspended in 2.5 ml PBS, and a total of 10–12 samples was pooled before use in cardiac perfusion experiments. The PMN preparations were >90% pure and >95% viable according to microscopic analysis and exclusion of 0.3% Trypan blue, respectively.

Determination of PMN Vascular Adherence and Infiltration Into Cardiac Tissue

Three rat hearts from each of the nine experimental groups were used for histological analysis. Ten similar areas of each heart section, ranging from the endocardium throughout the myocardium to the epicardium of the left ventricle, were counted for PMN vascular adherence and infiltration. The hearts were dehydrated in graded water for 30 s. The number of PMNs was counted by light microscopy onto glass slides. Sections were then placed in 100% ethanol for 5 min and were embedded in plastic, sectioned into 2.5-μm serial sections, and placed onto glass slides. Sections were then placed in 100% ethanol for 5 min to remove the plastic and rehydrated in tap water for 1 min. Subsequently, hematoxylin was applied to the sections for 7 min, and the sections were rinsed under running tap water for 30 s. Eosin stain was then applied for 2 min, followed by a second rinse under running tap water for 30 s. The number of PMNs was counted by light microscopy (41, 42). To determine the effect of the PKC-ζ peptide inhibitor on PMN adherence, the intravascular PMNs that adhered to the lumen of the coronary vascular endothelium were counted and expressed as adhered intravascular PMNs per millimeter squared. The total amount of PMNs counted within the heart tissue sections is expressed as total intravascular and infiltrated PMNs per millimeter squared area of cardiac tissue.

Measurement of NO Release From Rat Aortic Segments

Rat aortas were isolated after the rats were anesthetized with pentobarbital sodium (60 mg/kg). The excised aortas were immersed in warm oxygenated (95% O₂, 5% CO₂) Krebs-Henselit (K-H) buffer solution. The K-H buffer had the following composition (in mmol/l): 10 dextrose, 119 NaCl, 12.5 NaHCO₃, 2.5 CaCl₂, 4.8 KCl, 1.2 KH₂PO₄, and 1.2 MgSO₄. The aortas were cleaned of adherent fat and connective tissue, and rings 6–7 mm long (i.e., 10 mg wet wt) were prepared. The aortic rings were cut, spread open, and fixed by pins with the endothelial surface facing up in 24-well culture dishes containing 1 ml K-H solution (41). In other aortic segments, the endothelium was removed (denuded) from the vascular smooth muscle using mechanical isolation with a cotton swab to determine the source of NO release. After equilibration at 37°C, NO released into the buffer solution was measured after the administration of PKC-ζ peptide inhibitor (1–15 μM) to the aortic rings. Acetylcholine (200 nM) was used as a positive control to assess the viability of the endothelium. Basal rat aortic endothelial NO release was determined by placing the NO electrode in a well containing only K-H buffer and then placing the NO electrode in a well containing aortic tissue. The difference between the two readings determined the basal NO release for that aortic endothelial segment (41). After basal NO release, the effect of acetylcholine (200 nM) and PKC-ζ peptide inhibitor were determined, 400 μM L-NAME was added to the K-H buffer solution, and NO release was reassessed 30 min later in the presence of acetylcholine (200 nM) or PKC-ζ peptide (5 μM) inhibitor. The NO release was measured using a calibrated NO meter (Iso-NO, World Precision Instruments; Sarasota, FL) connected to a polygraph internally shielded NO electrode (12, 41). NO released into the medium was reported in picomoles per milligram of aortic tissue. Between 6 and 20 trials were performed for each group.

Measurement of Superoxide Radical Release From Rat PMNs

The superoxide anion release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c (42). The PMNs (5 × 10⁶) were resuspended in 450 μl PBS and incubated with ferricytochrome c (100 μM, Sigma Chemical) in a total volume of 900 μl PBS for 15 min at 37°C in spectrophotometric cells. PKC-ζ peptide inhibitor was added to the 900 μl PMN-ferricytochrome c suspension, mildly vortexed to yield a final concentration of 1, 2.5, 5, or 15 μM, and incubated at 37°C for 15 min in spectrophotometric cells. Control samples did not contain PKC-ζ peptide inhibitor. The PMNs were stimulated with 15 nM phorbol-12-myristate-13-acetate (PMA; Sigma Chemical) in a final reaction volume of 1.0 ml. Positive control samples were given superoxide dismutase (SOD; 10 μg/ml) just before the addition of PMA. Absorbance at 550 nm was measured every 30 s for up to 360 s (peak response), and the change (Δ) in superoxide anion release from PMNs was determined from time 0.

Limitations of the Study

A latex balloon was not inserted into the left ventricle, and therefore this study does not have a fixed preload volume as in other ex vivo isolated perfused rat heart studies using a similar protocol of 20-min global ischemia and 45-min reperfusion (16, 33). This limitation should be taken into consideration when the data are interpreted. However, the animals that were used in this study were within a specific weight range (275–325 g) for each group, and the initial baseline LVDP and +dP/dtmax values were similar among all of the study groups, and so therefore there should be similar preloads. The isolated rat heart was cannulated via the aorta onto a perfusion needle and was immersed in a water-jacketed reservoir that contained 160 ml Krebs buffer maintained at 37°C. The preload volume comes from Krebs buffer that fills the left ventricle upon insertion of the pressure transducer catheter via a small incision (i.e., 1 mm) in the base of the left side of the heart as in previous studies (41–43). The initial baseline left ventricular end-diastolic pressure was between 4 and 8 mmHg for all hearts in each study group. In the event that a procedural mistake was made and the perfusion needle penetrated the aortic valve, the heart experienced an increase in diastolic pressure (i.e., >10 mmHg) accompanied by arrhythmia within 5 min of perfusion. The preparation was therefore discarded and not included in any analyses. This ex vivo myocardial I/R model was designed for PMN-induced myocardial I/R injury and differed from the in vivo model, which requires 90 min of ischemia (left anterior descending coronary artery occlusion) followed by 270-min reperfusion before significant PMN infiltration in postreperfused myocardial tissue is observed (35, 42). The ex vivo model was intended to evaluate compounds that have the potential to attenuate PMN-induced myocardial I/R injury.

The aortic tissue NO release and PMN superoxide release are in vitro assays that may not be physiologically relevant to in vivo conditions. The results obtained from these assays were intended to...
suggest a NO and/or superoxide mechanism by which the PKC-ζ peptide inhibitor may exert its cardioprotective effects.

Statistical Analysis

All data in the text and figures are presented as means ± SE. 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.

On the basis of the number of planned groups for comparison coupled with the realistic constraints regarding the number of hearts that could be evaluated, it is recognized that a very strong effect is required to achieve statistical significance. Depending on the number of hearts and groups being compared, effect sizes between 2.5 and 3.2 are required to achieve statistical significance.

RESULTS

The time course of cardiac contractile function (i.e., LVDP) is shown in Figure 1. Data from the sham I/R, I/R, I/R + PMN + PKC-ζ peptide inhibitor (5 μM), and I/R + PMN groups illustrate the relative changes in LVDP during the 80-min perfusion period. As shown, the sham I/R group remained near or >100% of initial baseline values of LVDP for the entire perfusion period. The I/R hearts experienced a depression in LVDP at the beginning of reperfusion but recovered to 95 ± 7% of initial baseline values by the end of reperfusion. In contrast, the I/R + PMN hearts suffered severe cardiac contractile dysfunction, recovering to only 47 ± 7% of initial baseline values by 45 min postreperfusion. Conversely, the I/R + PMN + PKC-ζ peptide inhibitor (5 μM) hearts recovered to 84 ± 4% at 45 min postreperfusion.

To determine whether PKC-ζ peptide inhibitor produced direct inotropic effects on cardiac contractile function, nonischemic sham I/R hearts were perfused with the PKC-ζ peptide inhibitor (5 μM). Treatment of sham I/R hearts with PKC-ζ peptide inhibitor did not result in any significant change in LVDP (Fig. 2) or +dP/dt max (Fig. 3) during the 80-min perfusion period, demonstrating the PKC-ζ peptide inhibitor at 5 μM exerts no direct effect on cardiac contractile function. A 15 μM PKC-ζ peptide inhibitor concentration was initially tested because this concentration corresponded with an 82% inhibition of PMN superoxide release. However, 15 μM produced a cardiodepressant effect (50% reduction in LVDP in sham I/R hearts) and could not be used in these experiments (data not shown).

Figures 2 and 3 show the initial and final values for LVDP and +dP/dt max from isolated perfused rat hearts. The initial baselines were similar for all groups. However, the final LVDP and +dP/dt max (45 min postreperfusion) were significantly decreased (P < 0.01) by 47 ± 7% and 41 ± 7%, respectively, for I/R hearts reperfused with PMNs compared with initial baseline values. The PKC-ζ peptide inhibitor (2.5 and 5 μM concentrations) significantly attenuated the decrease in LVDP and +dP/dt max associated with posts ischemic reperfusion with PMNs. In the group receiving 5 μM of drug, hearts recovered to 84 ± 4% and 76 ± 5% for final LVDP and +dP/dt max compared with initial baseline values. The lowest effective dose was observed at 2.5 μM, and these hearts recovered to 83 ± 4% for LVDP and 75 ± 5% for +dP/dt max compared with initial baseline values. The cardioprotective effects of the PKC-ζ peptide inhibitor (5 μM) were blocked in the presence of L-NAME (50 μM). These hearts only recovered to 58 ± 7% and 54 ± 9% for LVDP and +dP/dt max, respectively, at 45 min postreperfusion compared with initial baseline values. These hearts were similar to the I/R + PMN group (47 ± 7% and 41 ± 7% LVDP and +dP/dt max). At 1 μM, PKC-ζ peptide inhibitor-treated hearts exposed to I/R + PMNs only recovered to 57 ± 10% and 46 ± 6% for LVDP and +dP/dt max, respectively, at 45 min postreperfusion compared with initial baseline values. These hearts were not significantly different from control I/R + PMN hearts at 45 min postreperfusion at this lower dose.

The cardiac injury associated with I/R in this model was closely correlated with the substantial number of PMNs infiltrating the myocardium within the 45-min reperfusion period. During reperfusion, a significant number of PMNs transmigrated into the myocardium, increasing from <25 PMNs/mm² in sham I/R hearts to >180 PMNs/mm² in I/R + PMN hearts.
at the end of the reperfusion period (Fig. 4A). In contrast, I/R + PMN + PKC-ζ peptide inhibitor-treated hearts experienced a 20 ± 6%, 46 ± 4%, and 48 ± 3% significant reduction in PMN infiltration into the postreperfused cardiac tissue at 1, 2.5, and 5 μM (P < 0.01), respectively, and this effect was blocked in the presence of L-NAME. Furthermore, the 2.5 and 5 μM-treated hearts had significantly fewer infiltrated PMNs compared with 1 μM-treated hearts (P < 0.01; Fig. 4A).

PMN adherence to coronary vascular endothelium was also evaluated within the assessment of total intravascular and infiltrated PMNs. As seen in Fig. 4B, the number of adherent PMNs to the coronary endothelium was not significantly reduced in I/R + PMN + PKC-ζ peptide inhibitor (5 μM)-treated hearts (43 ± 10%, P < 0.06; Fig. 4B).

NO release from rat aortic endothelium was measured to determine whether PKC-ζ peptide inhibitor provides cardio-
protection by a mechanism involving increased endothelial NO release. In Fig. 5, PKC-ζ peptide inhibitor-treated endothelium generated significantly more NO by 47 ± 2% (2.5 μM, P < 0.05), 54 ± 5% (5 μM, P < 0.01), and 91 ± 15% (15 μM, P < 0.01) compared with basal NO release. The numbers of PMNs adhering to the coronary vasculature were not significantly different from I/R + PMN. Hatched boxes represent non-PMN-perfused hearts, and solid boxes represent PMN-perfused hearts. **P < 0.01 from I/R + PMN. Hatched boxes represent non-PMN-perfused hearts, and solid boxes represent PMN-perfused hearts. All values are mean numbers of PMNs/mm² heart area ± SE. **P < 0.01 from I/R + PMN.

Acetylcholine (200 nM) was used as a positive control in the NO assay and significantly increased NO release by 67 ± 4% (P < 0.01) compared with basal NO release. The eNOS inhibitor L-NAME was used as another control to decrease basal release.
of NO to zero. Both the acetylcholine and PKC-ζ peptide inhibitor-induced production of NO were completely inhibited by treating the endothelium with L-NAME (400 μM). To attribute the source of the NO to the endothelium, experiments with endothelium-removed (denuded) rat aortic segments were incubated with the PKC-ζ peptide inhibitor (5 μM) and were not different from L-NAME-treated segments.

Another mechanism of the cardioprotective effects of PKC-ζ peptide inhibitor may be related to inhibition of superoxide release. The PKC-ζ peptide inhibitor significantly inhibited superoxide release by 33–82% (2.5–15 μM, *P* < 0.01) except at 1 μM, where there was no difference from suspensions of PMA-stimulated rat PMNs (Fig. 6). SOD (10 μg/ml) was used as a positive control in the superoxide assays and degraded...
superoxide release produced by the PMA-stimulated rat PMNs by 99% ($P < 0.01$; Fig. 6).

**DISCUSSION**

The major findings of this study were as follows: 1) I/R + PMN + PKC-ζ peptide inhibitor-treated rat hearts (5 μM) exerted maximal restoration of postreperfusion LVDP and $+\text{dP}/\text{d}t_{\text{max}}$ and was associated with maximal attenuation of total intravascular PMN infiltration in postperfused myocardium; 2) both of these effects were blocked in the presence of $\ell$-NAME, which was similar to I/R + PMN control hearts; 3) NO release in rat aortic segments was significantly increased by 54% in the presence of the PKC-ζ inhibitor (5 μM); 4) this effect was blocked by $\ell$-NAME and in denuded segments; and 5) PMA-stimulated PMN superoxide release was significantly inhibited by 40% in the presence of the PKC-ζ inhibitor (5 μM).

The PKC-ζ peptide inhibitor’s cardioprotective effects were characterized by a dose-dependent significant restoration of postreperfusion LVDP and $+\text{dP}/\text{d}t_{\text{max}}$. In addition, the cardioprotective effects of the PKC-ζ peptide inhibitor were blocked by $\ell$-NAME, suggesting that some of the cardioprotective effects were mediated by a NO mechanism (21, 27, 41).

The cardioprotective effect by the PKC-ζ inhibitor was associated with attenuating the PMN-induced cardiac dysfunction in this model of I/R (29, 41, 42). Our previous study (29) showed that sham I/R hearts perfused with the same number of PMNs do not demonstrate induced cardiac contractile dysfunction and PMN infiltration into the myocardium and suggests that I/R is required to elicit an appropriate chemotactic stimulus for PMN infiltration and cardiac contractile dysfunction. In addition, perfusion of untreated I/R hearts without PMNs does not result in prolonged cardiac contractile dysfunction, indicating that global ischemia alone was not the cause of sustained contractile dysfunction in this model of I/R (25, 36, 42, 43). Furthermore, sham I/R hearts and I/R hearts treated with the PKC-ζ peptide inhibitor did not show a direct effect on cardiac contractile function, indicating that the PKC-ζ peptide inhibitor imparts no direct effect on cardiac contractile function at the highest dose used in this assay.

In contrast to our results regarding PMN-induced myocardial I/R injury, previous studies (3, 18) suggest that PMNs do not significantly contribute to cardiac contractile dysfunction after I/R. This may be due to differences in the duration of ischemia between their study (i.e., 15 min) (18) and our study (i.e., 20 min). In our model of I/R, 20 min of ischemia is the time period required to induce a sufficient chemotactic stimulus for PMN infiltration (15, 29, 42). Additionally, a longer duration of ischemia (i.e., 30 min) results in significant cardiac contractile dysfunction, hearts do not recover to initial LVDP values in the absence of PMN reperfusion, and it is not appropriate to attribute the I/R dysfunction to PMNs (15, 42).

In our ex vivo I/R model, cardioprotection may be provided by a significant reduction in PMN infiltration into postischemic cardiac tissue (29, 41–43). The PKC-ζ peptide inhibitor dose dependently inhibited PMN infiltration, and this effect was blocked by $\ell$-NAME. However, intravascular coronary adherence was similar among all of the doses tested and was not significantly different from the I/R + PMN group. The fact that the majority of PMNs (i.e., 70%) had transmigrated into the postreperfused tissue suggests that differences in PMN vascular adherence between I/R + PMN + PKC-ζ peptide inhibitor-treated hearts would conceivably be observed at earlier time points. Furthermore, the reduced proportion of adhered PMNs per millimeter squared compared with total PMNs per millimeter squared would require a larger difference (i.e., 60%) between the I/R + PMN and treatment groups to achieve statistical significance (29).

The cardiac function results were further supported by the NO release and superoxide release assay results. The PKC-ζ peptide inhibitor dose dependently augmented NO release from the rat aorta and inhibited PMN superoxide release. Furthermore, the source of stimulated NO release by the PKC-ζ peptide inhibitor can be attributed to the aortic endothelium, because denuded segments blocked NO release to the same extent as $\ell$-NAME-treated segments. Although there is no direct link between the in vitro assays (i.e., NO release) and I/R hearts, these results tend to parallel the cardiac function data except for the 15 μM dose. This dose was most effective at augmenting NO release and inhibiting superoxide release yet exerted cardio depressant effects in non-PMN-perfused hearts. The negative effects observed in the isolated heart preparation may be due to differences in concentration sensitivity between a whole organ and isolated cells (e.g., PMNs) or vascular segments (e.g., aortic endothelium) (29).

During the early reperfusion period (5 min) of acute myocardial ischemia, PKC activation is enhanced (34). PKC activation inhibits eNOS; therefore, releasing the inhibition on this enzyme allows for an increase in NO production (14, 41). Previous studies (2, 17) have suggested that inhibition of the classical isoform PKC-β attenuates vascular dysfunction in humans and animal models with Type 1 diabetes. However, the compound used in those studies inhibited both PKC-β1 and -βII isoforms and was not selective for a specific isoform (17). In this study, the considerable increase in endothelial release of NO may be due to enhanced eNOS activity resulting from selective PKC-ζ inhibition (14). The doses of the PKC-ζ peptide inhibitor used in this study have been previously described to be specific for the PKC-ζ pseudosubstrate binding domain and do not cross-react with other PKC isoforms (7).

Activation of PKC-ζ is also associated with an increase in superoxide and hydrogen peroxide release from endothelial cells and PMNs (7, 10), suggesting that inhibition of superoxide release would enhance NO bioavailability (6). Previous studies (6, 28, 32) have shown that superoxide anion increases endothelial cell adhesion expression and quenches endogenous NO.

In this study, the PKC-ζ inhibitor attenuated the PMA-stimulated PMN superoxide response. A previous study (7) using the same type of PKC-ζ peptide inhibitor also showed 70% inhibition of the PMN superoxide response in a similar concentration range. However, the present results differ from the Dang et al. study in that the PMA-stimulated PMN superoxide response was inhibited in this study, whereas the N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated PMN superoxide response was inhibited in the Dang et al. study. This is most likely due to differences in the concentrations of PMA and fMLP used in the two studies.

Prior studies have associated PKC-ζ not only with PMN and endothelial cell superoxide release but also with PMN chemotaxis (7, 10, 19). Thus inhibition of PKC-ζ would attenuate...
PMN extravasation during the postreperfusion acute inflammatory response (41, 42).

In summary, these results show a cardioprotective effect on LVDP and +dP/dt max by the selective PKC-δ peptide inhibitor in the isolated perfused rat heart after I/R + PMNs. This cardioprotection appears to be related to attenuation of PMN infiltration into the myocardium along with an increase in endothelium-derived NO and inhibition of PMN-generated superoxide release. The net result would be attenuation cardiac contractile dysfunction in PMN-induced I/R injury.

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