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# Title Page

# Protein Kinase C beta II (PKC βII) peptide inhibitor exerts cardioprotective effects in rat cardiac ischemia/reperfusion injury

by

Didi Omiyi, Richard J. Brue, Philip Taormina II, Margaret Harvey, Norrell Atkinson and Lindon H Young

Department of Pathology, Microbiology and Immunology

Philadelphia College of Osteopathic Medicine

Philadelphia, PA 19131

**Running Title Page**

**Running title:** **PKC βII inhibition in Ischemia/Reperfusion**

**Corresponding Author:** Lindon H. Young Ph.D.

Department of Pathology, Microbiology and Immunology,

Philadelphia College of Osteopathic Medicine,

4170 City Avenue,

Philadelphia, PA 19131

Email: **LindonYo@pcom.edu**

Phone: (215) 871-6832

Fax: (215) 871-6869

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**List of nonstandard abbreviations:** $+dP/dt_{max}$, maximal rate of development of pressure; eNOS, endothelial nitric oxide synthase; fMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine, I/R, ischemia/reperfusion; L-NAME, $N^G$-nitro-L-arginine methyl ester; LVDP, left ventricular developed pressure; NO, nitric oxide; PKC-(α, βI, βII, δ, ε and ζ), protein kinase C-(alpha, beta I, beta II, delta, epsilon and zeta); PMNs, polymorphonuclear leukocytes; RACK-1, receptor for activated C kinase-1; SOD, superoxide dismutase.

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## Abstract

Ischemia followed by reperfusion (I/R) in the presence of polymorphonuclear leukocytes (PMNs) results in a marked cardiac contractile dysfunction. A cell permeable PKC βII peptide inhibitor was used to test the hypothesis that PKC βII inhibition could attenuate PMN-induced cardiac dysfunction by suppression of superoxide production from PMNs and increase nitric oxide (NO) release from vascular endothelium. The effects of the PKC βII peptide inhibitor were examined in isolated ischemic (20 min) and reperfused (45 min) rat hearts with PMNs. The PKC βII inhibitor (10 μM, n=7) significantly attenuated PMN-induced cardiac dysfunction compared to I/R hearts (n = 9) receiving PMNs alone in left ventricular developed pressure (LVDP) and the maximal rate of LVDP (+dP/dt max) cardiac function indices ($P<0.01$). The PKC βII inhibitor at 10 μM significantly increased endothelial NO release from a basal value of 1.85±0.18 picomoles NO/mg tissue to 3.49±0.62 picomoles NO/mg tissue from rat aorta. It also significantly inhibited superoxide release (i.e. absorbance) from N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated rat PMNs from 0.13±0.01 to 0.02±0.004 ($p<0.01$) at 10 μM. Histological analysis of the left ventricle of representative rat hearts from each group showed that the PKC βII peptide inhibitor treated hearts experienced a marked reduction in PMN vascular adherence and infiltration into the postreperfused cardiac tissue when compared to I/R+PMN hearts ($p<0.01$). These results suggest that the PKC βII peptide inhibitor attenuates PMN-induced post I/R cardiac contractile dysfunction by increasing endothelial NO release and by inhibiting superoxide release from PMNs.

# Introduction

In the setting of myocardial ischemia it is imperative that blood flow be restored as soon as possible. Early reperfusion remains the most effective way of limiting myocardial necrosis and improving ventricular function in experimental models and human patients (Forman et al., 1989). However, reperfusion results in a marked degree of cardiac contractile dysfunction and myocardial cell injury. A harmful cascade of events accelerates structural and functional changes in endothelial cells, resulting in a progressive decrease in microcirculatory flow (Forman et al., 1989; Tsao et al., 1990; Lefer and Lefer, 1996). These events occur sequentially and include a decreased endothelial release of nitric oxide (NO), up-regulation of adhesion molecules on the endothelial surface leading to enhanced leukocyte-endothelium interaction, infiltration of polymorphonuclear leukocytes (PMNs) into the myocardium, and subsequent release of superoxide radicals which are largely responsible for producing cardiac dysfunction and enhanced necrosis (Tsao and Lefer, 1990; Entman et al., 1992; Lefer and Lefer, 1996).

The time course of events are similar in the *ex vivo* and *in vivo* myocardial I/R models within the first 30 min of reperfusion with respect to PMN/endothelial interaction. However, the *in vivo* model requires a longer reperfusion period (ie, 270 min) to accumulate PMNs (Tsao and Lefer, 1990; Tsao et al., 1990; Ma et al., 1993; Young et al., 2001).

PMN activation by chemotactic substances such as IL-8 and complement fragment C5a results in the release of cytotoxic substances (i.e. oxygen-derived free radials such as superoxide) from the PMNs (Tsao and Lefer, 1990; Ma et al., 1991; Tsao et al., 1992). Damage to the endothelium by these free radicals is what contributes to the decrease in the endothelium-derived relaxing factor, NO (Tsao et al., 1990; Lefer and Lefer, 1996). As well as decreasing the

availability of NO, they contribute to reperfusion injury by initiating lipid peroxidation through hydrogen peroxide ($H_2O_2$) formation, and they alter membrane permeability to ions such as calcium ($Ca^{2+}$) (Lucchesi and Mullane, 1986; Rubanyi and Vanhoutte, 1986). Attenuation of the harmful effects of superoxide results in improved cardiac contractile function on reperfusion of ischemic tissue. Superoxide reduces the bioavailability of NO by combining with it to produce the peroxynitrite anion, therefore inhibiting the vasodilating effects of NO and resulting in endothelial dysfunction (Rubanyi et al., 1989; Tsao et al., 1992).

Protein kinase C (PKC) plays an important role in neutrophil activation. PKC activation results in an increase of superoxide release because it phosphorylates the cytosolic factor $p47^{phox}$ that is required for NADPH oxidase activation to produce superoxide from PMNs. (Xiao et al., 1998; Babior, 1999; Li et al., 2000). It also down regulates the activity of endothelial nitric oxide synthase (eNOS) leading to a reduction of endothelium derived NO and augments superoxide release from endothelial cells (Rubanyi et al., 1989; Hirata et al., 1995; Meyer et al.,1999).

PKC activity and expression are increased during ischemia and reperfusion in acute myocardial I/R models (Strasser et al., 1992). Therefore, PKC inhibition at the post-ischemic coronary endothelium will lead to the attenuation of superoxide production as a result of post-ischemia reperfusion injury and the preservation of NO bioavailability (Young et al., 2001; Peterman et al., 2004; Phillipson et al., *in press*).

Six different isoforms of PKC have been identified in rat neonatal cardiomyocytes (Dang et al., 1995). These isoforms include PKC α (alpha), PKC βI (beta I), PKC βII (beta II), PKC δ (delta), PKC ε (epsilon) and PKC ζ (zeta). Rat PMNs possess all of these isoforms except PKC ε (epsilon) (Korchak and Kilpatrick, 2001). PKC βII is essential for ligand-initiated assembly of the NADPH oxidase for generation of superoxide anion and is, in part, responsible for

superoxide release in activated PMNs (Dekker et al., 2000; Korchak and Kilpatrick, 2001). However, the role of PKC βII inhibition has not been previously characterized in myocardial ischemia/reperfusion (I/R).

In this study, the hypothesis being tested is that PKC βII inhibition will attenuate PMN induced cardiac contractile dysfunction following I/R. In addition, the hypothesis would predict that PKC βII inhibition would attenuate PMN superoxide release, PMN adhesion and transmigration into the post-ischemic myocardium and augment endothelial NO release. The selective PKC βII peptide inhibitor (M.W.= 1300) to be used in these assays is myristolated (fatty acid moiety) to allow for rapid cell permeability (within 10 sec). Mechanistically, the selective PKC βII peptide inhibitor may improve cardiac function by disrupting the binding of PKC βII to the Receptor for Activated C Kinase (RACK-1) region (Korchak and Kilpatrick, 2001).

# Methods

## Isolated rat heart preparation

Male Sprague Dawley rats (275-325 g, Ace Animals, Boyertown, PA) were anesthetized with 60 mg/kg pentobarbital sodium intraperitoneally (i.p.). Sodium heparin (1,000 U) was also administered i.p. The hearts were rapidly excised, the ascending aortas were cannulated, and retrograde filling of the heart was initiated with a modified Krebs buffer maintained at 37ºC at a constant pressure of 80 mmHg. The Krebs buffer had the following composition (in mmol/l): 17 dextrose, 120 NaCl, 25 $NaHCO_3$, 2.5 $CaCl_2$, 0.5 EDTA, 5.9 KCl, and 1.2 $MgCl_2$. The perfusate was aerated with 95% $O_2$-5% $CO_2$ and equilibrated at a pH of 7.3-7.4. The two side arms in the perfusion line proximal to the heart inflow cannula allowed PMNs, plasma without PKC βII peptide inhibitor (control hearts) or plasma containing different concentrations of PKC βII peptide inhibitor (1 -10μM) to be directly infused into the coronary inflow line. Coronary flow was monitored by a flowmeter (T106, Transonic Systems, Inc., Ithaca, NY). Left ventricular developed pressure (LVDP) and the maximal rate of LVDP ($+dP/dt_{max}$) were monitored using a pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX), which was positioned in the left ventricular cavity. Coronary flow, LVDP and $+dP/dt_{max}$ were recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO) in conjunction with a computer.

Figure 1 illustrates a schematic diagram of the protocol for ischemia/reperfusion in the isolated perfused rat heart. LVDP, $+dP/dt_{max}$, 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 X $10^6$ PMNs resuspended in 5 ml of Krebs buffer plus 5 ml of plasma at a rate of 1 ml/min. In some experiments, PKC βII peptide inhibitor (N-Myr-SLNPEWNET; M.W.= 1300;Genemed Synthesis, Inc., San Francisco, CA) was added to plasma at a final concentration of 1 μM, 5 μM or 10 μM. 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.

**Groups of Isolated Perfused Hearts**

Table 1 indicates the nine groups (control and treatment conditions) of isolated perfused rat hearts used in the study. Sham I/R hearts were not subjected to ischemia and were not perfused with PMNs. Previous studies showed that sham I/R hearts given PMNs exhibited no changes from initial control values (Peterman et al., 2004). In some sham I/R hearts, PKC βII peptide inhibitor (10 μM) was dissolved in plasma and infused at a rate of 1ml/min for 5 min after 35 min of perfusion.

**Isolation of Plasma**

In order to more closely simulate the conditions *in vivo*, the plasma used for infusion with the PMNs was isolated from the same rat from which the heart was isolated in each cardiac perfusion experiment. Blood was collected from the aorta in citrate phosphate buffer (Sigma Chemical Co., St. Louis, MO) over a period of 1 min just before isolation of the rat heart. The blood was centrifuged at 10,000 x g for 10 min at 4 $^{o}$C. Then the plasma was decanted and used for infusion in the I/R hearts. 5ml of plasma collected from a single rat was used for each perfused heart.

**Isolation of PMNs**

Male Sprague Dawley rats (350-400 g, Ace Animals, Boyertown, PA), used as PMN donors, were anesthetized with ethyl ether and were given a 16 ml i.p. injection of 0.5% glycogen (Sigma Chemical Co., St. Louis, MO) dissolved in phosphate buffered saline (PBS). The rats were reanesthetized with ethyl ether 16-18 hrs later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (Young et al., 2001). The peritoneal lavage fluid was centrifuged at 250 x g for 20 min at 4ºC. The PMNs were then washed in 20 ml of PBS and centrifuged at 250 x g for 10 min at 4ºC. Thereafter, the PMNs were resuspended in 2.5 ml of PBS and a total of 10-12 samples were pooled prior to 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 the cardiac tissue**

In this I/R model cardiac injury results from PMNs infiltrating the myocardium within the 45 min reperfusion period. Another component of the cardioprotective effects offered by the PKC βII peptide inhibitor may be associated with an inhibition of PMN infiltration and adherence to vascular endothelium. Three representative rat hearts from each of the 9 experimental groups were used for histological analysis. These hearts were representative of each group as their individual cardiac function data (LVDP and $+dP/dt_{max}$) were closest to the mean values of the entire group. The hearts were dehydrated in graded ice-cold acetone washes (50-100%). The heart tissue was then embedded in plastic and sectioned into 2.5 μm serial sections and placed onto glass slides. Sections were then stained with hematoxylin and eosin as

previously described (Young et al., 2001). The number of PMNs was counted by light microscopy in ten areas of the left ventricle. In order to determine the effect of PKC βII peptide inhibitor on PMN adherence, the intravascular PMNs that adhered to the coronary vascular endothelium were counted and expressed as adhered PMNs/$mm^2$. The effect of PKC βII peptide inhibitor on PMN transmigration was determined by counting the total intravascular and infiltrated PMNs, and expressed as total intravascular and infiltrated PMNs/$mm^2$ area of cardiac tissue. Slides of I/R+PMN control and PKC βII peptide inhibitor-treated hearts were viewed on a Nikon E800 epifluorescent microscope and the images were captured with the Spot RT camera and analyzed with the Phase 3 Image Pro plus 4.5 imaging software.

**Measurement of NO Release from Rat Aortic Segments**

NO release from rat aortic endothelium was measured to determine if PKC βII peptide inhibition provides cardioprotection by a mechanism involving increased endothelial NO release. Rat aortas were isolated after anesthetizing the rats with pentobarbital sodium (60 mg/kg). The aortic tissue came from the same rats that were used for hearts in the cardiac perfusion experiment. The excised aortas were immersed in warm oxygenated (95% $O_2$ – 5% $CO_2$) Krebs-Henselit (K-H) buffer solution. The K-H buffer had the following composition (in mmol/l): 10 dextrose, 119 NaCl, 12.5 $NaHCO_3$, 2.5 $CaCl_2$, 4.8 KCl, 1.2 $KH_2PO_4$, and 1.2 $MgSO_4$. The aortas were cleaned of adherent fat and connective tissue, and rings 6-7 mm long (ie.10 mg wet weight) were prepared. The aortic rings were cut, spread opened and fixed by pins with the endothelial surface facing up in 24-well culture dishes containing 1 ml K-H solution. After equilibration at 37ºC, NO released into the buffer solution was measured after administration of PKC βII peptide inhibitor (1 - 10 μM) to the aortic segments. NO release from rat aortic endothelium was compared to samples containing no drug. Acetylcholine (500 nM) was used as a positive control

to assess the viability of the endothelium for NO production/release (Tsao et al., 1992). Basal rat aortic endothelial NO release is 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 determines the basal NO release for that aortic endothelial segment. After basal NO release is determined, the effect of acetylcholine (500 nM) and PKC βII peptide inhibitor are then determined. Thereafter, 400 μM L-NAME is added to the K-H buffer solution, and NO release is reassessed 30 minutes later in the presence of acetylcholine (500 nM) or PKC βII peptide (10 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 (Guo et al., 1996). NO released into the medium was reported in picomoles per milligram of aortic tissue. Between 5 and 17 trials were performed for each group.

**Measurement of superoxide radical release from rat PMNs**

Another mechanism that may contribute to the cardioprotective effects (i.e. LVDP) of the PKC βII peptide inhibitor may be inhibition of PMN superoxide release. The superoxide anion release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech, El Cajon, CA) by the reduction of ferricytochrome C (Young et al., 2000). The PMNs ($5 \times 10^6$) were resuspended in 450 μl PBS and incubated with ferricytochrome C (100 μM, Sigma Chemical Co., St. Louis, MO) in a total volume of 900 μl of PBS for 15 min at 37ºC in spectrophotometric cells. PKC β II peptide inhibitor was added to the 900 μl PMN/ferricytochrome C suspension and mildly vortexed to yield a final concentration of 1, 5, 10, or 20 μM and incubated at 37 ºC for 15 min in spectrophotometric cells. Control samples did not contain PKC βII peptide inhibitor. The PMNs were stimulated with 200 nM fMLP (Sigma

Chemical Co., St. Louis, MO) in a final reaction volume of 1.0 ml. Positive control samples were given superoxide dismutase (SOD) (10 μg /ml) just prior to addition of fMLP. Absorbance at 550 nm was measured every 30 sec up to 90 sec (peak response) (Young et al., 2000) and the change (Δ) in superoxide anion release from PMNs was determined from time zero.

**Statistical Analysis**

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

## Results

Figure 2 shows the time course of cardiac contractile function (LVDP) for the sham I/R, I/R, I/R+PMN+PKC βII peptide inhibitor (10 μM) and I/R+PMN groups. It illustrates the changes in LVDP during the 80 min perfusion period.

The hearts in the sham I/R group remained at 103±4% of initial baseline values of LVDP for the entire duration of the perfusion period. Hearts in the I/R group experienced a depression in LVDP during the initial stages of reperfusion, but by the end of reperfusion they had recovered to 94±6% of initial baseline values, indicating that global ischemia alone was not the cause of sustained contractile dysfunction in this model of I/R.

However, the hearts in the I/R+PMN group exhibited severe cardiac contractile dysfunction, only recovering to 43±5% of initial baseline values by the end of reperfusion. By contrast the hearts in the I/R+PMN+PKC βII peptide inhibitor (10 μM), although initially showing a depression in LVDP of 61±10% of initial baseline values at 15 min into reperfusion, recovered to 82±9% of baseline.

In order to establish whether the PKC βII peptide inhibitor produced any direct inotropic effects on cardiac contractile function, sham I/R hearts were perfused with PKC βII peptide inhibitor 10 μM, which was the highest dose administered in this study. This group served as one of the controls for the study. These hearts did not show any significant change in LVDP (figure 3) or $+dP/dt_{max}$ (figure 4) at the end of the 80 min reperfusion period, hence indicating that at this dose the PKC βII peptide inhibitor has no direct effect on cardiac contractile function.

Figures 3 and 4 show the initial and final values for LVDP and $+dP/dt_{max}$ from isolated perfused hearts respectively. There were no significant differences 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 $+dP/dt_{max}$ for the sham I/R, Sham I/R+PKC βII peptide inhibitor (10 μM), I/R, and I/R+PKC βII peptide inhibitor (10 μM) groups. However, there was a significant difference between the initial and final values of LVDP and $+dP/dt_{max}$ for the I/R+PMN group ($p<0.01$). This group only recovered to 43±5% in LVDP and 42±6% in $+dP/dt_{max}$ from initial baseline at 45 min postreperfusion.

The presence of the PKC βII peptide inhibitor at a 5 μM and 10 μM dose attenuated the decrease in LVDP and $+dP/dt_{max}$ associated with the postischemic reperfusion with PMNs. The 10 μM dose the was the most cardioprotective as the hearts in the I/R+PMN+PKC βII peptide inhibitor (10 μM) recovered to 82±9% and 79±10% of initial baseline at 45 min postreperfusion for LVDP and $+dP/dt_{max}$ respectively. These values were significantly different from I/R+PMN at 45 min postreperfusion ($p<0.01$). The 5 μM dose was also cardioprotective, although not to the same extent as the 10 μM dose, as the hearts in the I/R+PMN+PKC βII peptide inhibitor (5 μM) recovered to 69±7% and 63±7% for LVDP and $+dP/dt_{max}$ of initial baseline at 45 min postreperfusion respectively. The LVDP values for the 5 μM dose were significantly different from I/R+PMN at 45 min postreperfusion ($p<0.05$). Although not shown in the results hearts treated with 2.5 μM (n=2) showed similar recovery with the hearts in the 5 μM group. The 1 μM dose of PKC βII peptide inhibitor was not cardioprotective as the hearts in the I/R+PMN+PKC βII peptide inhibitor (1 μM) group only recovered to 57±4% and 53±6% for LVDP and $+dP/dt_{max}$ respectively. The final values of LVDP and $+dP/dt_{max}$ at the 1 μM dose group were not significantly different from the final values of the I/R+PMN group. The cardioprotective effects of the PKC βII peptide inhibitor (10 μM) were blocked by the presence of L-NAME (50 μM) in the IR+PMN+PKC βII peptide inhibitor (10 μM)+L-NAME (50 μM) group, as the LVDP and

$+dP/dt_{max}$ values at the end of the 45 min reperfusion period were only 56±2% and 53±5% of the initial baseline values, respectively, and were not significantly different from the final values of the IR+PMN group (figures 3 and 4).

Figure 5 shows that segments of the endothelium treated with PKC βII peptide inhibitor generated significantly more NO when compared to the basal NO release for those segments (5 μM ($p<0.05$) and10 μM ($p<0.01$)).

The basal value of NO release was measured at 1.85±0.18 picomoles NO/mg tissue. There was a definite dose-response effect of stimulating the endothelium with PKC βII peptide inhibitor as the 1 μM, 2.5 μM, 5 μM and 10 μM produced an increase in NO release above basal of 0.75±0.19, 1.91±0.44, 2.54±0.29, and 3.49±0.62 picomoles NO/mg tissue respectively. Acetylcholine (500nM) was used as a positive control in this assay and stimulated the endothelium causing an increase of 3.75±0.58 picomoles NO/ mg tissue, above the baseline basal value. The NOS inhibitor, L-NAME was used as another control in order to decrease basal release of NO to zero. Both the acetylcholine and PKC βII peptide inhibitor stimulated release of NO were completely inhibited by treating the endothelium with L-NAME (400 μM).

PKC βII peptide inhibitor significantly inhibited superoxide release (i.e. absorbance) from suspensions of fMLP-stimulated rat PMNs from 0.13±0.01 to 0.05±0.009 ($p<0.01$), 0.02±0.004 ($p<0.01$), and 0.02±.007 ($p<0.01$) for 5 μM, 10 μM and 20 μM respectively (figure 6). There was no significant inhibition of superoxide at the 1 μM dose. SOD (10 μg/ml) was used as a positive control and it scavenged the superoxide released by the fMLP-stimulated rat PMNs reducing the response to 0.0016±.0006.

During reperfusion, a significant number of PMNs transmigrated into the myocardium, increasing from less than 20 PMN/$mm^2$ to more than 150 PMN/$mm^2$ at the end of the reperfusion

period in I/R+PMN hearts (figure 7). In the control groups Sham, Sham+PKC β II peptide inhibitor, IR and IR+ PKC β II peptide inhibitor the number of PMNs/mm$^2$ determined through histological analysis were 18.2±3.1, 19.6±3.5, 27.3±1.2 and 25.9±4.3 PMNs/mm$^2$ respectively. In the IR+PMN group this number increased to 155.4±5.6 PMNs/mm$^2$. However, in the treated groups of I/R+PMNs+ PKC β II peptide inhibitor at 1μM, 5μM and 10μM the number of PMNs/mm$^2$ decreased to 88.9±17.7, 80.5±14.1 and 76.3±14.2 PMNs/mm$^2$ respectively. Compared to I/R+PMN hearts, PKC βII peptide inhibitor treated hearts experienced a 43±11%, 48±9% and 51±9% reduction in PMN infiltration into the postreperfused cardiac tissue at 1 μM, 5 μM and 10 μM doses respectively (figure 7). In the I/R+PMN+ PKC β II peptide inhibitor 10μM+L-NAME group, where NO was inhibited, the number of PMNs/mm$^2$ was 158.9±20.5 PMNs/mm$^2$ which is almost identical to the number of PMNs/mm$^2$ in the I/R+PMN group.

In the control groups Sham, Sham+PKC β II peptide inhibitor, IR and IR+ PKC βII peptide inhibitor the number of adhered PMNs/mm$^2$ determined through histological analysis were 11.2±1.9, 10.5±1.2, 11.2±1.9 and 16.1±3.9 PMNs/mm$^2$ respectively. In the IR+PMN group this number increased to 53.9±9.4 PMNs/mm$^2$. But in the treated groups of I/R+PMNs+ PKC β II peptide inhibitor at1μM, 5μM and 10μM, the number of PMNs/mm$^2$ decreased to 21±3.6, 23.8±3.1 and 20.3±2.8 PMNs/mm$^2$ respectively. Again compared to I/R+PMN hearts, PKC βII peptide inhibitor treated hearts experienced a 61±7%, 56±6%, and 62±5% reduction in the number of adherent PMNs at 1 μM, 5 μM and 10 μM doses respectively (figure 8). In the presence of L-NAME the number of PMNs adhered was 67.2±6.3 PMNs/mm$^2$. Figure 9 is a photograph of a representative untreated and PKC βII treated I/R+PMN heart that shows the

relative differences in total intravascular and infiltrated PMNs in panels A and C. PKC βII treatment attenuated PMN adherence and infiltration indicated in panels B and D.

# Discussion

### *Summary of major findings*

The major findings of this study were as follows: 1) I/R+PMN+PKC beta II peptide inhibitor-treated rat hearts (10 μM) exerted maximal restoration of postreperfusion cardiac function (ie, LVDP and $+dP/dt_{max}$), and was associated with; 2) maximal attenuation of intravascular PMN adherence/infiltration in postreperfused myocardium; 3) Both of these effects were blocked in the presence of L-NAME which was similar to I/R+PMN control hearts; 4) NO release in rat aortic segments was significantly increased dose-dependently in the presence of PKC beta II inhibitor (1-10 μM), and this effect was blocked by L-NAME; 5) fMLP-stimulated PMN superoxide release was significantly inhibited by 85% in the presence of PKC beta II inhibitor (10 μM).

### *PKC βII peptide inhibitor effects on cardiac function*

The most effective dose of the PKC βII peptide inhibitor for attenuating the effects of PMN-induced cardiac contractile dysfunction was the 10 μM dose. The dose regiment used in this study was determined from the results of previous studies that found this range to be most effective in inhibiting the PKC βII isoform in cardiac myocytes (Ron et al., 1995). The I/R+PMN hearts + PKC βII peptide inhibitor clearly shows a dose-response effect in improving cardiac function (i.e. LVDP and $+dP/dt_{max}$) with the concentrations used in this study (1-10 μM, figures 3 and 4).

I/R hearts that were perfused with PMNs and treated with 10 μM of PKC βII peptide inhibitor exhibited maximal restoration of postreperfusion LVDP and $+dP/dt_{max}$, and there was

no significant difference between initial and final (45 min postreperfusion) LVDP and $+dP/dt_{max}$ values for hearts in this group (figures 3 and 4).

Hearts treated with the 5 μM dose also exhibited cardioprotection, but to a lesser extent (ie, 69±7% for LVDP), compared to the 10 μM dose. Whereas, the effects of a 1 μM dose showed minimal postreperfusion recovery (ie, 57% for LVDP), and was not significantly different from the hearts in the I/R+PMN control group (figures 3 and 4).

The hearts in the I/R+PKC βII peptide inhibitor (10 μM) and the Sham I/R+PKC βII peptide inhibitor (10 μM) showed no significant difference between initial baseline and final LVDP and $+dP/dt_{max}$ values (45 min postreperfusion), establishing that there were no cardiodepressant or cardiotonic effects of the PKC βII peptide inhibitor on cardiac contractile function. However, another study showed the PKC βII peptide inhibitor reversed PKC activated human cardiac Na+ channel current depression in Xenopus oocytes (Shin and Murray, 2001), suggesting that PKC beta II may be involved in electrophysiological regulation of cardiac function. Although differences in the regulation of cardiac Na+ channels between the whole organ preparation (ie, isolated perfused heart) and cardiac Na+ channel expression in Xenopus oocytes may account for the apparent discrepancy between the two studies.

***Mechanism of action related to cardioprotection***

***1. Endothelial-derived NO release***

This study established that PKC βII peptide inhibition results in cardioprotection of the isolated perfused rat heart from PMN-induced I/R injury. The significant restoration of postreperfusion LVDP and $+dP/dt_{max}$ is a direct indicator of these cardioprotective effects, which can be attributed to enhanced release of endothelium derived NO which quenches superoxide, improves vasodilation and inhibits PMN aggregation (Pabla et al., 1996, Lefer and Lefer, 1996).

This is further demonstrated by the absence of these cardioprotective effects in the I/R+PMN+PKC βII peptide inhibitor (10 μM) + L-NAME group of hearts, because the NO release was blocked by the presence of L-NAME (Phillipson et al., *in press*). The significant reduction in PMN adherence to the vascular endothelium and infiltration into the postreperfused myocardial tissue also demonstrates these cardioprotective effects (Scalia et al., 1996; Xiao et al., 1998), and suggests that the cardioprotection may be mediated, in part, by a NO mechanism, since L-NAME treatment in heart and aortic tissue resulted in significant increases in postreperfusion PMN vascular adherence and infiltration and decreases in PKC beta II peptide inhibitor stimulated NO release respectively.

Preserving endothelial NO release attenuates endothelial dysfunction and inhibits adherence and infiltration of PMNs into the coronary vasculature and surrounding tissue, thus attenuating cardiac contractile dysfunction (Ma et al., 1993; Pabla et al., 1996). The aortic NO release data (figure 5) show a direct relationship between the dose of PKC βII peptide inhibitor used to significantly augment NO release and cardioprotection of the hearts (figures 3 and 4), and establishes a correlation between the two assays.

***2. PMN superoxide release***

The inhibition of PKC βII peptide inhibits the activation of PMNs thereby inhibiting the release of superoxide and attenuating the cardiac contractile dysfunction associated with its release (Young et al., 2001). Similar to the NO release data, the doses of the PKC βII peptide inhibitor that significantly attenuated fMLP-stimulated PMN superoxide release (figure 6) show a direct relationship to the cardioprotective doses (figures 3 and 4), and suggest that the mechanism of cardioprotection may be due either to enhanced NO release (figure 5) or attenuated superoxide release (figure 6), or a combination of both (Lefer and Lefer, 1996; Yan and Novak, 1999,

Phillipson et al, *in press*). Oxygen-derived free radicals such as superoxide up-regulate endothelial cell adhesion molecules and quench endogenous NO (Patel et al., 1991; Davenpeck et al., 1994). NO inhibits the leukocyte-endothelial cell interaction by suppressing up-regulation of endothelial cell adhesion molecules (Davenpeck et al., 1994; Lefer and Lefer, 1996). Therefore the PKC βII peptide inhibitor that attenuates superoxide production from PMNs and increases the bioavailability of NO would attenuate the expression of endothelial cell adhesion molecules and this would effectively diminish the transmigration of PMNs into cardiac tissue and the subsequent release of superoxide radicals from transmigrated PMNs at or near cardiomyocytes (Young et al., 2000).

In this study, it is evident that the number of PMNs present in the postischemic reperfused myocardial tissue (figures 7, 8 and 9) is associated with cardiac contractile dysfunction in untreated I/R+PMN hearts, and this effect is attenuated by the PKC βII peptide inhibitor treatment (figure 9).

***Role of PKC isozymes in cardiac ischemia/reperfusion***

During the early reperfusion period (5 min.) of acute myocardial ischemia, PKC activation and cell membrane expression is enhanced (Strasser et al, 1992). PKC isozymes translocate on activation from one cellular compartment to another. RACKs are the receptor proteins responsible for translocation and subsequent function of a PKC enzyme (Ron and Mochly-Rosen, 1995). Activated PKC βII binds to the RACK 1 binding site, which increases the PKC βII isoform phosphorylation of substrates (e.g. NADPH oxidase, eNOS), by stabilizing the active form of PKC βII and translocating it to the cell membrane (Ron et al., 1995). NADPH oxidase activation in both PMNs and endothelial cells generates reactive oxygen species (e.g. superoxide), which cause oxidative damage and perpetuates cardiac contractile dysfunction (Ma

et al., 1991; Hansen, 1995). The PKC βII peptide inhibitor occupies the RACK 1 binding site preventing the binding of PKC βII, and therefore inhibiting the translocation of the activated PKC βII isoform and its function of activating NADPH oxidase (Korchak and Kilpatrick, 2001).

Other PKC isoforms have been shown to be either beneficial (e.g. PKC ε) or injurious (e.g. PKC ζ) when activated in cardiac and endothelial function (Hu et al., 2000; Das, 2003, Phillipson et al., *in press*). PKC ε activation has been associated with recovery of postreperfusion LVDP (Inagaki et al, 2003), but requires pretreatment of PKC ε activator prior to ischemia to elicit cardioprotection in contrast to the PKC βII inhibitor given at the beginning of reperfusion. PKC ζ has been implicated in superoxide release in PMNs and endothelial cells (Dang et al., 2001; Frey et al., 2002), adhesion molecule up-regulation (Rahman et al., 2000) and restoration of cardiac function in I/R (Peterman et al, 2004; Phillipson et al, *in press*). However, the PKC βII inhibitor restores early postreperfusion LVDP (ie, 10 min) sooner and does not exhibit cardiodepressant effects at higher doses compared to compounds that inhibit the PKC ζ isoform (Peterman et al, 2004; Phillipson et al., *in press*). An interesting prospective study would be to characterize the potential cardioprotective effects of a combination of PKC ζ and PKC βII peptide inhibitors, on cardiac and endothelial function.

In summary, these results show a cardioprotective effect of a selective PKC βII peptide inhibitor on LVDP and $+dP/dt_{max}$ on PMN-induced myocardial ischemia-reperfusion injury in the isolated perfused rat heart. The cardioprotection appears to be related to an increase in endothelial-derived NO along with inhibition of PMN-generated superoxide release and PMN adherence to the vascular endothelium, resulting in fewer PMNs infiltrating into the cardiac tissue. The resulting decrease in superoxide radical release at the level of cardiomyocytes leads to an attenuation of cardiac contractile dysfunction in PMN-induced I/R injury.

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# Footnotes

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Reprint Requests to: Lindon H. Young, Ph.D., Department of Pathology, Microbiology and Immunology, Philadelphia College of Osteopathic Medicine, 4170 City Avenue, Philadelphia, PA 19131-1694

# Legends for Figures

Figure 1. Schematic diagram of the ischemia/reperfusion protocol in the isolated perfused rat heart model.

Figure 2. Time course of LVDP in sham, I/R, I/R+PMNs and I/R+PMN+βII peptide inhibitor (10 μM) perfused rat hearts. LVDP data at initial (baseline) and reperfusion from 0 to 45 min following 20 min ischemia. The sham group (n=6) maintained the same LVDP throughout the 80 min. protocol. The I/R+PMN group (n=9) exhibited a significant and sustained reduction in LVDP compared to I/R (n=6) and I/R+PMN+βII peptide inhibitor (n=7) groups. All values are expressed as mean ± SEM. *$p<0.05$ and **$p<0.01$ from I/R+PMNs.

Figure 3. Initial and final LVDP expressed in mmHg from isolated perfused rat hearts before ischemia (I) (initial) and after 45 min post reperfusion (R) (final). Hearts were perfused in the presence or absence or PMNs. PMNs induced a significant cardiac contractile dysfunction, which was attenuated by the protein kinase C (PKC) βII peptide inhibitor, but was significantly blocked by the presence of L-NAME. All values are expressed as mean + SEM. Numbers of hearts are at the bottom of the bars. *$p<0.05$ and **$p<0.01$ from final I/R+PMNs.

Figure 4. Initial and final +dP/dt max expressed in mmHg/s in isolated perfused rat hearts before ischemia (I) and after reperfusion (R). Hearts were perfused in the presence or absence of PMNs. PMNs induced a significant cardiac contractile dysfunction, which was attenuated by a protein kinase C (PKC) βII peptide inhibitor, but was blocked by L-NAME. All values are expressed as means +SEM. Numbers of hearts are at the

bottom of the bars. *p<0.05 and **P<0.01 from final I/R + PMNs.

Figure 5. Measurement of NO release from rat aortic segments. Endothelial NO release was significantly increased from basal NO release in PKC βII peptide inhibitor treated segments (1, 2.5, 5 and 10 μM), as well as acetylcholine (Ach 500nM). NO release was significantly reduced in both groups given 400 μM L-NAME. All values are expressed as means +SEM, numbers at bottom of bars are numbers of separate experiments. *p<0.05, **p<0.01 from basal values.

Figure 6. Superoxide release from rat PMNs. Superoxide release was measured from 5 X $10^6$ PMNs after fMLP (200 nM) stimulation. SOD (10 μg/ml) was employed as a positive control. The change in absorbance (Δ) was measured 90 sec after fMLP addition (peak response). Superoxide release was significantly inhibited by the PKC βII peptide inhibitor (*p<0.05 and **p<0.01). All values are means ± SEM; numbers at bottom of bars are from numbers of separate experiments.

Figure 7. Histological assessment of total intravascular and infiltrated PMNs in isolated perfused rat heart samples taken from 3 rats per group and 10 areas per heart. All values are mean numbers of PMNs/$mm^2$ of heart area ± SEM. (*p<0.05, **p<0.01 from basal values)

Figure 8: Histological assessment of intravascular PMNs that adhered to the coronary vasculature in isolated perfused rat heart samples taken from 3 rats per group and 10 areas per heart. All values are mean numbers of PMNs/$mm^2$ of heart area ± SEM. (*p<0.05, **p<0.01 from basal values).

Figure 9: Light Microscopy photograph of hematoxylin and eosin stained rat heart tissue subjected to 20 min ischemia (I) and 45 min reperfusion (R) reperfused with

polymorphonuclear leukocytes (PMN). Panel A) I/R + PMN control, illustrates a coronary blood vessel with PMN vascular adherence and tissue infiltration. Panel B) Higher magnification of panel A. Arrow indicates infiltrated PMN and arrowhead indicates PMN adherence to coronary vasculature. Panel C) I/R + PMN + PKC beta II peptide inhibitor (10 μM) treated hearts exhibit a significant reduction in PMN vascular adherence and tissue infiltration compared to control (Panel A). Panel D) higher magnification of Panel C. Arrow indicates infiltrated PMN. Panels A: Mag Bar = 10 μm.

**Tables**

Table 1. Groups of rat hearts and treatment received at the beginning of reperfusion.

| Group | Control (no drug) | PKC beta II (1 μM) | PKC beta II (5 μM) | PKC beta II (10 μM) | PKC beta II (10 μM) + L-NAME |
|---|---|---|---|---|---|
| Sham I/R | N = 6 | | | N = 6 | |
| I/R | N = 6 | | | N = 6 | |
| I/R + PMN | N = 9 | N = 6 | N = 7 | N = 7 | N = 6 |

**Figure 1**



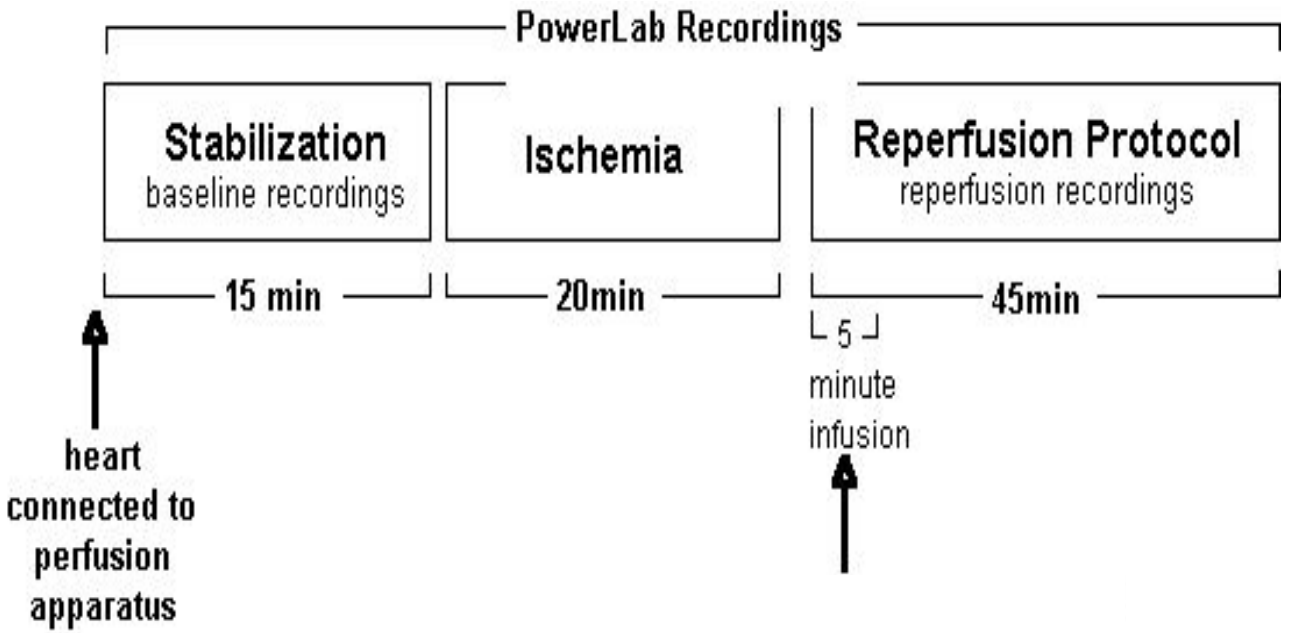

**Figure 2**



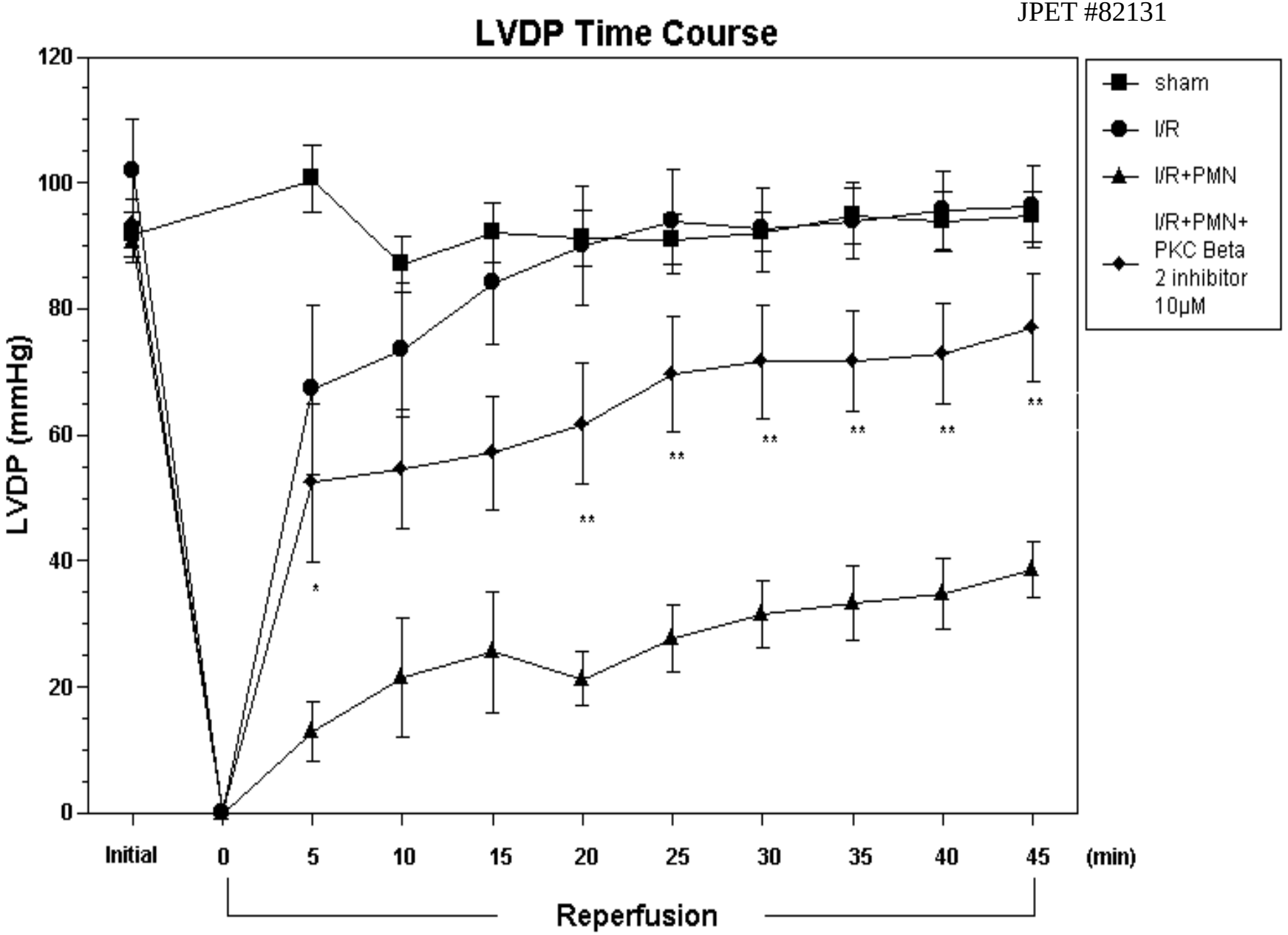

**Figure 3**



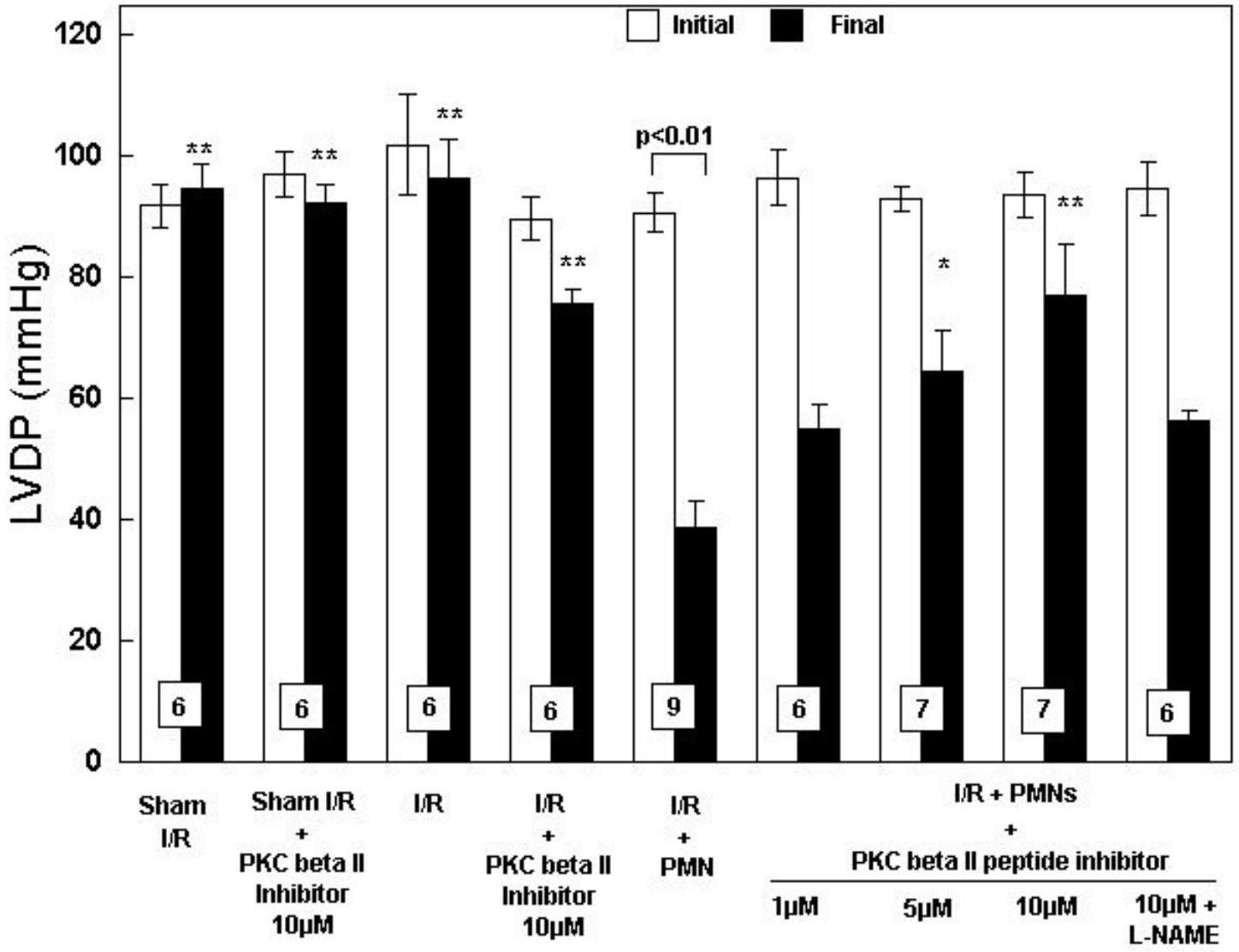

**Figure 4**



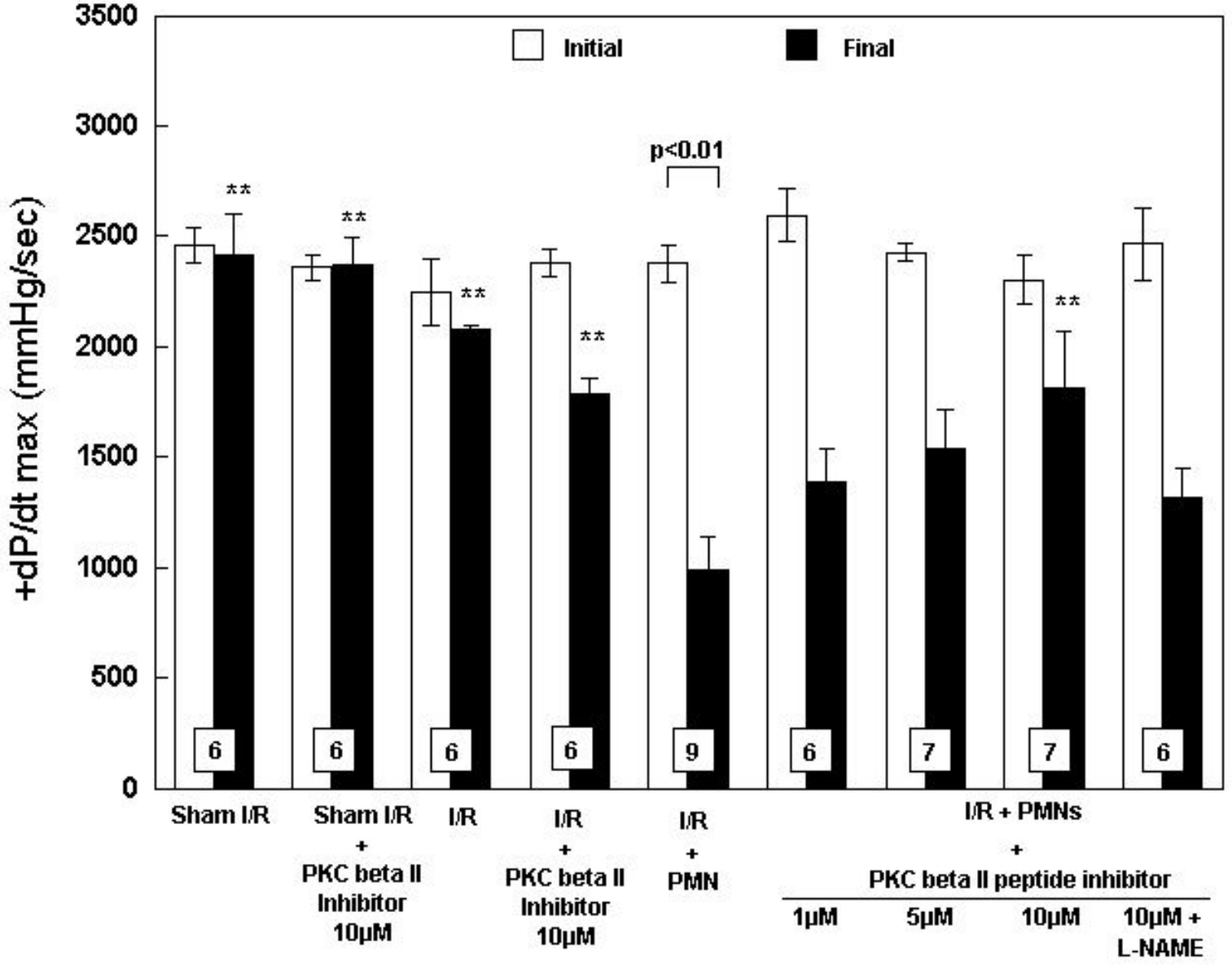

**Figure 5**



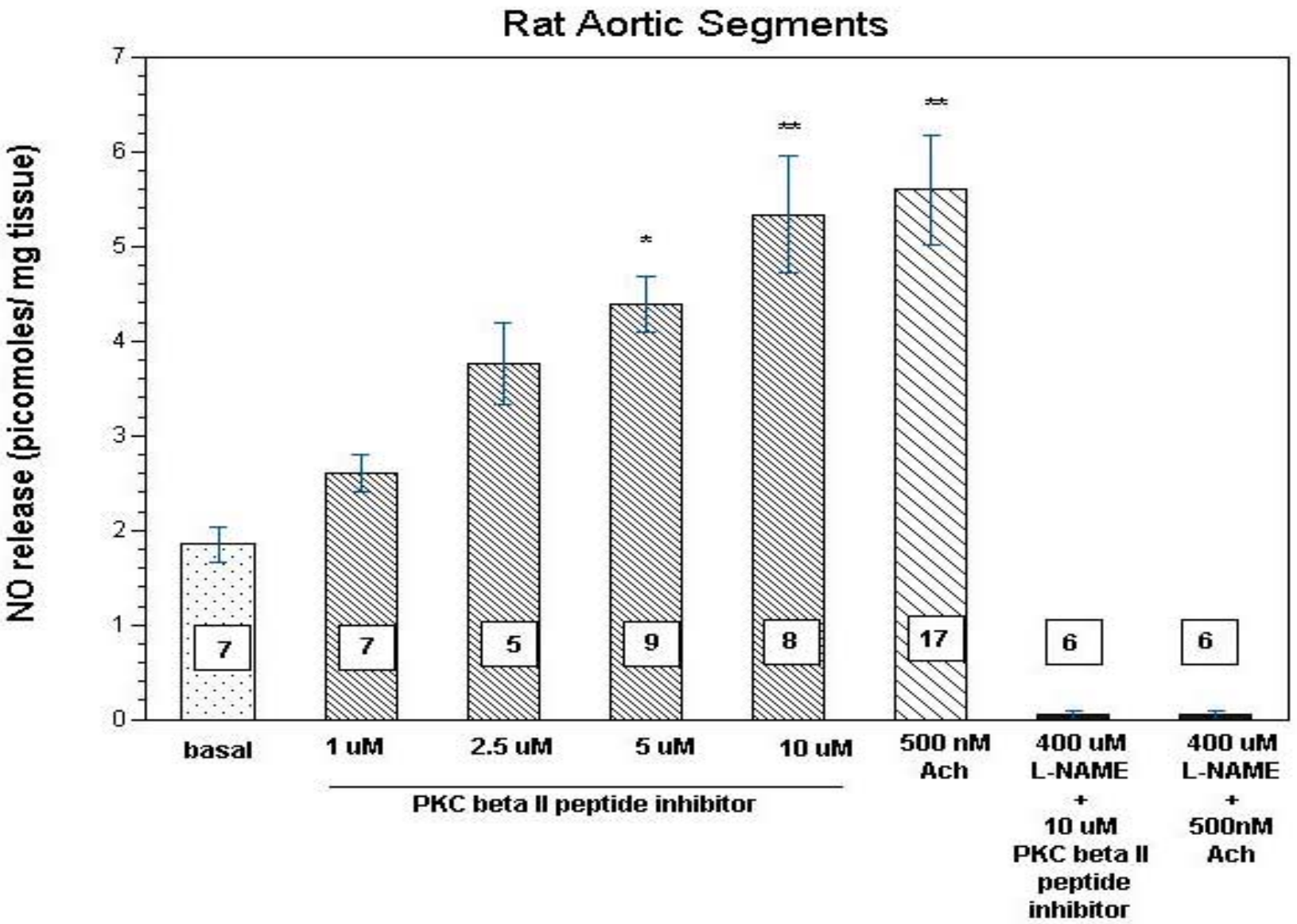

**Figure 6**



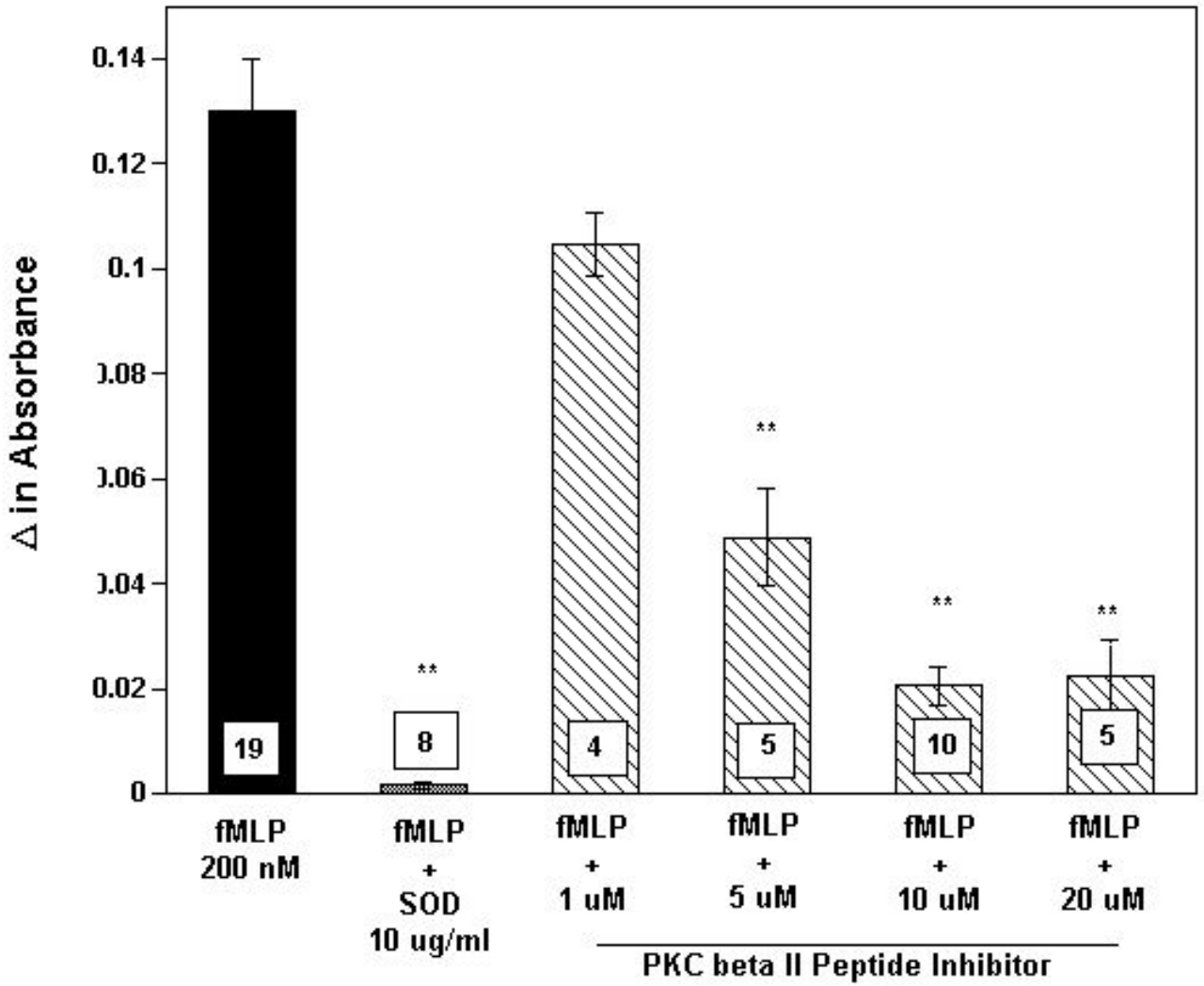

**Figure 7**

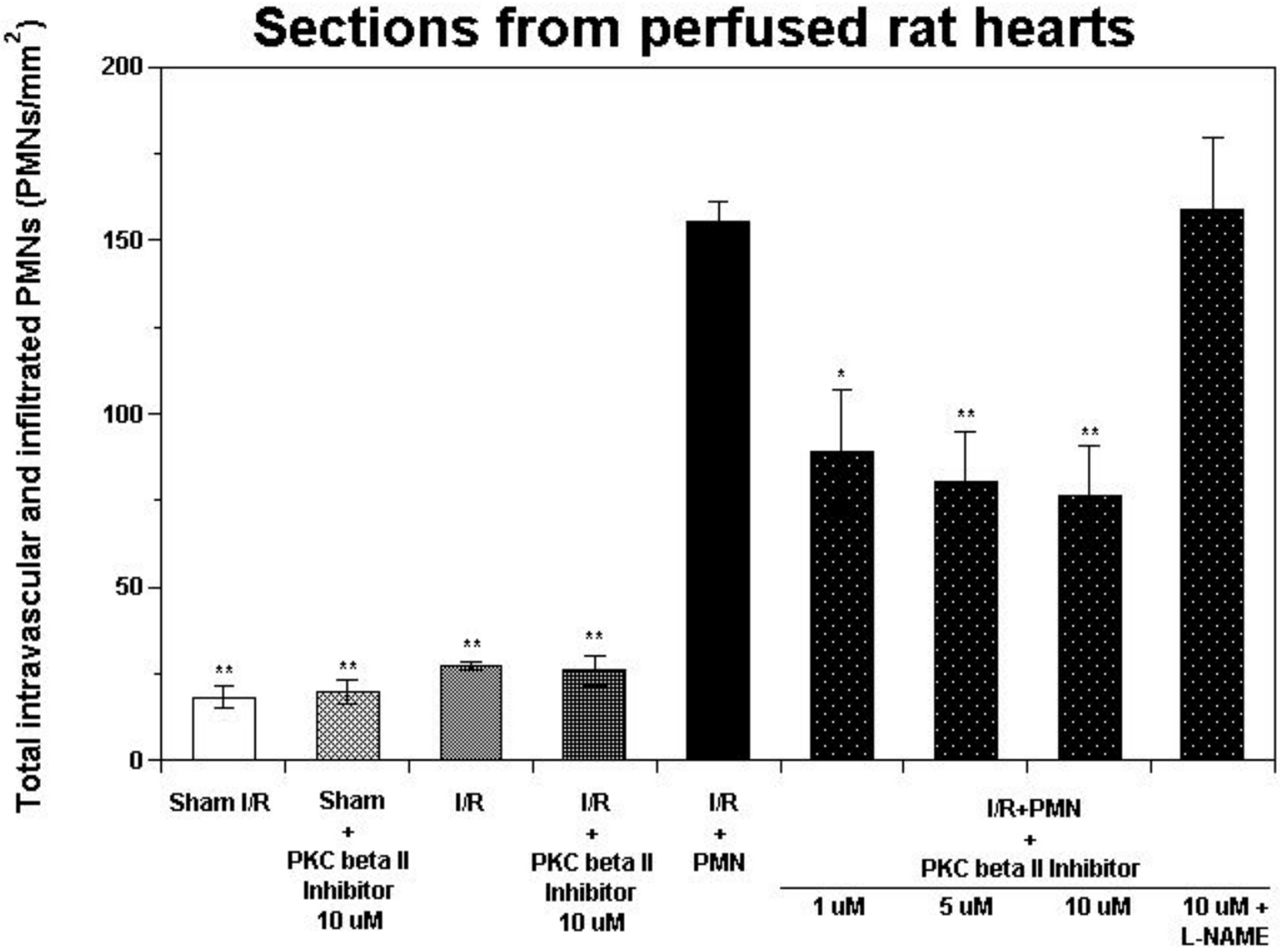

**Figure 8**



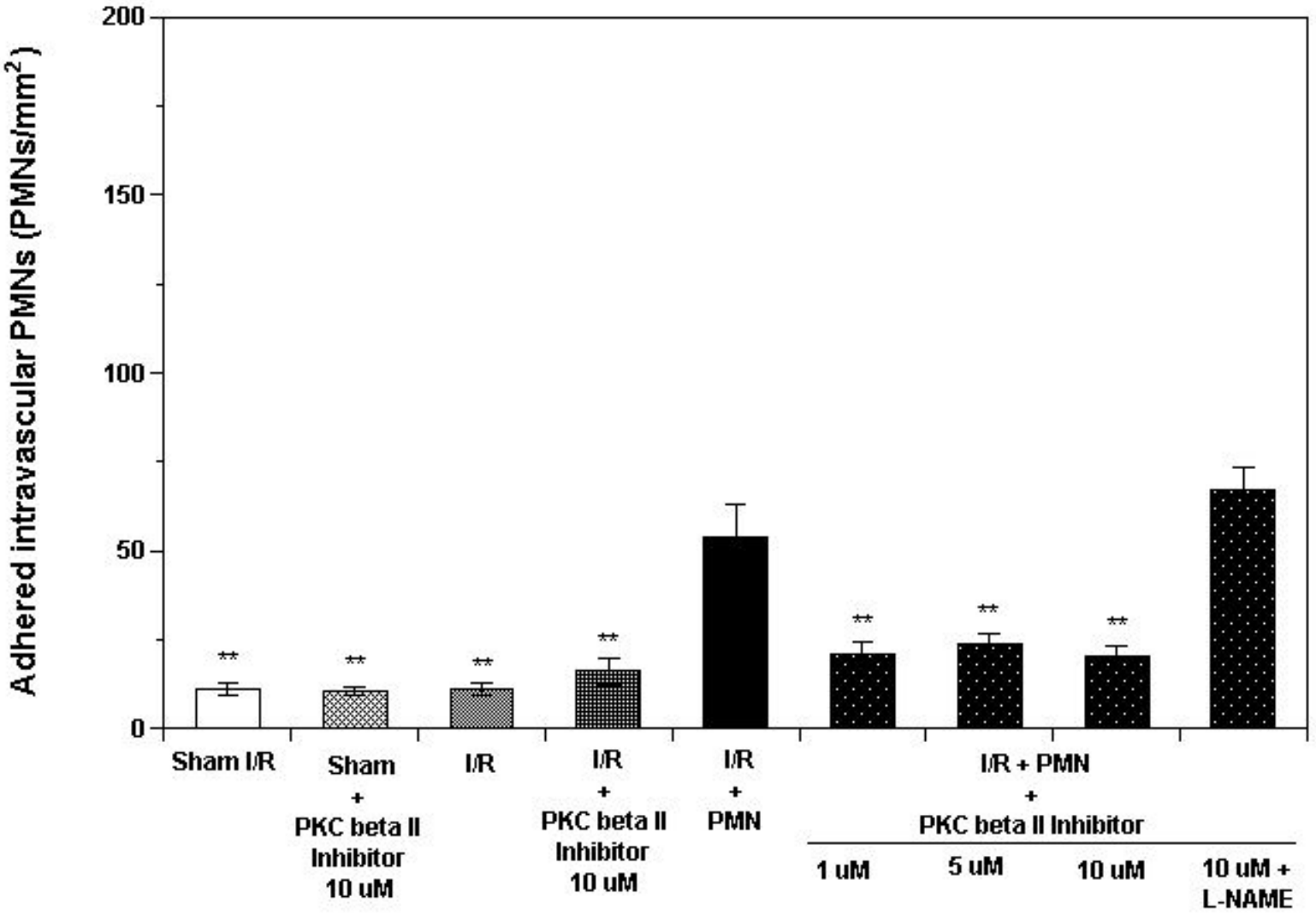

**Figure 9**