

Protein Kinase C Beta II (PKC β II) Peptide Inhibitor Exerts Cardioprotective Effects in Myocardial Ischemia/Reperfusion Injury

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Introduction

Coronary heart disease is the leading cause of death worldwide, and is primarily attributable to the detrimental effects of tissue infarct after an ischemic insult. The most effective therapeutic intervention for reducing infarct size associated with myocardial ischemia injury is timely and effective reperfusion of blood flow back to the ischemic heart tissue. However, the reperfusion of blood itself can induce additional cardiomyocyte death that can account for up to 50% of the final infarction size. Currently, there are no effective clinical pharmacologic treatments to limit myocardial ischemia/reperfusion (MI/R) injury in heart attack patients [1]. Reperfusion injury is initiated by decreased endothelial-derived nitric oxide (NO) which occurs within 5 min of reperfusion [2], and may in part be explained by PKC β II mediated activation of NADPH oxidase, which occurs upon cytokine release during MI/R [3]. PKC β II activity is increased in animal models of MI/R and known to exacerbate tissue injury [4,5]. PKC β II is known to increase NADPH oxidase activity in leukocytes, endothelial cells and cardiac myocytes *via* phox47 phosphorylation, and decrease endothelial NO synthase (eNOS) activity *via* phosphorylation of Thr 495 [6-8]. NADPH oxidase produces superoxide (SO) and quenches endothelial derived NO in cardiac endothelial cells. Moreover, PKC β II phosphorylation of p66Shc at Ser 36 leads to increased mitochondrial reactive active oxygen species (ROS) production, opening of the mitochondrial permeability transition pore (MPTP), and pro-apoptotic factors leading to cell death and increased infarct size [9] (Figure 1 left). Therefore, using a pharmacologic agent that inhibits the rapid release of PKC β II mediated ROS, would attenuate endothelial dysfunction and downstream pro-

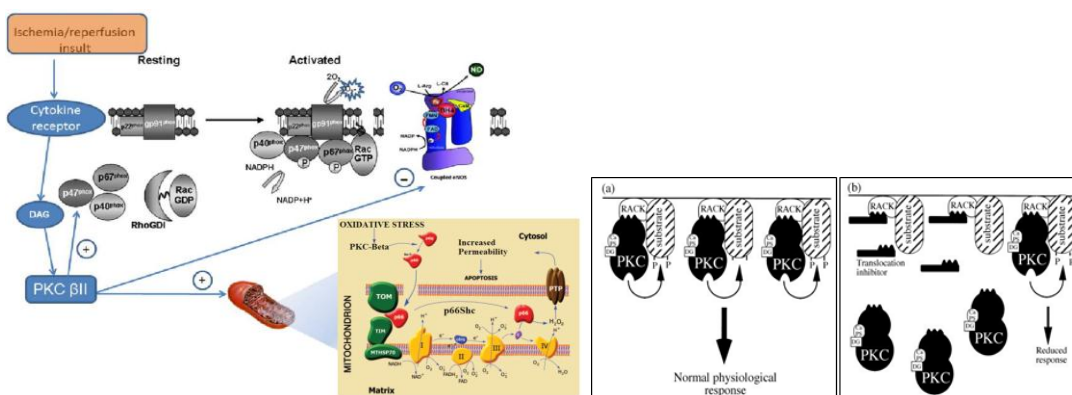


Fig. 1. Left: Schematic representation of PKC β II mediated activation in myocardial ischemia and reperfusion (MI/R). MI/R induces cytokine receptor activation within minutes leading to activation of PKC β II via diacylglycerol (DAG). PKC β II increases reactive oxygen species (ROS) release from damaged mitochondria and NADPH oxidase, respectively, and reduces coupled eNOS activity. (Adapted from [9]). Right: Mechanism of action of PKC β II peptide inhibitor. PKC β II peptide inhibitor attenuates the translocation of PKC β II to cellular substrates (e.g., NADPH oxidase) by competing for the receptor for activated C kinase (RACK), adapted from [10].

apoptotic pathways when given during reperfusion and should be an ideal candidate to attenuate MI/R injury. PKC β II peptide inhibitor mechanism of action (Figure 1 right) is to inhibit PKC β II translocation to cellular substrates such as eNOS, NADPH oxidase, and mitochondrial p66Shc protein that increase ROS leading to opening of the MPTP which in turn leads to consequent release of pro-apoptotic factors into the cytosol [9,10]. We've previously shown that PKC β II peptide inhibitor restored post-reperfused cardiac function and reduced polymorphonuclear leukocyte (PMN) infiltration in isolated rat hearts subjected to MI(20min)/R(45min) reperfused with PMNs [8]. In addition, the use of PKC β II peptide inhibitor (10-20 μ M) correlated with the inhibition of SO release from isolated leukocytes suggesting that this dose range maybe effective in attenuating ROS production [11].

We extended our research in the current study by using a MI (30min)/R (90min) isolated perfused rat heart model. A cell permeable PKC β II peptide inhibitor (10-20 μ M) was given at the beginning of reperfusion for five minutes. Post-reperfused cardiac function and infarct size were measured and compared to untreated control MI/R hearts.

Hypothesis

We hypothesize that PKC β II peptide inhibitor will improve post-reperfused cardiac function and reduce infarct size in isolated perfused rat hearts (*ex vivo*) subjected to global MI/R compared to non-drug control MI/R hearts in MI(30min)/R(90min) studies.



Fig. 2. PKC β II peptide inhibitor location resides in the Ca²⁺ binding domain (C2-4 region) of RACK (N-Myr-SLNPEWNET), adapted from [8].

Methods

Isolated Rat Heart Preparation

Male Sprague Dawley rats (275-325g, Charles River, Springfield, MA) were anesthetized with pentobarbital sodium (60mg/kg) and anti-coagulated with sodium heparin (1,000 U) injection intraperitoneally. Hearts were rapidly excised and perfused at a constant pressure of 80 mm Hg with a modified physiological Krebs' buffer aerated with 95% O₂-5% CO₂ maintained at 37°C and pH 7.3-7.4 by Langendorff preparation. Hearts were subjected to 15 min of baseline perfusion, 30 min of ischemia, and a 90 min reperfusion period [8]. Five ml of plasma (control MI/R hearts), or plasma containing cell-permeable PKC β II peptide inhibitor (N-Myr-SLNPEWNET, MW=1300 g/mol, 10-20 μ M Genemed Synthesis Inc., San Antonio, TX) (Figure 2) were infused during the first 5min of reperfusion by a side arm line proximal to the heart inflow at a rate of 1ml/min. Coronary flow, left ventricular developed pressure (LVDP), maximal and minimal rate of LVDP (+dP/dt_{max} and -dP/dt_{min}), and heart rate were taken every 5min during baseline and reperfusion using a flow meter (T106, Transonic Systems, Inc., Ithaca, NY) and pressure transducer (SPR-524, Millar Instruments, Inc., Houston, TX), respectively. Data were recorded using a Powerlab Station acquisition system (ADInstruments, Grand Junction, CO).

Sham hearts experienced no ischemia, received no drug and were infused with plasma at the same time point as I/R hearts. To evaluate tissue viability, the left ventricle was isolated at the end of the cardiac function experiment and cross sectioned into five 2mm thick slices from apex to base. The slices were subjected to 1% triphenyltetrazolium chloride (TTC) staining for 15min at 37°C (viable tissue stained red, infarct left unstained (white)). Infarct size was expressed as the percentage of dead tissue to the total tissue weight.

Statistical Analysis

All data in the text and figures are presented as means \pm S.E.M. Analysis of variance using post hoc analysis with the Student-Newman-Keuls test was used for heart function and infarct size in the MI(30min)/R(90min) study. Probability values of <0.05 are statistically significant.

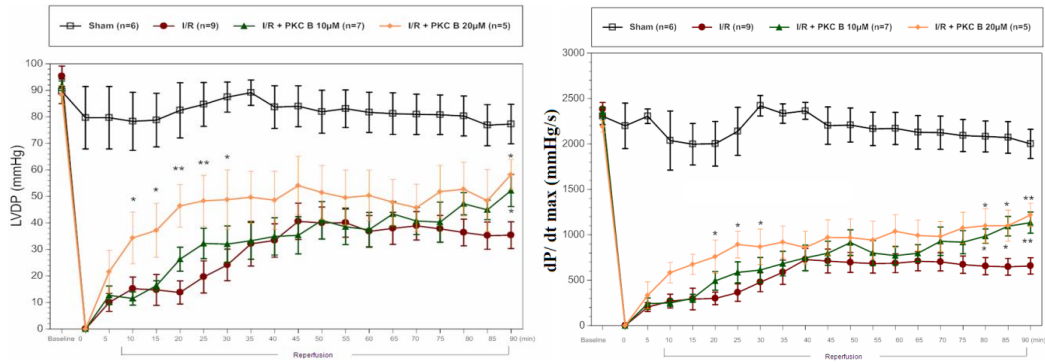


Fig. 3. Time course of LVDP (left) and dP/dt_{max} (right) in sham, control MI/R and MI/R+PKC β II inhibitor (10 and 20 μ M) perfused rat hearts. LVDP and dP/dt_{max} data at initial (baseline) and reperfusion from 0 to 90 min following 30 min ischemia are shown. (* p <0.05; ** p <0.01 compared to untreated control MI/R hearts).

Results and Discussion

This study focused on the inhibition of PKC β II in MI/R injury. Figure 3 shows that PKC β II inhibitor (10 and 20 μ M) significantly improved cardiac function compared to untreated control MI/R hearts. Sham hearts ($n=6$) maintained cardiac function throughout the experimental protocol (i.e., $87\pm 9\%$ of initial LVDP and $89\pm 8\%$ of initial dP/dt_{max}). MI/R+PKC β II inhibitor hearts (10 μ M, $n=7$; 20 μ M, $n=5$) exhibited a significant improvement in LVDP $66\pm 8\%$ and dP/dt_{max} $56\pm 8\%$ (20 μ M) and $57\pm 7\%$ and $48\pm 5\%$ (10 μ M) compared to control MI/R hearts ($n=9$) that only recovered to $38\pm 6\%$ (LVDP) and $28\pm 4\%$ (dP/dt_{max}) at 90 min post-reperfusion of initial baseline. MI/R+PKC β II inhibitor hearts (20 μ M) significantly improved post-reperfused LVDP at 10-30 and 90 minutes and dP/dt_{max} at 20-30 and 80-90 minutes compared to untreated MI/R controls. MI/R+PKC β II inhibitor hearts (10 μ M) significantly improved post-reperfused LVDP at 90 minutes and dP/dt_{max} at 80-90 minutes compared

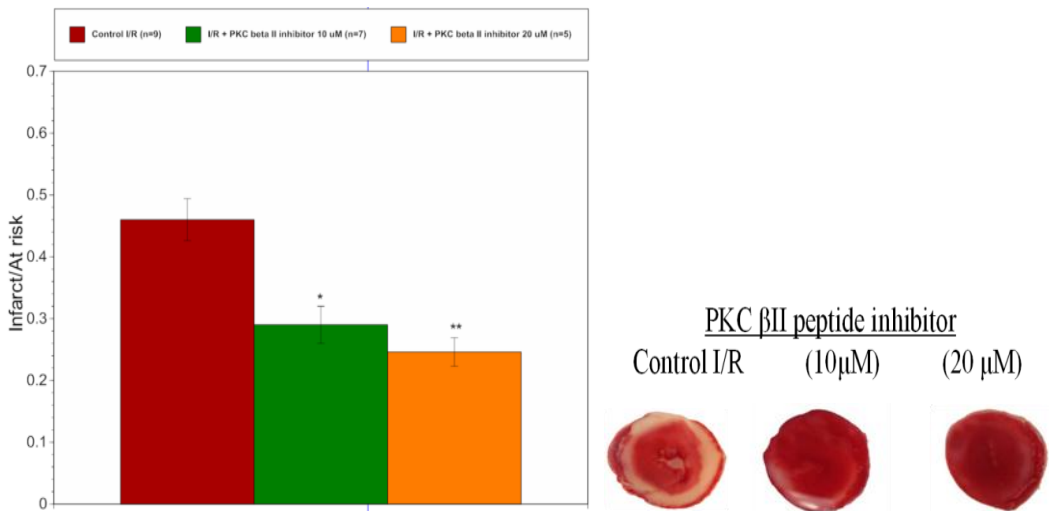


Fig. 4. Representative TTC stained heart sections displayed above from control MI/R and MI/R+PKC β II inhibitor hearts were assessed after cardiac function experiments to determine infarct size. Viable tissue stained red and infarcted tissue was unstained (white). (* p <0.05 and ** p <0.01 compared to I/R control).

to untreated MI/R controls. Also, PKC β II inhibitor hearts displayed a significantly reduced infarct size ($29\pm 3\%$, $10\ \mu\text{M}$; $25\pm 3\%$, $20\ \mu\text{M}$) and compared to untreated I/R hearts that had an infarct size of $46\pm 3\%$ (Figure 4). Sham hearts had minimal cell death ($<0.05\%$) at the end of the experimental protocol (data not shown).

In conclusion, PKC β II peptide inhibitor was shown to improve post-reperused cardiac function and decrease infarct size. Reperfusion injury following myocardial ischemia has been shown to be a pathologic condition resulting in contractile dysfunction and myocardial cell death in animal models and patients suffering from a myocardial infarction. PKC β II peptide inhibitor given at the beginning of reperfusion significantly improved contractile function and decreased infarct size compared to MI/R untreated controls at 90 min post-reperfusion following 30 min global ischemia. These data suggest that PKC β II inhibition during reperfusion attenuates MI/R injury by improving cardiac function and salvaging heart tissue. These effects may be related to inhibiting ROS release in MI/R. Therefore, PKC β II inhibitor will be an effective therapeutic tool to ameliorate cardiac contractile dysfunction and tissue damage in heart attack, coronary bypass, and organ transplant patients.

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