Protein Kinase C Beta II (PKC βII) 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 βII mediated activation of NADPH oxidase, which occurs upon cytokine release during MI/R [3]. PKC βII activity is increased in animal models of MI/R and known to exacerbate tissue injury [4,5]. PKC βII 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 βII 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 βII mediated ROS, would attenuate endothelial dysfunction and downstream pro-

Fig. 1. Left: Schematic representation of PKC βII mediated activation in myocardial ischemia and reperfusion (MI/R). MI/R induces cytokine receptor activation within minutes leading to activation of PKC βII via diacylglycerol (DAG). PKC βII 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 βII peptide inhibitor. PKC βII peptide inhibitor attenuates the translocation of PKC βII 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 βII peptide inhibitor mechanism of action (Figure 1 right) is to inhibit PKC βII 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 βII 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 βII peptide inhibitor (10-20 μM) 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 βII peptide inhibitor (10-20 μM) 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 βII 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 βII peptide inhibitor location resides in the Ca²⁺ binding domain (C2-4 region) of RACK (N-Myr-SLNPEWNET), adapted from [8].](image)

**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 βII peptide inhibitor (N-Myr-SLNPEWNET, MW=1300 g/mol, 10-20μM 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 ± 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.
Results and Discussion

This study focused on the inhibition of PKC βII in MI/R injury. Figure 3 shows that PKC βII inhibitor (10 and 20 μM) 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±9% of initial LVDP and 89±8% of initial dP/dt_{max}). MI/R+PKC βII inhibitor hearts (10 μM, n=7; 20 μM, n=5) exhibited a significant improvement in LVDP 66±8% and dP/dt_{max} 56±8% (20 μM) and 57±7% and 48±5% (10 μM) compared to control MI/R hearts (n=9) that only recovered to 38±6% (LVDP) and 28±4% (dP/dt_{max}) at 90 min post-reperfusion of initial baseline. MI/R+PKC βII inhibitor hearts (20 μM) 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 βII inhibitor hearts (10 μM) significantly improved post-reperfused LVDP at 90 minutes and dP/dt_{max} at 80-90 minutes compared to untreated MI/R controls.

Fig. 3. Time course of LVDP (left) and dP/dt max (right) in sham, control MI/R and MI/R+PKC βII inhibitor (10 and 20 μM) 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).

Fig. 4. Representative TTC stained heart sections displayed above from control MI/R and MI/R+PKC βII 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 βII inhibitor hearts displayed a significantly reduced infarct size (29±3%, 10 μM; 25±3%, 20 μM) and compared to untreated I/R hearts that had an infarct size of 46±3% (Figure 4). Sham hearts had minimal cell death (<0.05%) at the end of the experimental protocol (data not shown).

In conclusion, PKC βII peptide inhibitor was shown to improve post-reperfused 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 βII 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 βII 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 βII 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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