Myristoylated Protein Kinase C Beta II Peptide Inhibitor Exhibits Robust Attenuation of Myocardial Ischemia/Reperfusion Injury in Rats

Ian T. Madison, Melinda A. Beale, Daphne Metellus, Jeremy Castro, Michael F. Lloyd, Redona Gjoka, Tameka Dean, Arjun Nair, Rose M. Martorana, Anahi McIntyre, Christina Lipscombe, Chinyere Ebo, Qian Chen, Robert Barsotti, and Lindon Young

Department of Bio-Medical Sciences, Philadelphia College of Osteopathic Medicine, 4170 City Avenue, Philadelphia, PA 19131

Young Therapeutics, LLC

Introduction

Heart disease remains the leading cause of death in adults in the United States and worldwide, with coronary artery disease being the most common form that often leads to myocardial infarction. Though rapid restoration of coronary blood flow is crucial to preserving cardiac tissue function, it also results in an additional insult known as myocardial ischemia/reperfusion (MI/R) injury. MI/R injury may be attenuated by inhibiting the generation of reactive oxygen species (ROS) upon cardio-angioplasty following a heart attack. Protein kinase C βII (PKCβII) is a key signaling molecule in generation of ROS and I/R injury (Figure 1)(1). MI/R induces cytokine receptor activation, leading to PKCβII activation via second messengers diacylglycerol (DAG) and calcium. Activated PKCβII binds to its selective receptor for activated C kinase (RACK). RACK enhances PKCβII translocation to the cell membrane to activate NADPH oxidase (NOX-2), which produces copious ROS during reperfusion (Figure 2)(2-4). PKCβII activation and ROS production further contribute to additional damage via mitochondrial dysfunction and reduced nitric oxide (NO) bioavailability (Figure 1).

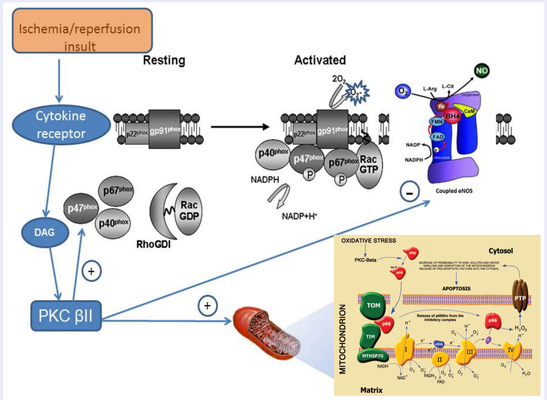


Figure 1. Schematic representation of PKCβII mediated activation of mitochondrial ROS and NOX-2 superoxide (O_2-) release along with decreased NO release from endothelial NO synthase (eNOS) in MI/R (adapted from [5,6]). MI/R induces cytokine receptor activation leading to activation of PKC βII via diacylgycerol (DAG). Activated PKCβII increases ROS and O_2- release from damaged mitochondria and NOX-2, respectively, and decreases eNOS activity. It also stimulates mitochondrial p66Shc protein, a component in the pathway resulting in opening of the mitochondrial permeability transition pore (PTP), which in turn leads to release of proapoptotic factors into the cytosol to further promote tissue injury during reperfusion.

Inhibition of tissue NOX-2 attenuates inflammation-mediated vascular injury seen in various diseases, including diabetes and myocardial infarction (4). Previously, a myristoylated (myr-) selective PKCβII peptide inhibitor (*N*-myr-SLNPEWNET; myr-PKCβII-) was found to dose-dependently inhibit superoxide (SO) release and MI/R injury via the mechanism depicted in Figure 2 (3, 6, 7). The effects of PKCβII peptide activator (*N*-myr-SVEIWD: myr-PKCβII+), in MI/R is unknown. Its proposed action is to enhance MI/R injury by prolonging PKCβII kinase action directed towards NOX-2 activation (Figure 2). Myristoylation of peptides is known to augment entry into the cell via simple diffusion through the cell membrane to affect PKC activity (8), but prior studies did not explore the possibility that myr-conjugation contributes to the attenuation of MI/R injury (9). Therefore, we tested the effectiveness of myr-PKCβII- compared to scrambled myr-PKCβII- (*N*-myr-WNPESLNTE; myr-PKCβII-scram) and plasma controls to evaluate whether myristoylation plays a role in the cardioprotective effects of myr-PKCβII- in I/R injury (10).

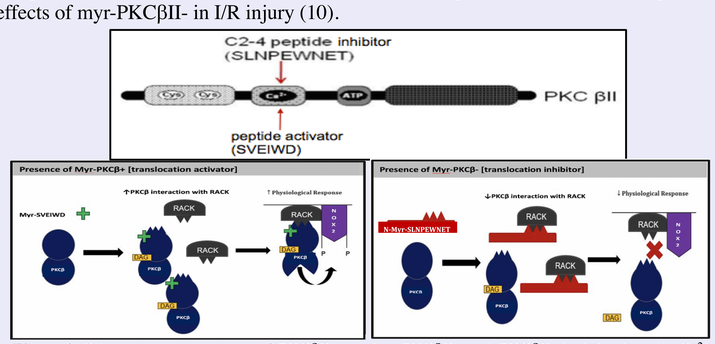


Figure 2. Schematic representation of PKCβII peptides. PKCβII+ and PKCβII- both bind to the Ca^{2+} binding domain within the RACK binding site (i.e., C2-4 region;) of PKCβII to regulate its translocation to the cell membrane (top; Adapted from [3]). PKCβII+ mechanism of action is to increase PKCβII translocation to the cell membrane via RACK binding and its interaction with substrates, like NOX-2, while PKCβII- inhibits that interaction (bottom; Adapted from [2]). Myr-PKCβII-scram should exert neither a preventative nor stimulatory effect.

Hypothesis

We hypothesize that 1) Myristic acid conjugation itself is not responsible for the improved post-reperfused cardiac function and reduced infarct size as previously reported with myr-PKCβII- (6) 2) Myr-PKCβII- will improve post-reperfused cardiac function and decrease infarct size compared to all groups. 3) Myr-PKCβII-scrambled peptide (myr-control) will be similar to non-drug treated controls. 4) Myr-PKCβII+ treated hearts will exacerbate MI/R injury as measured by decreased post-reperfused cardiac function and increased infarct size compared to all other groups.

Research Design

Male Sprague-Dawley rats (~300g, Charles River, Springfield, MA) were anesthetized with I.P. pentobarbital (60mg/kg) and anticoagulated with 1000U of heparin. The heart was then removed and placed on a perfusion needle of the Langendorff apparatus and perfused with Krebs' buffer. A pressure transducer was placed into the left ventricle to measure cardiac function, as previously described (6,7). A schematic of the MI/R protocol is depicted below:

myr-PKCβII+ (20 uM in plasma); myr-PKCβII – (20 uM in plasma); myr-PKCβII -scram (20μM in plasma) or untreated control (plasma alone) for first 5min of reperfusion

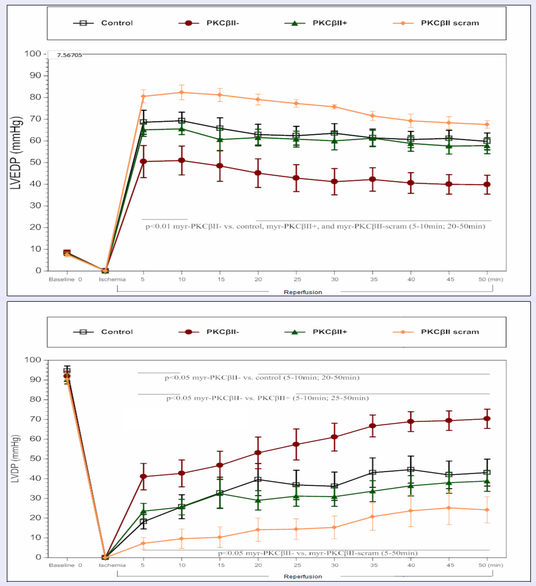
Baseline (15min.) → Ischemia (30min.) → Reperfusion (50min.) → TTC staining

At the end of the reperfusion period, all hearts were frozen at -20 ºC for 30 min, sectioned into 2mm slices and incubated at 37ºC in 1% triphenyltetrazolium chloride (TTC). The percentage between dead heart tissue (i.e., unstained) weight to total heart tissue weight was calculated for infarct size.

Statistical Analysis

All data in the text, figures, and table are presented as means ± S.E.M. The data were analyzed by ANOVA using Bonferroni-Dunn post-hoc analysis. Probability values of <0.05 are considered to be statistically significant.

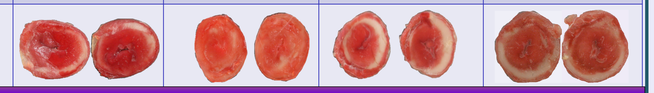
Results



myr-PKCβII- (n=14)
myr-PKCβII-scram (n=6)
myr-PKCβII+ (n=13)
Control (n=13)

Figure 3. Time course of left ventricular end diastolic pressure (LVEDP) for control, myr-PKCβII+/-/-scram MI/R studies (top) and LV developed pressure (LVDP)(bottom). Myr-PKCβII- treated I/R hearts significantly improved LVEDP (an index of myocardial relaxation) throughout the 50 minute reperfusion time course compared to Control, Myr-PKC βII+, and Myr-PKC-β-scrambled treated I/R hearts (top). Myr-PKCβII- treated I/R hearts significantly improved LVDP (the difference between LV end systolic pressure and LVEDP) throughout most of the 50 minute reperfusion period compared to Control, Myr-PKCβII+, and Myr-PKC-β-scrambled I/R hearts; this effect is attributed to improved LVEDP.

Table 1. Initial and final cardiac function values and infarct size for control, myr-PKCβII+; myr-PKC βII-; myr-PKCβII-scram MI/R studies; *p<0.05, **p<0.01 vs. non-drug treated controls; #p<0.05, ##p<0.01 vs. myr-PKCβII+; †p<0.05, ††p<0.01 vs. myr-PKC βII-scram. Representative sections shown are both sides of a 2mm mid-wall section for each MI/R study group. $+dP/dT_{max}$ = contractility, dP/dT_{min} = relaxation, LVDP=left ventricular developed pressure, LVESP=left ventricular end systolic pressure, LVEDP=left ventricular end diastolic pressure.

Cardiac Function and Infarct Size Indices	Control (n= 13)	PKCβII Inhibitor (n=14)	PKCβII Activator (n=13)	PKCβII scrambled (n=6)
Initial Flow (mL/min)	19±1	18±1	18±1	19±3
Final Flow (mL/min)	10±1	10±1	10±1	7±1
Initial $+dP/dt_{max}$ (mmHg/sec)	2437±64	2390±55	2391±54	2331±67
Final $+dP/dt_{max}$ (mmHg/sec)	851±117	1575±97**##††	893±122	543±99
Initial $-dP/dt_{min}$ (mmHg/sec)	-1694±75	-1715±65	-1625±57	-1623±84
Final $-dP/dt_{min}$ (mmHg/sec)	-765±96	-1085±85**##††	-716±84	-428±96
Initial LVDP (mmHg)	95±2	92±3	90±2	90±3
Final LVDP (mmHg)	43±7	70±5**##††	41±5	24±7
Initial LVESP (mmHg)	103±3	100±3	98±2	98±4
Final LVESP (mmHg)	103±4	110±5	99±3	92±5
Initial LVEDP (mmHg)	8±1	8±1	8±1	8±1
Final LVEDP (mmHg)	60±4	40±4**##††	58±4	68±2
Initial Heart Rate (BPM)	281±8	280±5	275±9	265±12
Final Heart Rate (BPM)	269±7	249±4	255±10	287±24
Infarct Size (%)	24±4	13±2**#††	21±3	25±2
Representative Sections				

Conclusions

Infarct size

Myr-PKCβII- treated hearts had significantly reduced infarct size compared to all other treatment groups. There was no significant difference between untreated control I/R hearts, myr-PKCβII- scram, and myr-PKCβII+. It is possible that further PKCβII activation by myr-PKCβII+ does not result in additional tissue injury due to maximal activation of NOX-2 mediated ROS generation by tissue cytokines during reperfusion.

Cardiac function

Myr-PKCβII- improved post-reperfused cardiac function vs. control, myr-PKCβII+, and myr-PKCβII- scram treated hearts. The significant improvement in final post-reperfusion LVDP in myr-PKCβII- treated hearts is attributed to the significant reduction in final LVEDP values (i.e. ~40mmHg) compared to control and myr-PKCβII+ hearts (i.e. ~58mmHg) and myr-PKCβII-scram hearts (~68mmHg). This is reflected in the significant restoration of the final maximal rate of contractility ($+dP/dt_{max}$) and relaxation ($-dP/dt_{min}$). Surprisingly, myr-PKCβII-scram exhibited the most deleterious effects on the heart by an unknown mechanism. Further experiments are underway to determine this mechanism.

These results suggest that: 1) Myr-PKCβII- significantly improves post-reperfusion cardiac function in isolated rat hearts as measured by LVDP, LVEDP, $+dP/dt_{max}$, and $-dP/dt_{min}$ **2)** Myr-conjugation is not responsible for the cardioprotective effects observed with myr-PKC βII-. Therefore, treatment with myr-PKCβII- may be an effective strategy to limit MI/R injury in heart attack patients upon reperfusion via fibrinolytic therapy, angioplasty or coronary artery bypass surgery.

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