Protein Kinase C Beta II Peptide Inhibitor Conjugated to a Novel Myristic Acid-Trans-Activator of Transcription – Tandem Rapidly Attenuates Superoxide Release in Isolated Rat Polymorphonuclear Leukocytes through Superior Intracellular Delivery of Cargo

Sunit G. Singh, Tameka C. Dean, Alexis Verwoert, Qian Chen, Robert Barsotti, Lindon Young

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

Introduction

Polymorphonuclear leukocyte (PMN) respiratory burst, characterized by marked superoxide (SO) generation has been shown to contribute to cardiac contractile dysfunction and left ventricular remodeling following ischemia-reperfusion (I/R) injury.[1] NADPH oxidase (NOX-2) is a principal producer of superoxide (SO) in PMNs following phosphorylation by activated protein kinase C beta II (PKCβII), as shown in **Fig. 1.**[2,3] Following activation with diacylglycerol (DAG) and calcium (Ca^{+2}), PKCβII binds to its selective receptor for activated C kinase (RACK-1) and translocates from the cytosol to phosphorylate protein targets, such as NOX-2, shown in **Fig. 2B & Fig. 3.**[2,3]
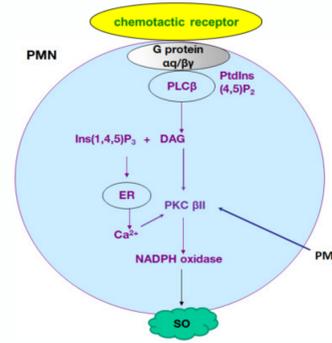

Figure 1. Schematic representation of PKCβII role in stimulating SO release in PMNs.

Ca^{2+} and DAG directly activate PKCβII. Activated PKCβII phosphorylates NOX-2, which then releases SO. Phorbol 12-myristate 13-acetate (PMA), a well-known lipid-soluble DAG mimetic exhibits broad-spectrum PKC agonist activity by directly activating multiple PKC isoforms, such as PKC delta (PKCδ) and PKCβII to diminish and augment PMN SO release, respectively[2,3,4,5,6] (adapted)[3].
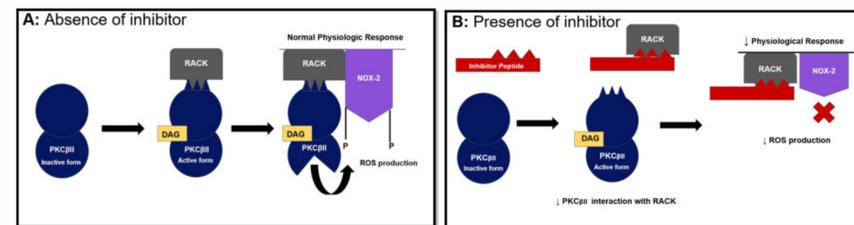

Figure 2. Panel A: The normal physiological response, without an inhibitor present. The normal physiological response is for PKCβII translocation via RACK binding to interact with substrates, like NOX-2. Panel B: PKCβII peptide inhibitor impedes interaction of PKCβII and RACK-1 (adapted).[7]
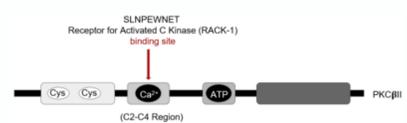

Figure 3. Illustration of PKCβII. PKCβII binds to the Ca^{2+} binding domain within the RACK-1 binding site (i.e., C2-4 region;) of PKCβII to regulate its translocation to cellular proteins to phosphorylate its substrate (e.g. NOX-2) (adapted).[3]

NOX-2 inhibition has been shown to attenuate myocardial infarct size following global I(30 min)/R(45min) and attenuate PMA-induced SO release from PMNs in Sprague-Dawley rats.[4] Additionally, myristic acid (myr) and trans-activation of transcription (Tat) conjugation have independently demonstrated enhanced intracellular delivery of peptide cargo via simple diffusion and endocytosis respectively, shown in **Fig. 4.**[8] In previous studies, Tat conjugated NOX-2 peptide inhibitor (100μM, Nox2ds-tat) and myristoylated NOX-2 peptide inhibitor (10 μM, myr-Nox2ds) resulted in ~35% and ~70% inhibition of PMA-induced SO release, respectively.[9] Thus, selective inhibition of PKCβII translocation, an upstream regulator of NOX-2 activity, may mitigate inflammatory SO damage involved in I/R injury (mechanism shown in **Fig. 2B**) By combining anchoring (myr) and endocytic (Tat) mechanisms for synergistic intracellular delivery, we propose a dual myr-Tat conjugated PKCβII peptide inhibitor (myr-Tat-PKCβII-; N-myr-Tat-CC-SLNPEWNET) for optimal attenuation of PMA-induced SO release.
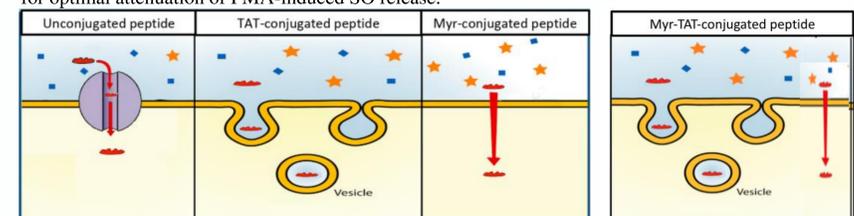

Figure 4. Mechanisms of entry for unconjugated, Tat-, and myr-conjugated peptide cargo. Unconjugated peptide uses facilitated diffusion, requiring carrier protein for intracellular delivery.[10] Tat-conjugated peptide cargo uses endocytosis-mediated entry as positive charge in Tat reacts with negative charge on cell membrane. CC/GG spacers facilitate liberation of peptide cargo intracellularly. Myr-conjugated peptide cargo uses simple diffusion into intracellular space. Myr-Tat conjugated peptides are proposed to enhance cargo delivery by employing synergistic mechanisms of myr and Tat conjugation, thereby increasing the potency of cargo effects. (adapted).[8]

Aims/Hypothesis

This study aims to compare the effects of myr conjugated, myr-Tat conjugated and unconjugated PKCβII- (SLNPEWNET) on PMA-induced PMN SO release. Additionally, we aim to test a scrambled control PKCβII- (WNPESLNTE; myr-PKCβII-scram) to further evaluate whether the proposed mechanism of action (i.e. inhibition of PKCβII translocation) is influenced by myr-conjugation.

We hypothesize the following:
- Myr-Tat-PKCβII- and myr-PKCβII- should attenuate PMA-induced PMN SO release compared to non-drug and myr-PKCβII-scram controls.
- Scrambled myr-PKCβII inhibitor should not differ from non-drug treated controls following PMA stimulation.

Experimental Design

Isolation of PMNs. Male Sprague-Dawley rats (350-400g, Charles River, Springfield MA) under anesthesia of 2.5% isoflurane were injected intraperitoneally (I.P.) with 16 ml of 0.5% glycogen for induction of rat peritonitis and PMN recruitment. After 16–18h, rats were re-anesthetized with 2.5% isoflurane, and the PMNs were harvested by peritoneal lavage. SO release was measured spectrophotometrically as the change in absorbance at 550 nm over 390 sec via ferricytochrome c reduction after PMA stimulation (100nM), as previously described.[10]

Statistical Analysis. All data in the text and figures 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.
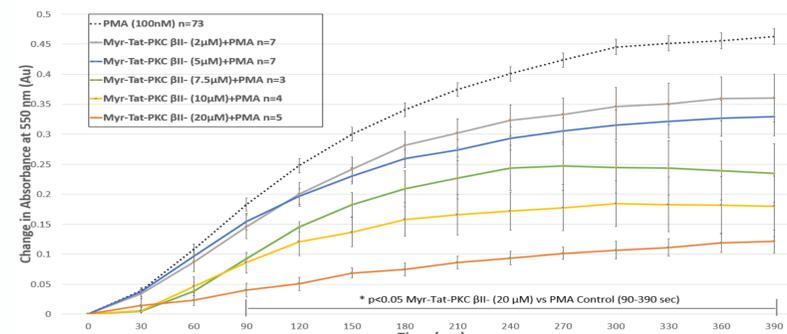
Results

Figure 5. Time course of PMA induced PMN SO release at various concentrations. Myr-Tat-PKCβII- attenuated PMA-induced PMN SO release in a concentration dependent manner (2μM-20μM) throughout the time course.
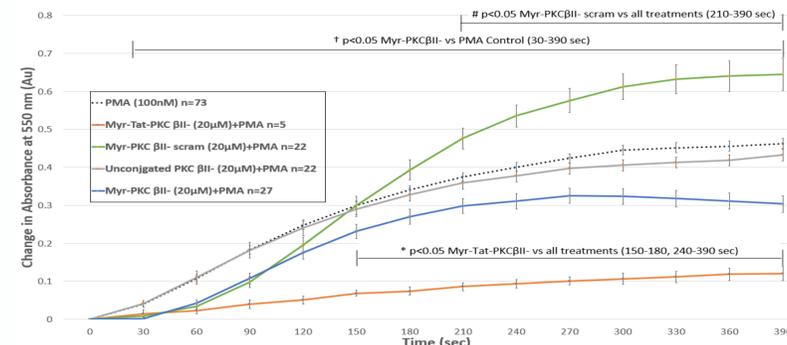

Figure 6. Time course of PMA induced PMN SO release. Myr-Tat-PKCβII- reduced SO release from 240-390 sec compared to all study groups (*p<0.05). Cell viability determined by trypan blue exclusion (not shown) was similar in all groups (94±2%).
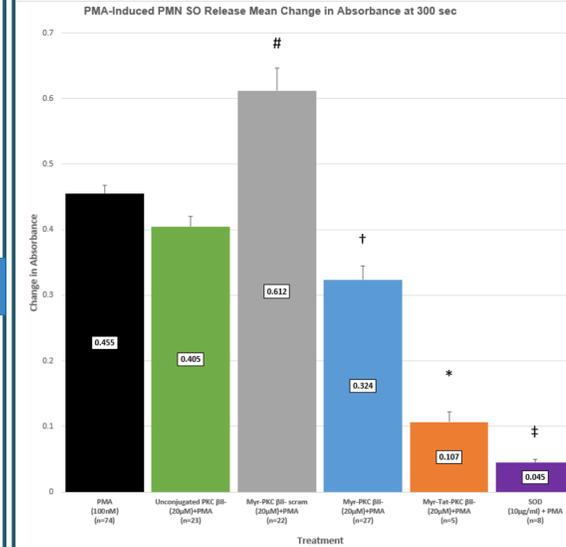
Results

Figure 7. PMA induced PMN SO release at peak absorbance (300 sec) in non-treated control, myr-Tat-conjugated, myr-conjugated, and unconjugated PKCβII- peptides treatment groups.

PMA induced PMN SO release increased absorbance in non-treated controls (0.455±0.01). Myr-PKCβII- significantly reduced absorbance to 0.324±0.02 compared to PMA (†p<0.05). Myr-Tat-PKCβII- (0.107±0.02) significantly attenuated SO release compared to all study groups (*p<0.05). Myr-PKCβII-scram significantly increased absorbance (0.612±0.03) compared to all groups (#p<0.05). Unconjugated PKCβII- (0.405±0.02) was not different from non-drug controls. SOD-treated samples (0.045±0.01) reduced SO release by ~90% and was significantly different compared to all groups, except myr-Tat-PKCβII- (‡p<0.05).

Discussion

Dual conjugated myr-Tat-PKCβII- exerted the greatest attenuation of PMA-induced PMN SO release compared to all groups except SOD (positive control). SOD, which rapidly converts SO to H_2O_2, significantly reduced absorbance by ~90%. Unconjugated PKCβII- did not significantly alter PMN SO release compared to non-treated control. Unexpectedly, myr-PKCβII-scram significantly stimulated the highest increase in absorbance compared to all groups.

These results suggest that:
1. Both myr and myr-Tat conjugations enhance intracellular delivery of peptide cargo for significant attenuation of PMA-induced SO release compared to unconjugated PKCβII inhibitor.
2. Myr-Tat dual conjugation is superior to myr conjugation alone.
3. The marked reduction of absorbance with SOD indicates that increases in absorbances are mainly due to PMA-induced PMN SO release.

Conclusion: Myr-Tat-PKCβII inhibitor (5 μM) is 4x more potent than myr-PKCβII inhibitor (20 μM) in attenuating PMA-induced PMN SO release. These results suggest that dual myr-Tat conjugation increases potency of cargo peptides for optimal response. Selective potent inhibition of PKCβII may be an effective strategy to limit inflammation-induced (e.g. PMNs) tissue damage in I/R with potential applications in coronary angioplasty following an acute myocardial infarction or organ transplantation.

Future studies will:
1. Compare Tat-PKCβII and myr-Tat-PKCβII scrambled inhibitor peptides and myr-Tat to other groups in PMA-induced PMN SO release.
2. Evaluate PKCβII translocation via western blot analysis of PMN cell lysates to determine whether myr-PKCβII-scram augments absorbance by a different mechanism.

References

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