Protein Kinase C Epsilon Inhibitor Conjugated with Myristic Acid and Trans-Activator of Transcription Elicits Superior Cargo Delivery and Cardioprotective Effects in Rat Myocardial Ischemia Reperfusion Injury

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Introduction

Rapid restoration of coronary blood flow is crucial to preserving cardiac tissue function following myocardial infarction, but it also results in additional insult known as myocardial ischemia/reperfusion (MI/R) injury.[1] MI/R injury may be attenuated by inhibiting reactive oxygen species (ROS) formation upon reperfusion during coronary angioplasty. Following cytokine-mediated activation, Protein Kinase C epsilon (PKCε) binds to its selective receptor for activated C kinase (RACK-1) and translocates from the cytosol to phosphorylate transmembrane protein targets, such as eNOS, shown in **Fig. 1A & Fig. 2.** Activated PKCε has been shown to increase reactive oxygen species (ROS) release, in part, by its stimulation of increased uncoupled endothelial nitric oxide synthase (eNOS) activity and opening of mitochondrial potassium ATP channels, shown in **Fig. 1**.[2,3,4,5]

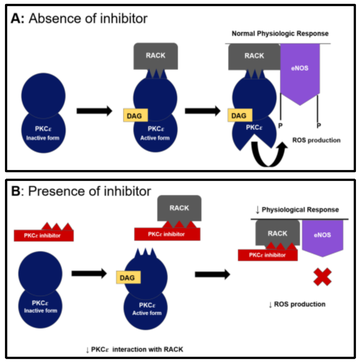


Figure 1. Panel A: the normal physiological response, without an inhibitor present. The normal physiological response is for PKCε translocation via RACK binding to interact with substrates, like eNOS. Panel B: PKCε inhibitor impedes interaction of PKCε and RACK-1 (adapted).[6]

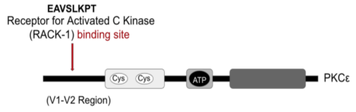
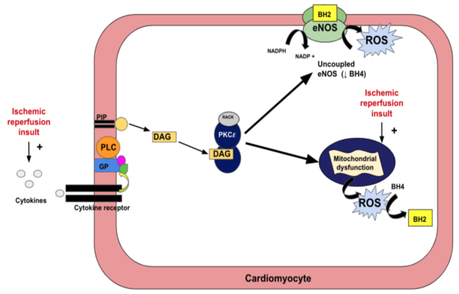


Figure 2. Illustration of PKCε. PKCε binds to the variable region within the RACK-1 binding site (i.e., V1-V2 region) of PKCε to regulate its translocation to cellular proteins to phosphorylate its substrate (e.g. eNOS) (Adapted). [7]

Figure 3. Schematic representation of PKCε regulation of eNOS and mitochondrial-derived ROS release in myocardial I/R. I/R insult activates PKCε and opening of mitochondrial potassium ATP channels, resulting in excess mitochondrial ROS production. [4] Oxidative stress leads to oxidation of tetrahydrobiopterin (BH4) to dihydrobiopterin (BH2) which in turn promotes eNOS uncoupling. Activated PKCε phosphorylation increases uncoupled eNOS activity resulting in excess ROS production instead of nitric oxide.



Myristic acid (Myr) and trans-activator of transcription (Tat) conjugation have independently demonstrated enhanced intracellular delivery of peptide cargo in animal studies via simple diffusion and endocytosis respectively, shown in **Fig. 4.**[8] In separate pre-clinical studies, Myr-conjugation and Tat-conjugation with an inhibitory peptide of PKCε-(EAVSLKPT) have been shown to mitigate myocardial I/R injury via inhibiting ROS formation and cytokine release.[9,10,11] Myr-PKCε- was effective at 5μM to 20 μM concentrations, but was not effective at 1μM concentration in reducing infarct size and restoring cardiac function in *ex vivo* rat hearts subjected to global I(30 min)/R(90 min).[9] However, Tat-PKCε-(0.46 mg/kg or ~5μM in blood) was determined to be ineffective in clinical studies.[12,13] The absence of clinical significance with Tat-PKCε- may be due to limited penetration of the peptide cargo. By combining anchoring (Myr) and endocytic (Tat) mechanisms for synergistic intracellular delivery, we propose a dual Myr-Tat conjugated PKCε peptide inhibitor (Myr-Tat-PKCε-; N-Myr-Tat-CC-EAVSLKPT) for optimal cardioprotection.

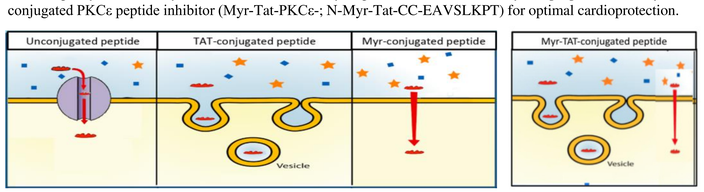


Figure 4. Mechanisms of intracellular entry for unconjugated, Tat, Myr, and Myr-Tat-conjugated peptide cargo. Unconjugated peptide uses facilitated diffusion, requiring carrier protein for intracellular delivery. Tat-conjugated peptide cargo uses endocytosis-mediated entry as positive charge in Tat reacts with negative charge on cell membrane. CC or 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 cardioprotective effects of Myr conjugated, Tat-conjugated and Myr-Tat conjugated PKCε- (EAVSLKPT). Additionally, we aim to test PKCε- scrambled controls (LSETKPAV), Myr-PKCε-scram and Myr-Tat-PKCε-scram to further evaluate whether the proposed mechanism of action (i.e. inhibition of PKCε- translocation) is influenced by Myr- or Myr-Tat conjugation.

We hypothesize the following:

- Myr-Tat-PKCε- should demonstrate the most robust cardioprotection, as indexed by infarct size reduction and improve post-reperfused cardiac function (i.e., maximal rise of left ventricular contraction, dP/dT max)
- PKCε- scrambled controls should not differ from non-drug treated controls.

Experimental Design

Male Sprague-Dawley rats (275-325g) were anesthetized with sodium pentobarbital (60 mg/kg) and anticoagulated with heparin 1000 units intraperitoneally. Hearts were isolated and studied using a modified Langendorff heart preparation as previously described.[9,10] PKCε-treatments were prepared in 28% dimethyl sulfoxide (DMSO) and further dissolved in Krebs' buffer and infused during the first 5 min of reperfusion via a syringe pump at 1 ml/min, following a 30 min period of global ischemia and then 50 min of reperfusion. All hearts were frozen at -20 °C for 30 min, sectioned into 2mm slices and incubated at 37° C in 1% 2,3,5-Triphenyltetrazolium chloride (TTC) to determine infarction size per area at risk (i.e. entire heart).

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 <0.05 were considered to be statistically significant.

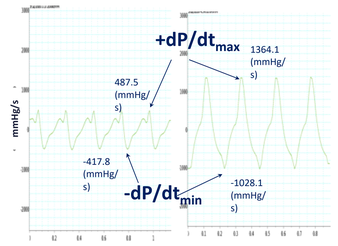


Figure 5. Representative tracings of the maximal rise of left ventricular developed pressure (LVDP) [+dP/dt$_{max}$; mmHg/s] and the maximal rate of decline of LVDP [-dP/dt$_{min}$;mmHg/s] for control I/R (left) and I/R + Myr-Tat PKCε inhibitor (100 nM) (right) treated hearts at 50-min reperfusion.

Results

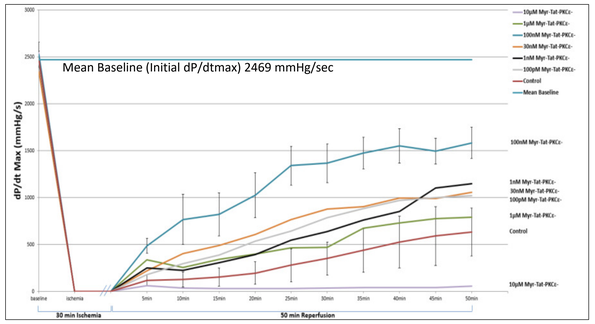


Figure 6. Time course of Myr-Tat PKC epsilon inhibitor effects on cardiac function (dP/dT max) in rat hearts subjected to global I(30min)/R(50min).

100nM Myr-Tat-PKCε inhibitor hearts significantly restored post-reperfused dP/dtmax compared to control I/R hearts p<0.05.

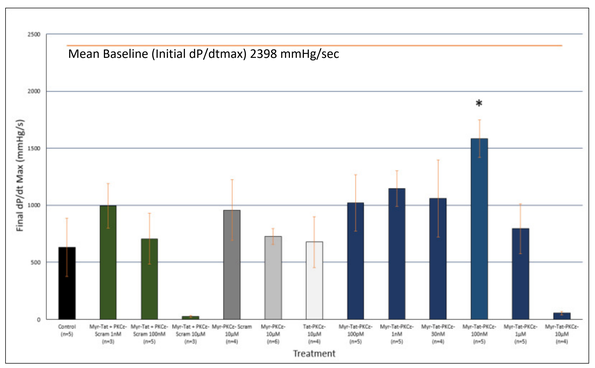


Figure 7. Comparison of Myr, Tat and Myr-tat PKC epsilon inhibitor effects on cardiac function (final dP/dT max) in rat hearts subjected to global I(30min)/R(50min).

100nM Myr-Tat-PKCε inhibitor hearts significantly restored dP/dtmax compared to control I/R hearts p<0.05.

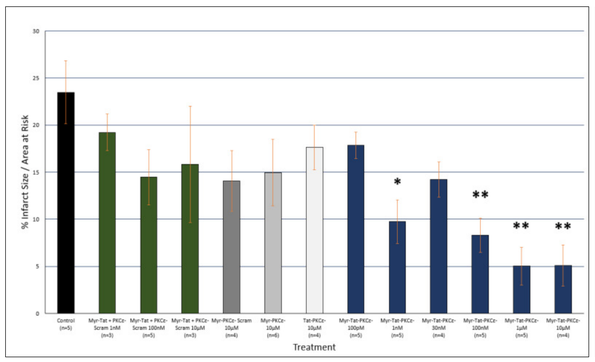


Figure 8. Comparison of Myr, Tat and Myr-Tat PKC epsilon inhibitor effects on infarct size/area at risk (%) in rat hearts subjected to global I(30min)/R(50min).

10μM, 1μM, 100nM and 1nM Myr-Tat-PKC epsilon inhibitor significantly reduced infarct size compared to control hearts. Myr-Tat conjugation is superior to Myr or Tat conjugation to PKCε- in significantly reducing infarct size. *p<0.05 vs. control I/R hearts, **p<0.01 vs. control I/R hearts.

Results

Table 1. Cardiac Function and Infarct Size Indices. Cardiac function initial (baseline) and final values for control I/R, I/R + Tat-PKCε- (10 μM), I/R + Myr-PKCε- (10 μM) and I/R + Myr-Tat-PKCε- hearts (10 μM - 1 nM). Myr-Tat-PKCε-treated hearts (100 nM) exhibited significant improvement in post-reperfused cardiac function and recovered to 63±4% for dP/dt$_{max}$ compared control I/R hearts that only recovered to 26±4% of initial baseline values respectively at 50 min R. Initial and final coronary flow was approx 19±2 ml/min and 10±2 ml/min , respectively, with the exception of 10 μM Myr-Tat PKCε- (not shown). *p < 0.05 vs. control I/R, **p<0.01 vs. control I/R .

Cardiac Function and Infarct Size Indices	Control (n =5)	Tat-PKCε Inhibitor 10 μM (n = 4)	Myr-PKCε Inhibitor 10 μM (n = 6)	Myr-Tat PKCε Inhibitor 10 μM (n = 4)	Myr-Tat PKCε Inhibitor 1 μM (n = 5)	Myr-Tat PKCε Inhibitor 100 nM (n = 5)	Myr-Tat PKCε Inhibitor 1 nM (n = 5)	Myr-Tat PKCε Inhibitor 100 pM (n = 6)
Initial +dP/dt$_{max}$ (mmHg/sec)	2478±87	2408±126	2296±88	2540±158	2493±128	2528±133	2441±72	2457±37
Final +dP/dt$_{max}$ (mmHg/sec)	632±254	678±223	727±68	54±14	794±220	1585±164*	1147±156	1021±246
Initial -dP/dt$_{min}$ (mmHg/sec)	-1693±115	-1735±149	-1543±75	-1772±141	-1786±97	-1807±125	-1674±106	-1726±49
Final -dP/dt$_{min}$ (mmHg/sec)	-464±163	-576±176	-701±137	-56±14	-616±122	-981±135	-733±68	-688±142
Initial LVDP (mmHg)	93±3	86±5	89±4	92±3	94±5	89±4	91±4	89±3
Final LVDP (mmHg)	33±12	37±12	46±6	1±0.2	40±15	66±6	49±8	47±11
Infarct Size: % Total Weight	23±3	18±2	15±4	5±2**	6±1**	8±2**	9±1*	17±1
Representative Mid-wall Heart Sections								

Discussion

Conclusions:

- Myr-Tat-PKCε- significantly salvages cardiac tissue over a wide concentration range (10,000x) compared to Myr- or Tat-conjugated PKCε peptide inhibitor.
- Myr-Tat may be a platform technology to conjugate other types of cargo to facilitate intracellular delivery.

Future Studies will:

- Assess Myr-Tat vehicle without peptide cargo as a control in the pharmacological assessment of other Myr-Tat-conjugated peptides (e.g. Myr-Tat-PKCε or Myr-Tat-PKCβII) in PMN SO release and myocardial I/R models.
- Western blot and immunohistochemistry analysis to determine PKCε membrane localization in the presence of Myr-Tat PKCε peptide inhibitor compared to controls in ex-vivo I/R animal models.

References

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