Myristoylated PKC β II Peptide Inhibitor Exerts Dose-Dependent Inhibition of N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) Induced Leukocyte Superoxide Release

Chinyere B. Ebo, Carly Schmidgall, Christina Lipscombe, Harsh Patel,
Qian Chen, Robert Barsotti, and Lindon H. Young

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

Introduction

Phosphorylation of polymorphonuclear leukocyte (PMN) NADPH oxidase by protein kinase C (PKC) is essential to generate superoxide (SO) release. Inhibition of PMN SO release attenuates inflammation mediated vascular tissue injury during myocardial ischemia/reperfusion (MI/R) injury. PMNs express five isoforms of PKC (α (α), β I (β I), β II (β II), δ (δ) and ζ (ζ)) and their role regulating this response have not been fully elucidated. PKC α , β II and ζ are thought to positively regulate PMN SO release, whereas PKC δ negatively regulates PMN SO release. [1,2] PKC β I, in contrast to the other four isoforms, translocates to the nucleus after second messenger stimulation [1]. PKC β II, a classical isoform, is activated by calcium and diacylglycerol (DAG) following PMN chemotactic receptor stimulation with fMLP peptide (Figure 1) [1]. Activated PKC β II will phosphorylate PMN NADPH oxidase to produce SO. Selective PKC β II peptide inhibitor has been developed based on its binding sites to receptor for activated C kinase (RACK) domain (Figure 2) [3]. RACK shuttles cytosolic PKC β II to interact with cell membrane substrates (e.g., NADPH oxidase).

Fig. 1. Schematic representation of PKC activation generating SO release in PMN. PMN chemotactic G-protein receptors are activated by fMLP. The G-protein subunits α and β dissociate after stimulation and activate phospholipase C beta (PLC β) to produce inositol 1,4,5 trisphosphate (Ins(1,4,5)P₂) plus DAG respectively from phospholipids phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P₂). Ins(1,4,5)P₂ stimulates Ca²⁺ release from the endoplasmic reticulum (ER). Ca²⁺ and DAG directly activate PKC β II while PKC ζ is activated by phosphatidyl inositol trisphosphate (PtdIns(3,4,5)P₂). Activated PKC β II phosphorylates NADPH oxidase to release SO (Adapted from Young, et al.).

Myristoylation of peptides is known to be an effective strategy to enable simple diffusion through cell membranes to affect PKC function [4,5]. The cell permeable myristoylated (myr) PKC β II peptide inhibitor is known to inhibit PMN SO release at doses that correlated with restoration of post-reperused cardiac function following global MI(20 min)/R(45 min) in leukocyte mediated cardiac MI/R dysfunction and more recently in prolonged MI(30 min)/R(90 min) in isolated rat hearts [1,6,7]. However, a full dose-response curve with Myr-PKC β II peptide inhibitor has not been indicated previously. Characterizing the full dose-response effects is essential in identifying putative mechanisms responsible for attenuating vascular and tissue injury following I/R.

Hypothesis

We hypothesized that Myr-PKC β II peptide inhibitor would dose-dependently attenuate fMLP induced PMN SO release, and these effects would not be associated with a decrease in cell viability.

Results and Discussion

Isolation of PMNs

Male Sprague-Dawley rats (350-400 g, Charles River), used as PMN donors, were anesthetized with 2.5% isoflurane and given a 16 ml intraperitoneal injection of 0.5% glycogen (Sigma Chemical; St. Louis, MO) dissolved in PBS. Rats were reanesthetized with isoflurane 16-18 h later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described [4,6].

Measurement of SO Release from Rat PMNs

The SO release from PMNs was measured spectrophotometrically (model 260 Gilford, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c [4,6]. The PMNs (5×10^6 cells) were resuspended in 450 μ l PBS and incubated with ferricytochrome c (100 μ M, Sigma Chemical; St. Louis, MO) in a total volume of 900 μ l PBS in the presence or absence of myr PKC β II inhibitor (0.2 to 20 μ M; N-myr-SLNPEWNET, 1300 g/mol) for 15 min at 37°C in spectrophotometric cells. The PMNs were stimulated with 1 μ M fMLP (MW= 438 g/mol Calbiochem; La Jolla, CA) in a final reaction volume of 1.0 ml. Absorbance at 550 nm was measured every 30 sec for up to 120sec for fMLP and the change in absorbance (SO release) from PMNs was determined relative to time 0. Cell viability among all study groups was determined microscopically by 0.3% trypan blue exclusion at the end of the SO release assay. Viable cells remained unstained and non-viable cells stained blue.

Statistical Analysis

All data in the text and figures are presented as means $\pm$ S.E.M. The data were analyzed by ANOVA using post hoc analysis with Fisher's PLSD test. Probability values of <0.05 are considered to be statistically significant.

Figures 3 and 4 illustrate the dose-dependent effects of Myr-PKC β II peptide inhibitor (0.5-20 μ M) on fMLP-stimulated PMN SO release. Untreated fMLP control stimulates SO release which increases absorbance and the reaction reached plateau at 60-90 sec. As shown in Figure 3, Myr-PKC β II peptide inhibitor dose dependently inhibited SO release from untreated controls by 21-26 $\pm$ 12% (0.5 μ M; 30-120 sec), 43-46 $\pm$ 19% (1 μ M; 30-120 sec), 58-69 $\pm$ 9% (2 μ M; 30-120 sec), 71-75 $\pm$ 12%

Fig. 3. The time course response of fMLP (1 μ M)-induced SO release in PMNs. * $p<0.05$ compared to untreated controls; # $p<0.05$ (5 to 20 μ M) compared to the lowest dose group (0.2 μ M); n=number of trials.

Fig. 2. Top: Illustration of PKC β II peptide inhibitor. The Ca^{2+} binding domain for PKC β II peptide inhibitor (i.e., C2-4 region) is unique for PKC β II translocation to the cell membrane when activated (Adapted from Young et al.) Bottom: PKC β II peptide inhibitor mechanism of action. The peptide inhibitor is intended to inhibit PKC β II translocation from cytosol to the sites where PKC β II interacts with substrates such as leukocyte NADPH oxidase (Adapted from Csukai and Mochly-Rosen).

Fig. 4. fMLP (1μM)-induced peak response (120 sec) SO release in PMNs. * $p<0.05$, ** $p<0.01$ compared to untreated controls; # $p<0.05$ compared to the lowest dose group (0.2 μM).

Fig. 5. The effects of Myr-PKC βII peptide inhibitor (0.2-20 μM) on cell viability in fMLP (1 μM)-induced SO release in PMNs. Cell viability ranged between 94-96% ± 1 and was not different amongst study groups, suggesting that the inhibition of leukocyte SO release at higher doses was not due was not related to cell death.

(5-20 μM; 30-120 sec). Myr-PKC βII peptide inhibitor (1 to 20 μM) significantly reduced the PMN SO release compared to the control (all, $p<0.05$) Furthermore, PKC βII peptide inhibitor high dose groups (5-20 μM) were significantly different from the low dose group (0.2 μM) from 30-120 sec. Myr-PKC βII peptide inhibitor dose-dependently inhibited the fMLP-induced SO release from PMNs. A linear decrease in PMN SO release was observed when the doses increased from 0.2 to 2 μM, whereas the inhibition reached a plateau phase of 71-75±12% in the 5 to 20 μM dose range. Moreover, the higher dose range (5-20 μM) had no toxicity on PMN cell viability. These results suggest that activation of NADPH oxidase via PKC βII is the dominant pathway following stimulation of the PMN chemotactic receptor, and that PKC βII peptide inhibitor functions as an anti-inflammatory agent that can be used in vascular complications associated with I/R, hypertension and diabetes.

Acknowledgments

This study was supported by the Center for Chronic Disorders of Aging and the Department of Bio-Medical Sciences at PCOM. Special thanks to PCOM DO/Biomed students Stephanie Liu, Joseph Venditto, and Gregory Stoner for assistance with editing the manuscript.

References

- Young, L., et al. *Cardiovasc. Drug Rev.* **23**, 255-272 (2005), <http://www.ncbi.nlm.nih.gov/pubmed/16252018>
- Kilpatrick, L., et al. *Am. J. Physiol. Cell Physiol.* **279**, C2011-C2018 (2000), <http://www.ncbi.nlm.nih.gov/pubmed/11078718>
- Csukai, M, Mochly-Rosen, D. *Pharmacol. Res.* **39**, 253-259 (1999), <http://www.ncbi.nlm.nih.gov/pubmed/10208754>
- Perkins, K.A., et al. in Michal Lebl (Editor), *Proceedings of the 22nd American Peptide Symposium*, American Peptide Society, San Diego, 288-289, 2011.
- Eichholtz, T., et al. *J. Biol. Chem.* **268**, 1982-1986 (1993), <http://www.ncbi.nlm.nih.gov/pubmed/8420972>
- Omiyi, D., et al. *J. Pharm. Exp. Ther.* **314**, 542-551 (2005), <http://www.ncbi.nlm.nih.gov/pubmed/15878997>
- Lipscombe, C., et al., in Ved Srivastava, Andrei Yudin, and Michal Lebl (Editors), *Proceedings of the 24th American Peptide Symposium*, American Peptide Society, San Diego, 2015. *In press*.