Myristoylated PKC β II peptide inhibitor exerts dose-dependent inhibition of N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) induced leukocyte superoxide release

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Introduction

Phosphorylation of polymorphonuclear leukocyte (PMN) NADPH oxidase by protein kinase C (PKC) is essential to generate superoxide (SO) release. Inhibition of leukocyte SO release attenuates inflammation mediated vascular tissue injury (e.g. myocardial ischemia/reperfusion (MI/R)). There is 11 isofoms of PKC and the role of PKC isoforms that mediate this response has not been fully elucidated. PKC beta II (PKC βII), a classical isofom that is activated by calcium and diacylglycerol (DAG), following PMN chemotactic receptor stimulation with fMLP peptide (Fig 1). Activated PKC βII will activate NADPH oxidase and stimulate SO release. Selective PKC βII peptide inhibitor has been developed based on its binding sites to receptor for C kinase (RACK) domain (Fig 2). Myristoylation of peptides is known to be an effective strategy to enable simple diffusion through cell membranes to affect PKC function (3, 4).

Myristoylated (Myr) PKC βII peptide inhibitor is known to inhibit PMN SO release at doses that correlated with restoration of post-reperfusion cardiac function following global MI(20min)/R(45min) in leukocyte medicated cardiac MI/R dysfunction (1,5) and more recently in prolonged MI(30min)/R(90min) in isolated rat hearts (See Poster P 204). However, a full dose-response curve with Myr-PKC βII peptide inhibitor (0.2-20 µM) has not been indicated previously. The peptide attenuates PKC βII translocation to the cell membrane by inhibiting the interaction with the RACK domain (Fig 2). 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 βII peptide inhibitor (0.2-20 µM) would dose-dependently attenuate iMLP induced PMN SO release. We further predict that 5 to 20 µM doses would further angiotensin (A) activation of iMLP induced PMN SO release compared to non-drug or low drug treated (0.2 and 0.5 µM) PMNs and these effects would not be associated with a decrease in cell viability.

Methods

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% glycopyrrolate (Sigma Chemical) dissolved in PBS. Rats were anesthetized with isofoflurane 16–18 h later, and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (2,3). The peritoneal lavage fluid was centrifuged at 200 g for 10 min at 4 °C. The PMNs were then washed in 20 ml PBS and centrifuged at 200 g for 10 min at 4 °C. Thereafter, the PMNs were resuspended in 2.3 ml PBS and density was calculated. The PMNs preparation were >90% pure and >95% viable according to microscopic examination and exclusion of 0.3% trypan blue, respectively. Cell viability among all study groups was determined by 0.3% trypan blue exclusion.

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 (1,3). The PMNs (5 x 10⁶) were resuspended in 450 µl PBS and incubated with ferricytochrome c (100 µM, Sigma Chemical) in a total volume of 900 µl PBS in the presence or absence of myr PKC βII inhibitor (0.2 to 20 µM, N-myristoyl-SLNPEWNET [300 g/mol] for 15 min at 37 °C in spectrophotometric cells. The PMNs were stimulated with 1 µM fMLP (MW= 434 g/mol) (Calbiochem) in a final reaction volume of 1.0 ml. Absorbance at 550 nm was measured every 30 sec for 120sec for fMLP and the change in absorbance (SO release) from PMNs was determined relative to time 0.

Statistical Analysis

All data in the text and figures are presented as means ± S.E.M. The data were analyzed by analysis of variance using post hoc analysis with the Fisher’s LSD test. Probability values of <0.05 are considered to be statistically significant.

Results

Fig 3. The time course response of fMLP (1µM)-induced SO release. Myr-PKC βII peptide inhibitor (0.5-20 µM)-dose dependently inhibited SO release from peritoneal controls (*p<0.05; **p<0.01) by 26-81% (1,5 µM); 30-60 sec, 51-81% (1 µM); 10-60 sec, 88-97% (0.5 µM); 60-120 sec, 71-87% (0.2 µM); 10-60 sec. PKC βII peptide inhibitor high dose groups (5-20 µM) were significantly different from the low dose group (0.2 µM) (p<0.01) from 10-60 sec.

Fig 4. fMLP (1µM)-induced peak response (120 sec) SO release in PMNs. Myr-PKC βII peptide inhibitor dose dependently (0.5-20 µM) attenuated SO release compared to peritoneal controls (*p<0.05; **p<0.01) by 26-81% (0.5 µM), 41-19% (1 µM), and 60-75% (2-10 µM). PKC βII peptide inhibitor high dose groups (5-20 µM) were significantly different from the low dose group (0.2 µM) (p<0.05).

Fig 5. The effect of Myr-PKC βII peptide inhibitor (0.2-20 µM) on cell viability in fMLP (1µM)-induced SO release in PMNs. Cell viability ranged between 94-99%, ± 1 and was not different amongst study groups, suggesting that the inhibitors of leukocyte SO release was not related to cell death.

Conclusions

Myr-PKC βII peptide inhibitor dose-dependently inhibited fMLP-induced SO release by about 70%, suggesting that activation of NADPH oxidase via PKC βII is the dominant pathway following stimulation of the leukocyte chemotactic receptor. This study suggests that PKC βII peptide inhibitor maybe anti-inflammatory agent that can be used in vascular complications associated with I/R.

References


Figure 1. Schematic representation of PKC activation generating SO release in PMN. PKC interacts with G protein-coupled receptors and tyrosyl phosphorylised G protein subunits a and βγ which disassociate after stimulation and activate phospholipase C β (PLCβ) to produce inositol 1,4,5-triphosphate (Ins1,4,5P3) plus DAG respectively from phosphatidylinositol 4,5 bisphosphate (PtdIns(4,5)P2). Ins1,4,5P3 stimulates Ca2+ release from the endoplasmic reticulum (ER). Ca2+ and DAG directly activate PKC βII. Activated PKC βII phosphorylates NADPH oxidase to release SO (Adapted from Young et al.)