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Comparing the Effectiveness of TAT and Myristoylation of gp91ds on Leukocyte Superoxide (SO) Release

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Introduction

SO release from leukocytes via NADPH oxidase activation contributes to oxidative stress under various diseases, such as ischemia/reperfusion (I/R) injury and vascular complications in diabetes. NADPH oxidase has seven isoforms with NOX2 being the predominant isoform of NADPH oxidase in polymorphonuclear leukocytes (PMNs). Activation of NOX2 requires the assembly of cytosolic subunits ($p47^{phox}$, $p40^{phox}$, $p67^{phox}$, Rac) to plasma membrane subunits ($gp91^{phox}$ and $p22^{phox}$) [1]. NADPH oxidase is activated during I/R injury via cytokine receptor stimulation or chemotactic factor (N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, MW= 438 g/mol) and utilizes molecular oxygen to produce SO [2] (Figure 1).

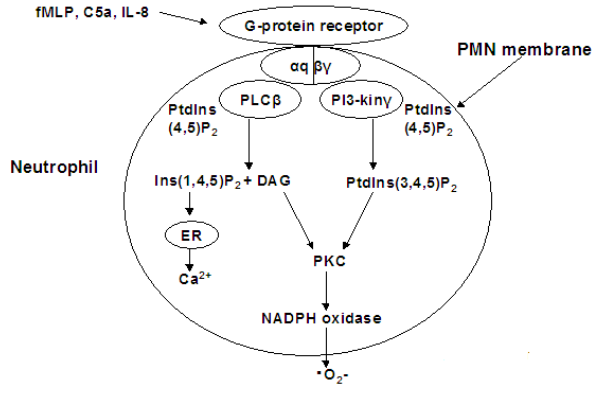

Fig. 1. Schematic representation of PKC activation generating SO release in PMN. PMN chemotactic G-protein receptors are activated by fMLP, C5a and interleukin 8. The G-protein subunits αq and βγ disassociate after stimulation and activate phospholipase C beta (PLCβ) and phosphatidyl inositol-3-kinase gamma (PI-3kinγ) to produce inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_2$) plus DAG and $PtdIns(3,4,5)P_2$ respectively from phospholipids phosphatidylinositol 4,5 bisphosphate ($PtdIns(4,5)P_2$). $Ins(1,4,5)P_2$ stimulates Ca^{2+} release from the endoplasmic reticulum (ER). Ca^{2+}/DAG and $PtdIns(3,4,5)P_2$ directly activate PKC. Activated PKC phosphorylates NADPH oxidase to release SO anion ($•O_2$-). Adapted from Young, et al. [2].

Gp91ds conjugated with tat or myristric acid + pegylated linker (myr-peg) are cell permeable peptide formulations which selectivity inhibit NADPH oxidase assembly by blocking $p47^{phox}$ interaction with $gp91^{phox}$ (Figure 2).

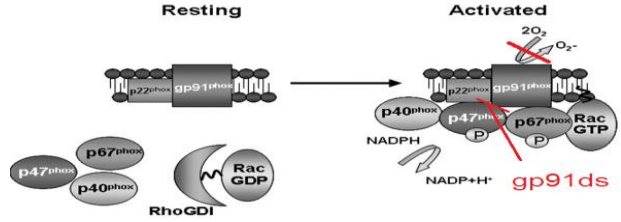

Fig. 2. Schematic showing the inactive and active forms of NADPH oxidase. Red-lines denote areas of inhibition performed by gp91ds. Adapted from Wilkinson, et al. [1]

It is well known that adding myristic acid or a tat carrier peptide to native peptides facilitates cell membrane permeability which is required for effectively targeting intracellular substrates. The

addition of a glycine-glycine (gg) spacer between the tat and cargo portion of the peptide is reported to facilitate delivery of the cargo sequence (i.e., CSTRIRRQL) [3,4] (Figure 3).

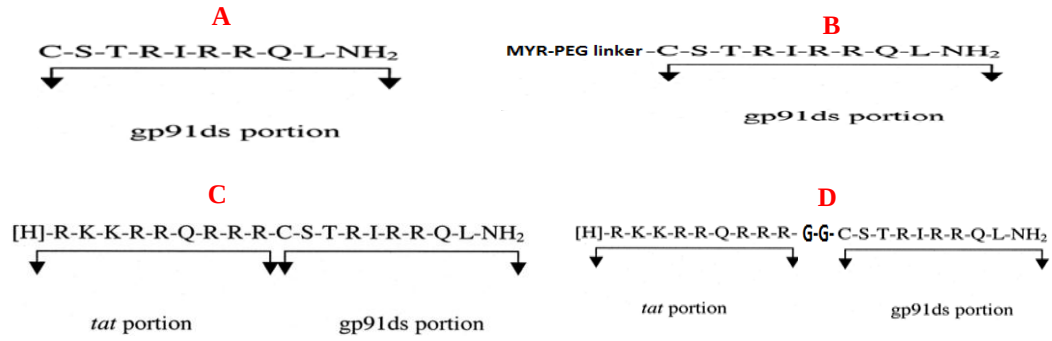

Fig. 3. A: native gp91ds (MW=1131 g/mol). B: myr-peg linker-gp91ds (MW=1486 g/mol). C: gp91ds-tat sequence (MW= 2452 g/mol). D: gp91ds-tat gg spacer (MW=2566 g/mol). Adapted from Rey, et al. [3].

We have previously shown that myristic acid conjugated caveolin-1 and protein kinase C (PKC) beta II and zeta peptide inhibitors significantly attenuated fMLP-induced SO release compared to their native counterparts [5]. However, it is not known if differences exist in the effectiveness of myristic acid versus tat conjugated gp91 ds-tat peptides compared to their native counterparts or untreated controls. We hypothesized that myr-gp91ds (2-10 μM) would dose-dependently attenuate fMLP induced PMN SO release at lower concentrations compared to tat conjugated or tat conjugated gg spacer-gp91ds peptides. Moreover, we also predict that both myristic acid and tat conjugated gg spacer gp91ds peptides would significantly attenuate fMLP-induced leukocyte SO release compared to native or untreated controls without affecting 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) dissolved in PBS. Approximately 16 h later, the rats were reanesthetized with isoflurane and the PMNs were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described [2,5].

Measurement of SO Release from Rat PMNs

The SO release from PMNs was measured spectrophotometrically (Gilford model 260, Nova Biotech; El Cajon, CA) by the reduction of ferricytochrome c [2,5]. The PMNs (5 x10^6 cells) were suspended in 450 μl PBS and incubated with 100 μM ferricytochrome c (Sigma Chemical; St Louis, MO) in a total volume of 900 μl PBS in the presence or absence of myr-peg conjugated (2 to 10 μM), tat conjugated (80 μM) or native gp91ds (80 μM) for 15 min at 37°C in spectrophotometric cells. Thereafter, absorbance at 550 nm was measured every 30 sec for up to 120 sec following fMLP 1 μM (Calbiochem; La Jolla, CA) stimulation and the change in absorbance (SO release from PMNs) was determined relative to time 0 in a final reaction volume of 1 ml. Cell viability among all study groups was determined by 0.3% trypan blue exclusion at the end of the SO release assay.

Myr-peg gp91 (5 & 10 µM) significantly attenuated untreated and native gp91ds induced SO release by 56-57 ± 8% and 52-54 ± 9 % respectively. Myr-peg gp91 (2 µM), gp91ds-tat (80 µM), and gg spacer (80 µM) significantly inhibited untreated by 36 ± 11% compared to untreated controls and was not different from native gp91ds (see Figure 4). Concentrations of tat conjugated peptides ≤40 µM did not exert significant inhibition of PMN SO release. (Data not shown)

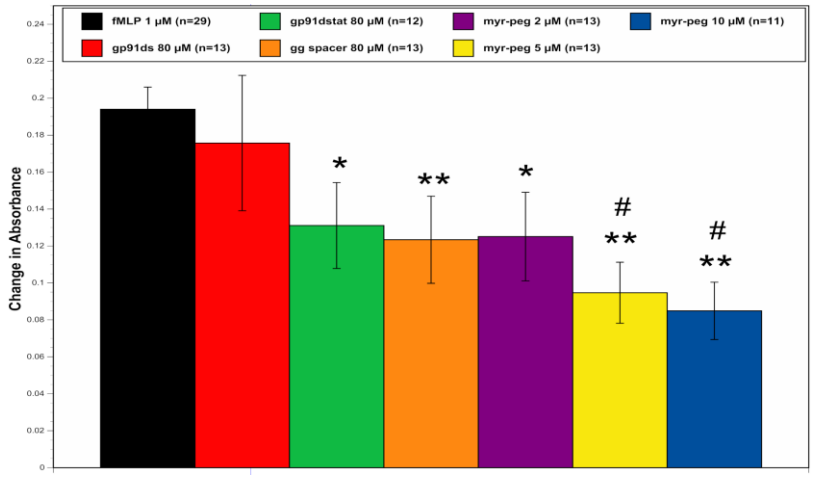

*Fig. 4. fMLP (1µM)-induced peak response SO release in PMNs. Probability values of <0.05 are considered to be statistically significant (*p<0.05, **p<0.01 compared to untreated controls; #p<0.05 compared to native gp91ds). All data in the text and figures are presented as means ± S.E.M. The data were analyzed by analysis of variance using post hoc analysis with the Fisher's test and n= number of trials.*

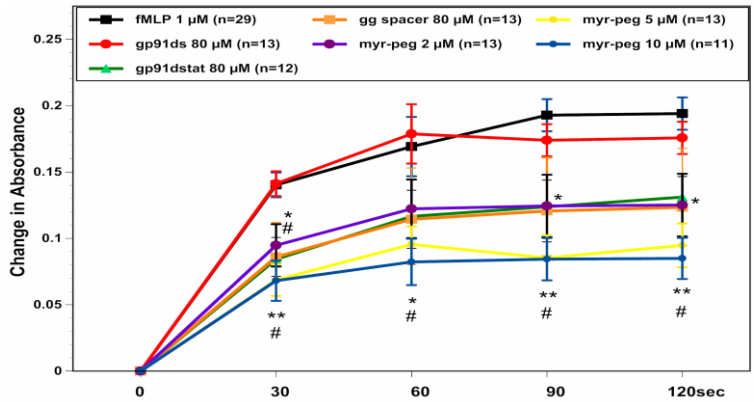

*Fig. 5. The time course response of fMLP (1µM)-induced SO release. *p<0.05, **p<0.01 compared to untreated controls; #p<0.05 compared to native gp91ds.*

Myr-peg gp91 (5 & 10 µM) significantly inhibited untreated and native gp91 by 44-56% and 46-52% respectively from 30-120 sec. Myr-peg gp91ds (2 µM), gp91dstat (80 µM), and gp91ds-tat with gg spacer (80 µM) significantly untreated by 33-36% from 30-120 sec and was only different from native gp91 at 30 sec (Figure 5).

We also observed that TAT (R-K-K-R-R-Q-R-R-R; MW=1321g/mol, n=7) alone decreased fMLP-induced absorbance by 48 ± 9% and cell viability was 92 ± 1% *(data not shown).* Although cell viability was not significantly different from untreated and other tat conjugated gp91ds groups, we observed a significant degree of cell clumping, which in turn would decrease the absorbance. Therefore

the measured decrease in absorbance was likely due to the effect of cell clumping rather than a decrease in fMLP induced SO release. By contrast, cell clumping was not observed in the other tat conjugated gp91ds groups suggesting that in these groups leukocyte SO release was attenuated. These results suggest that tat alone may potentiate cell aggregation.

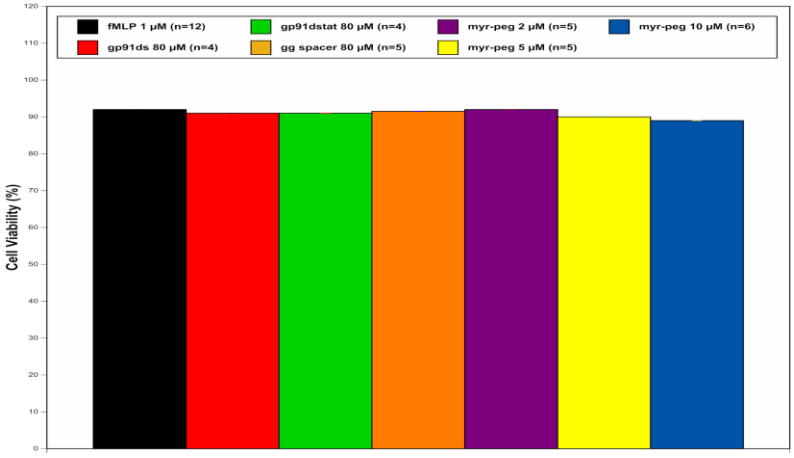

Fig. 6. The effects of native, tat, and myr-peg conjugated gp91ds on cell viability in fMLP (1 µM)-induced SO release in PMNs. Cell viability ranged between 90-93± 1% and was not different amongst study groups indicating that the reduction in SO release was not related to cell death.

Unconjugated native sequence did not inhibit the fMLP induced SO response at the highest dose tested (80 µM). Myr-peg gp91ds NADPH oxidase peptide inhibitor significantly attenuated leukocyte SO release dose dependently compared to untreated or native sequence (myr-peg-gp91ds; 2-10 µM). The tat conjugated gp91ds inhibitors (both 80 µM) significantly attenuated fMLP-induced leukocyte SO release, but to a lesser extent than the myr-peg linked inhibitor and were not different from native gp91ds at 60-120 sec. Moreover, we expected to see greater inhibition with gp91ds-tat with gg spacer compared to gp91ds-tat since the gg spacer is reported to facilitate delivery of the cargo sequence. However, this subtle difference in cargo sequence delivery may be masked with respect to inhibition of leukocyte SO release in this assay. Collectively, these results suggest that native gp91ds requires conjugation of myr-peg or tat in order to effectively inhibit the intracellular target of $p47^{phox}$ and gp91 interaction. Additionally, myr-peg-gp91ds is more cell permeable and therefore can inhibit fMLP-induced SO release from leukocytes at lower doses compared to gp91ds-tat and gg spacer gp91ds-tat. Future studies will be aimed at comparing myr and tat conjugated peptides that have other intracellular targets i.e. mitochondria in myocardial ischemia-reperfusion.

Acknowledgments

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