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 (p47phox, p40phox, p67phox, Rac) to membrane subunits (gp91 phox and p22phox) (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) (fig1). Gp91ds-tat is a peptide which selectivity inhibits NADPH oxidase assembly by blocking $p47^{phox}$ interaction with $gp91^{phox}$. It is well known that adding myristic acid or tat carrier peptide to native peptides will facilitate cell membrane permeability required for targeting intracellular substrates. The addition of a glycine-glycine (GG) spacer between the tat and cargo portion of the peptide is reported to facilitate better delivery of the cargo sequence (i.e., CSTRIRRQL) (3,4). 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.

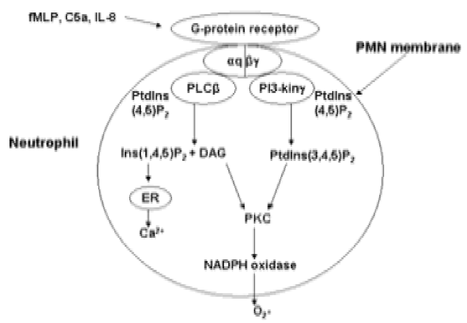

Figure 1. Schematic representation of PKC activation generating SO release in PMN. PMN chemotactic G-protein receptors are activated by fMLP. The G-protein subunits αq and βγ disassociate after stimulation and activate phospholipase C beta (PLCβ) and phosphatidyl inositol-3-kinase gamma (PI-3kinγ) to 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. Adapted from Young et al. (2)

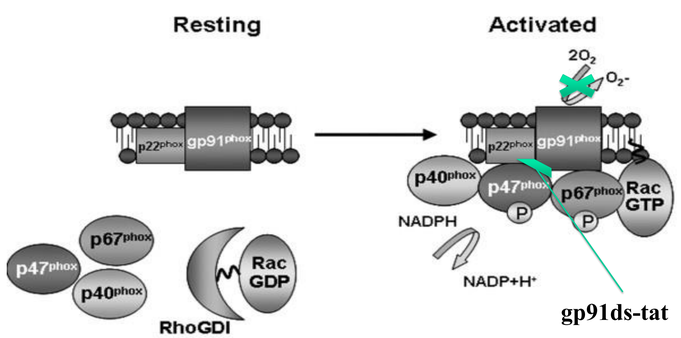

Figure 2. Schematic showing the inactive and active forms of NADPH oxidase. Green-lines denote areas of inhibition performed by gp91ds-tat. Adapted from Wilkinson et al. (1)

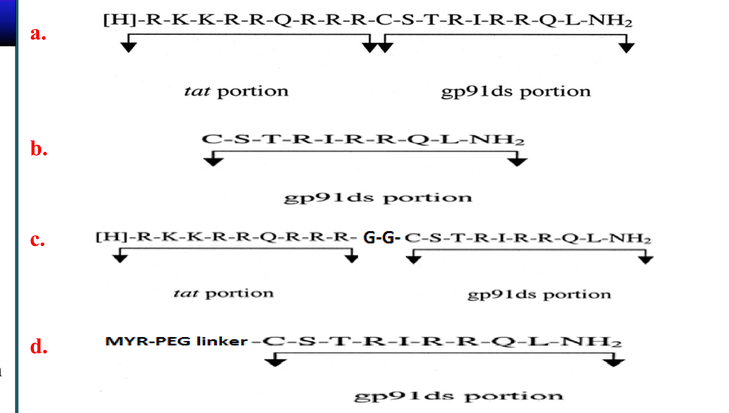

Figure 3. a- gp91ds-tat sequence (MW= 2452 g/mol). **b-** native gp91ds (MW=1131 g/mol). **c-**gg spacer (MW=2566 g/mol) **d-** myristoylated (Myr-peg linker) (MW=1486 g/mol). Adapted from Rey et al. (3)

Hypothesis

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.

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% glycogen (Sigma Chemical) 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 (2,5). 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 2.5 ml PBS and density was calculated. The PMNs preparation were >90% pure and >95% viable according to microscopic analysis 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 (2,5). The PMNs (5 $x10^6$) 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 myristric acid conjugated (2 to 10 μM), tat conjugated (80 μM) or native gp91ds (80 μM) for 15 min at 37° C in spectrophotometric cells. The PMNs were stimulated with 1 μM fMLP (Calbiochem) in a final reaction volume of 1.0 ml. Absorbance at 550 nm was measured every 30 sec for up to 120 sec for fMLP and the change in absorbance (SO release) from PMNs was determined relative to time 0.

Cell Viability

Cell viability was determined by combining 0.5 ml of the samples from spectrophotometric analysis using trypan blue exclusion (0.3%). Then, 20 μl of the combined sample was placed on to a hemocytometer, and 100-150 cells were subsequently counted using microscopic analysis.

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 test. Probability values of <0.05 are considered to be statistically significant.

Results

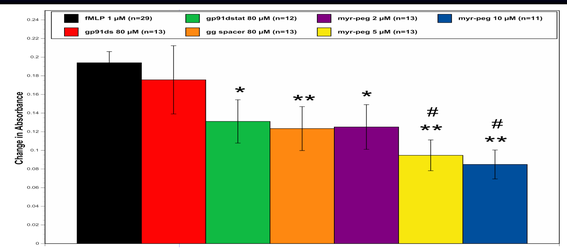

Figure 4. fMLP (1μM)-induced peak response SO release in PMNs. Myr-peg gp91 (5 & 10 μM) significantly attenuated untreated (*p<0.01) and native gp91 (# p<0.05) induced SO release by 56-57 ± 8% and 52-54 ± 9 % respectively. Myr-peg gp91 (2 μM), gp91dstat (80 μM), and gg spacer (80 μM) significantly inhibited untreated by 36 ± 11 % compared to untreated controls (*p<.05) and was not different from native gp91. Concentrations of tat conjugated peptides ≤40 μM did not exert significant inhibition of PMN SO release. (data not shown)

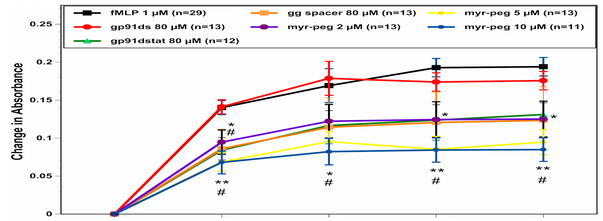

Figure 5. The time course response of fMLP (1μM)-induced SO release. Myr-peg gp91 (5 & 10 μM) significantly inhibited untreated (**p<0.01, *p<0.05) and native gp91 (# p<0.05) by 44-56% and 46-52% respectively from 30-120 sec. Myr-peg gp91 (2 μM), gp91dstat (80 μM), and gg spacer (80 μM) significantly untreated (*p<0.05) by 33-36% from 30-120 sec and was only different from native gp91 at 30 sec (#p<0.05).

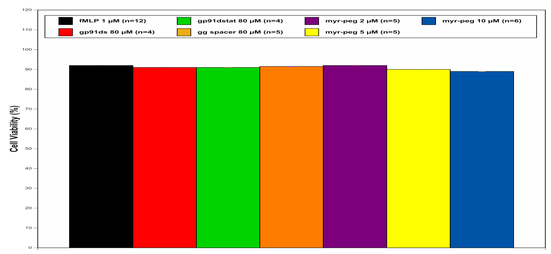

Figure 6. The effects of native, tat, and myristoylated conjugated gp91 on cell viability in fMLP (1 μM)-induced SO release in PMNs. Cell viability ranged between 90-93% ± 1 and was not different amongst study groups indicating that the reduction in SO release were not related to cell death.

Conclusions

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; 5-10 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. These results suggest that myr-peg-gp91ds is more cell permeable and therefore can inhibit fMLP-induced SO release from leukocytes at lower doses compared to tat gp91ds.

References

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