

Detection of NADPH oxidase-dependent extracellular superoxide production in neutrophils using Turner BioSystem's GloRunner™ Luminometer

This application note was submitted to Turner BioSystems under the "Tell us your story program" by Jim Burritt of Montana State University.

INTRODUCTION

The polymorphonuclear neutrophil (PMN) is the most numerous white blood cell type in the blood of humans, and several types of PMN defect are associated with increased susceptibility to infection. PMN rely on various defensive mechanisms to kill invading microbes, and some of these mechanisms remain to be fully characterized. One such mechanism is phagocyte NADPH oxidase that produces microbicidal superoxide. Though several methods of detecting superoxide in PMN to study this mechanism have been described, they differ in regard to both sensitivity and specificity to NADPH oxidase activity. To measure extracellular superoxide produced by the NADPH oxidase in alveolar PMN from normal C57Bl/6 and knockout gp91^{phox-/-} mice (that lack a functional NADPH oxidase), a luminometry assay was utilized.

MATERIALS REQUIRED

- GloRunner™ Microplate Luminometer (P/N 9000-000)
- 96-well plates, white (E&K Scientific EK 25075)
- 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one (MCLA, Molecular Probes-Invitrogen # M-23800)
- Superoxide dismutase (SOD, Sigma-Aldrich #S-2515)
- HBSS (Cambrex Bio Science, Walkersville, MD #10-547F)
- Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich #P-8139)

EXPERIMENTAL PROTOCOL

Superoxide liberated from murine LPS-elicited alveolar PMN was examined in white 96-well plates, using 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazol[1,2-a]pyrazin-3-one, a luminometry reagent that has optimal sensitivity for detecting low-level superoxide generation by phagocytes³.

To recruit PMN to the lungs of mice so they could be harvested in lung lavage fluid and examined by luminometry, 9-11 week old C57Bl/6 and gp91^{phox-/-} mice were first exposed to aerosolized LPS 12 hours prior to collection of lavage fluid as described¹, producing samples that contained >85% PMN. Following harvest of lavage fluid, cells were counted by hemocytometer and Wright stained to determine the percentage of PMN in the sample. PMN were then resuspended at 10⁷ cells per ml in HBSS, then 10 µl containing 10⁵ PMN were mixed in 100 µl of 5 µM MCLA in 140 mM NaCl per well in the presence of 1.7 µM phorbol 12-myristate 13-acetate. Data for superoxide liberation by PMN were collected at 25°C using a Turner Biosystems GloRunner™ luminometer (1 sec datapoints collected each minute for 30 minutes). Representative samples were also examined that contained 310 U/well superoxide dismutase, which abolished the signal and confirmed the specificity of the signal to superoxide detection.

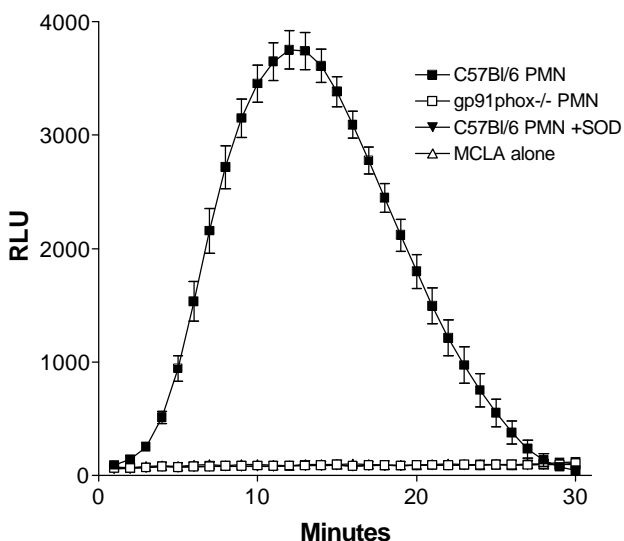
RESULTS


Figure 1. Data show luminometry signal in relative light units (RLU), due to superoxide produced by PMN collected from either C57Bl/6 or gp91^{phox-/-} mice, then exposed to 1.7 μ M PMA in the presence of MCLA. Each condition (except MCLA alone) represents 10⁵ PMN. Data are shown for PMN collected from C57Bl/6 (■) and gp91^{phox-/-} (□) mice, and PMN from C57Bl/6 mice stimulated with PMA in the presence of SOD (▼). For reference, the signal produced by MCLA alone is shown, which averaged about 75 RLU (△). The data represent the average for 5 mice (or reagent replicates) for each condition (n=5), and error bars indicate \pm SEM.

CONCLUSION

PMN from C57Bl/6 showed significantly greater signal following exposure to PMA when compared to that of gp91^{phox-/-} mice ($P=0.00002$ at 13 minutes). Specificity of luminometry signal to that of superoxide production was validated using SOD in representative wells containing PMN from C57Bl/6 mice. Comparison of signal from C57Bl/6 mice to that of gp91^{phox-/-} NADPH oxidase mice as a negative control confirms the source of superoxide to be NADPH oxidase activity. Because the PMN plasma membrane is not permeable to either SOD or MCLA, the signal represents extracellular and not intracellular superoxide detection. Luminometry reagents that are membrane permeable are available to identify intracellular superoxide production². Using the method described above, we have observed that as few as 20 RLU can identify significant differences in NADPH

oxidase-dependent signal generated from cells in some conditions (data not shown). This application can be modified to test the effect of different activators and inhibitors of NADPH oxidase directly in PMN and some other types of white blood cells that utilize this superoxide-generating system.

REFERENCES

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