NOX2 Activation Measured by ROS Production Using L-012 Chemiluminescent Probe

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- **Although much is known about NOX2, understanding regulation of its activity is still useful**
- **A chemiluminescent assay detecting ROS production by NOX2 in a 96-well plate cell-based assay is described**
- **The CLARIOstar® provides excellent detection of a kinetic luminescence assay**

Introduction

The NADPH oxidase family member, NOX2, has been extensively studied based on its association with phagosomes[1]. Among its functions, NOX2 has been shown to play a central role in regulation of antigen presentation on dendritic cells. More specifically, NOX2 is involved in alkalinization of dendritic cell phagosomes, thus limiting protease activity, resulting in only partial degradation of the antigen. The antigen then undergoes further processing before it is presented to CD8+ T cells. More recently it was shown that NOX2 undergoes activation mediated by PKCδ leading to pinocytosis of antigens[2].

NOX2 activity is known to be regulated by numerous cytosolic factors (Figure 1). In order to more completely understand the regulation of, and to seek out possible inhibitors of, NOX2 activity, the appropriate tools must be employed. Here, we describe the use of a chemiluminescent probe suitable for the detection of NOX2 activity in a microplate-based assay.

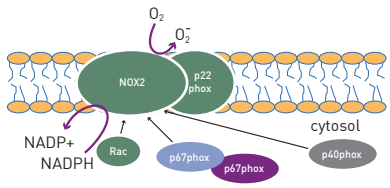

Fig. 1: Regulation of NOX2 activity.

Assay Principle

The luminol analog L-012 has been shown to be more sensitive at detecting ROS than its predecessor[3]. Furthermore, it has a higher luminescence yield compared to other superoxide probes as luminol and lucigenin[3]. Figure 2 shows the proposed reaction from interaction between L-012 and reactive oxygen (and nitrogen) species.

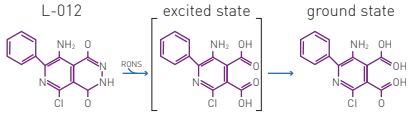

Fig. 2: L-012 Assay Principle
Structure of L-012 and indicated states are displayed (adapted from Conroy et al.4) RONS = reactive oxygen and nitrogen species.

Materials & Methods

- CLARIOstar
- L-012 from Wako Chemical (120-04891)
- other chemicals obtained from commercially available sources

Experimental Procedure

Bone Marrow-Derived immature Dendritic Cells (BMiDCs) were prepared from wild type and NOX2 knockout mice and CD11c positivity determined as described in Singla et al2. Fifty thousand cells/well were plated in sterile phosphate buffered saline (PBS) containing 400 µM L-012. Cells were stimulated with 1 µM PMA (phorbol 12-myristate 13-acetate) and in some cases, further addition of superoxide dismutase (SOD) at a concentration of 150 U/mL provides confirmation of specificity of this test for O_2-. Luminescence was read as indicated below. Evaluation was performed using BMG LABTECH's MARS data analysis software.

CLARIOstar settings

Optic settings	Luminescence, plate mode kinetic	
	Gain	3500
Kinetic settings	Number of cycles	60
	Cycle time	120 s
	Measurement interval	0.2 s
Incubation	37 °C	

Results & Discussion

Figure 3 shows the kinetic response of BMiDCs over a 2 hour period based on the light intensity produced by L-012 chemiluminescence. In vehicle treated cells a stable low level of light output is observed. Treatment with the protein kinase C activator PMA induces a strong increase in luminescent signal that peaks at ~1 hour. PMA stimulation in the presence of SOD fails to elicit a response indicating that the light production by L-012 is dependent on the presence of O_2- in this system.

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Keywords: enzyme activity, NOX2, oxygen, ROS

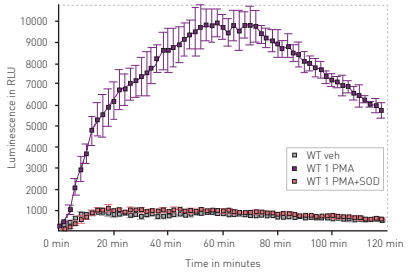

Fig. 3: Kinetic measurement of superoxide by L-012. Chemiluminescent response of L-012 in BMiDCs cultures are shown. Treatment with PMA (■) promotes a strong response compared to vehicle (□). Additional treatment with SOD (■) provides evidence that the response is O_2- dependent. Average (n=3) +/- 1 standard deviation are shown. RLU = relative luminescent units.

Figure 4 compares WT BMiDCs with BMiDCs from NOX2 KO background based on their ability to promote light output from L-012 chemiluminescence. After 1 hour of treatment, PMA stimulated a greater than 9-fold increase in RLU in WT cells compared to vehicle. By contrast cells from the Nox2 KO did not respond to PMA stimulation.

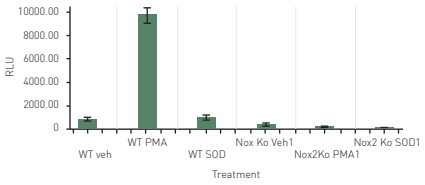

Fig. 4: Superoxide production measured by L-012 in WT and NOX2 KO cells. BMiDCs from the indicated backgrounds were treated as shown and luminescent signal assessed at a 1 hour post-treatment time point. Average (n=3) are presented.

Additional results indicated that PKC δ is the predominant PKC isoform present in BMiDCs and that plays a functional role in PMA-dependent O2- production.

Conclusion

The results indicate that L-012 is a very useful tool for the detection of ROS using a microplate reader-based assay. Sensitive detection using the CLARIOstar aids in the utility of this assay format. The results shown here indicate that there is a PKC-dependent activation of NOX2 in dendritic cells leading to O2- production important for their role in immune response.

References

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