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Kinetic Luminescent Measurement of ROS (Reactive Oxygen Species) production with ABEL® Cell activation ROS kit with PHOLASIN® on the FDSS/μCELL platform

Introduction

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in leucocytes, which is located in the cell plasma membrane and in the membrane of phagocytic vesicles inside the cell, transfers an electron of NADPH to outside of the cell or inside of the phagocytic vesicles across the membrane when it is activated. The electron reduces oxygen directly to generate superoxide, which leads to a number of reactive oxygen species (ROS) via a cascade of reactions. Such ROS production by leucocytes occurs when they are activated in the inflammation sites, and is a very important reaction in the immune system.

The processes involved in the generation of ROS can be monitored using luminescence. Luminol and lucigenin have been used to measure ROS produced by the NADPH oxidase. However, their sensitivity is poor to require the isolation of ROS-producing cells from other cells in blood when the ROS production from leucocytes in blood is measured. The process of isolation of the correspondent cells changes the behavior of these cells making the relevance of these investigations less valuable for drug discovery. Also, the ROS production activity of the ROS-producing cells is influenced by the non ROS-producing cells in blood. Therefore, it is essential to measure ROS production in the presence of a mixed population of cells, a whole blood sample.

Principle

PHOLASIN®(1) is a photoprotein derived from the bioluminescent mollusc Pholas dactylus and directly reacts with a wide range of ROS to emit light. It reacts with peroxidase enzymes such as myeloperoxidase. Also, PHOLASIN® on its own does not react directly with hydrogen peroxide but in the presence of peroxidase enzymes hydrogen peroxide fuels an intense luminescent reaction. PHOLASIN® is the most sensitive detector of superoxide under physiological conditions and is 100 to 1000 times more sensitive than luminol and lucigenin.
The ABEL® (Analysis By Emitted Light)(2) cell activation test kit for whole blood and isolated cells (Knight Scientific(3)) uses PHOLASIN® to probe the production of free radicals and other reactive species by leucocytes that occurs during the activation of the superoxide-generating NADPH oxidase system in leucocytes, the so-called respiratory burst. The kit contains PHOLASIN® as well as cell stimulants, PMA (phorbol-myristate-acetate) and fMLP (formyl-methionyl-leucyl-phenylalanine), together with all necessary reagents, buffers, and materials for performing the tests. The tests may also be used to monitor superoxide production by other types of cells that contain the NADPH oxidase system (e.g. fibroblasts, chondrocytes, mesanglial cells) as well as the production of superoxide by enzyme systems such as xanthine/xanthine oxidase. Additionally, the tests can be used to monitor degranulation in leucocytes and to identify cells that are prone to degranulate.

Experiments

We performed kinetic luminescence measurements of monitoring ROS productions by leucocytes upon its activation in blood using the sensitive PHOLASIN® technology of Knight Scientific, “ABEL® Cell activation ROS kit with PHOLASIN® ”, on an imaging plate reader FDSS/μCELL (Hamamatsu Photonics). Capillary blood collected in EDTA coated tubes was diluted and dispensed to a 96-well plate (black, Greiner optical button plate). Luminescence signals after addition of PHOLASIN® and a cell stimulant (fMLP or PMA) were collected by the FDSS/μCELL, a camera-based imaging plate reader for kinetic cell-based assays. FDSS/μCELL is equipped with an injection head (96/384-well) which enables simultaneous injection of compounds into all wells during the measurements. The measurements were performed in 0.67 second of integration time and 1 second of sampling time with maximum gain.
The luminescence signals are produced when PHOLASIN® contacts with superoxide generated by the NADPH oxidase of mainly neutrophils in the blood. The first column (blue) of Figure 1 shows the time-profiles of luminescence intensity when fMLP was added to stimulate NADPH oxidase of neutrophils. fMLP binds to its receptors on the cell plasma membrane of neutrophils, which leads to a cascade of reaction to activate NADPH oxidase. As shown in a blue line in the right figure, the luminescence intensity began to increase within several seconds after addition of fMLP and increased sharply up to the maximum intensity for about one minute. The luminescence went back close to the base line within about five minutes.
The second column (red) shows the time-profiles of luminescence intensity when PMA was added as the cell stimulant. PMA penetrates the cell membrane into cells and directly binds to Protein Kinase C (PKC), leading to the activation of the NADPH oxidase. It is known that the PMA activation route, unlike receptor-mediated activation, does not switch off NADPH oxidase activity. In this case, as shown in a red line in the right figure, the luminescence intensity increased gradually after addition of PMA and the luminescence lasted more than ten minutes.

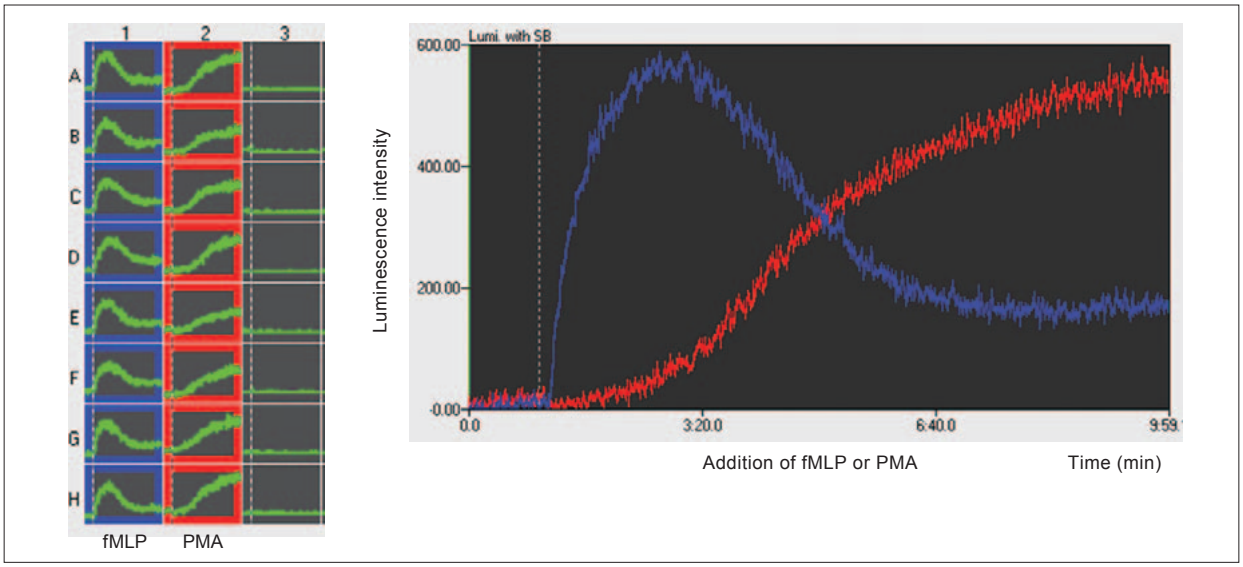

Figure 1: Luminescence signals upon activation of the NADPH oxidase in leucocytes in blood

Conclusion

This work demonstrates the ability of FDSS/μCELL to monitor activation of NADPH oxidase using ABEL® Cell activation Kit with PHOLASIN® technology in the 96-well format using a Hamamatsu camera. As shown in Figure 1, we can clearly distinguish between luminescence caused by different molecular routes of activation of NADPH oxidase, a receptor-mediated process and a process via direct activation of PKC, by performing kinetic luminescence measurements. Such kinetic assays could be very useful in investigations of subjects involving inflammation and ROS, in particular investigations of the activation of the NADPH oxidase in leucocytes.

Notes

(1) PHOLASIN® was originally named luciferin by Raphael Dubois in 1895 and the enzyme it reacts with in the mollusc, luciferase. Because of confusion now with firefly luciferin and luciferase, which has no chemical similarity with the system in the mollusc, we do not use that terminology.
(2) ABEL® is the registered trade mark of Knight Scientific Limited and is used to describe most of our assay. It is an acronym for Analysis By Emitted Light.
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