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Chemiluminescence

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Introduction

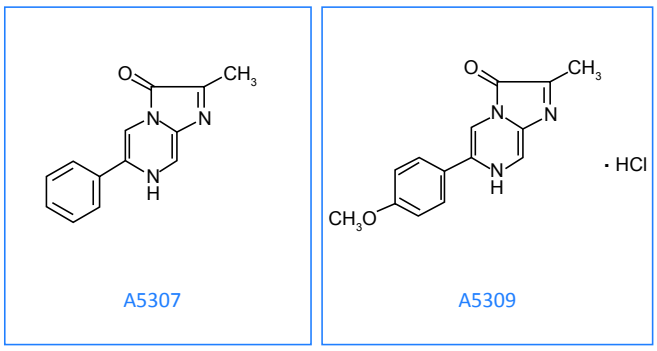
Inflammatory cells such as neutrophils, eosinophils, and macrophages produce a multitude of reactive oxygen species (ROS) when they are activated. Of the reactive oxygen species, superoxide anion radical (SOD, $\cdot O_2^-$), a precursor of several oxidants such as hydrogen peroxide (H_2O_2), hydroxy radical ($OH\cdot$), singlet oxygen ($^1O_2$), and hypochlorite (HOCl), can be formed in, and released from, each of three cell lines. On the other hand, $^1O_2$ (singlet oxygen, metastable oxygen) and hypohalides can be generated by myeloperoxidase (MPO)-mediated reactions in the neutrophil phagosome at pH 4.5, and by the eosinophil peroxidase-mediated reaction in eosinophils at physiological pH. It is, therefore, important to detect and measure the generation of $\cdot O_2^-$ and $^1O_2$ in small amounts in such biological systems. Because production of highly bactericidal agents such as $^1O_2$ and HOCl is a result of myeloperoxidase activity, simple and sensitive methods to measure this enzyme activity are required. Herein, we describe the measurement of $\cdot O_2^-$, $^1O_2$ generation, and myeloperoxidase activity, in leukocytes by chemiluminescene using cypridina luciferin analogues as chemiluminescence probes.

$\cdot O_2^-$ Production by Stimulated Leukocytes

When granulocytes or macrophages are stimulated, they produce and release $\cdot O_2^-$. The $\cdot O_2^-$.released from these cells reacts efficiently with cypridina luciferin analogue (CLA, 2-methyl-6-phenyl-3, 7-dihydroimidazo[1,2-α]pyrazin-3-one. MCLA, 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazol[1,2-α]pyrazin-3-one) (Figure 1) to produce an excited carbonyl which emits light in the visible region; i.e., maximum emission at 380 nm for CLA and at 457 nm for MCLA [1-3].

Protocol

1. Dissolve powdered Hanks' balanced salt solution (HBSS) for tissue culture (Nissui Pharmaceutical Co. Ltd, Tokyo) in distilled water (9.8 g/L) and adjust to pH 7.4 by use of aqueous Na_2HPO_4 solution (280 mOsm).
2. Prepare granulocytes or macrophages by standard methods.
3. Dissolve MCLA ($E_{430\ nm}$=9600 M/cm) or CLA ($E_{410\ nm}$ = 8900 M/cm) in distilled water at a concentration of 40 μM.
4. Dilute granulocytes or macrophages with HBSS at 5×10^4 cells for MCLA or 4×10^5 cells for CLA.
5. Add MCLA or CLA (50 μL, final concentration: 1 μM).
6. Preincubate at 37°C for 3 min.
7. Add opsonized zymosan (OZ, in water, 20 mg/mL, 80 μL), formyl-methionyl-leucyl-phenylalanine (fMLP in Ca^{2+} - and Mg^{2+} -free HBSS/ DMSO = 1:1, v/v, 200 μM, 10 μL), or 4β-phorbol myristate acetate (PMA in Ca^{2+}-and Mg^{2+}-free HBSS/DMSO = 1:1, *v/v*, 2 μg/ mL, 20 μL) (Total volume 2.0 mL).
8. Measure chemiluminescence.



A5307 CLA,
A5309 MCLA

Figure 1: Chemical structure of CLA and MCLA.

MPO in granulocytes $^1O_2$ measurements

When peripheral leukocytes derived from bone marrow are separated the neutrophils contain abundant granule myeloperoxidase as a host enzyme. Under acidic conditions, myeloperoxidase (MPO) catalyses the production of HOBr and HOC1 in the presence of Br^- and Cl^-, respectively (Reaction 1). The HOBr or HOCl can efficiently react with H_2O_2 to generate $^1O_2$ (Reaction 2) [4].

$$H_2O_2 + HBr\ (Cl) \xrightarrow{MPO,\ pH\ 4.5\text{-}5.0} HOBr\ (Cl) + H_2O_2 \quad (1)$$

$$HOBr\ (Cl) + H_2O_2 \rightarrow {}^1O_2 + H_2O + HBr\ (Cl) \quad (2)$$

MCLA reacts not only with $\cdot O_2^-$ but a1so with $^1O_2$ to emit 1ight with maximum intensity at 457 nm. The addition of 0.5 μMSOD to the system, in which both $\cdot O_2^-$ and $^1O_2$ are generated, completely quenches the $\cdot O_2^-$ dependent luminescence leaving only the $^1O_2$-dependent luminescence.

Protocol

1. Mixing human neutrophils and hexadecyltrimethylammonium bromide (0.02% w/v) in potassium phosphate buffer (pH 6.0, 50 mM) for 10 min.
2. Sonicate the mixture on ice for 30 s (0.5-s bursts at 0.5-s

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intervals) at an output level of 30 W (Branson Sonifier 250, Branson Ultrasonics Corporation, Danbury, CT, USA).

3. Centrifuge at 40000 g for 20 min at 4°C (The supernatant is used as the sample).

4. Preincubate a standard solution containing MCLA (in water, 200 μM, 100 μL) SOD (in water, 50 μM, 20 μL), KBr (in water, 50 μM, 20 μL), desferrioxamine (in water, 2 mM, 20 μL), H_2O_2 (40 mM, 25 μL), and acetate buffer (0.2 M, 1 mL) (Total volume 1.95 mL).

5. Measure chemiluminescence until baseline is constant.

6. Inject samples rapidly (50 μL) by means of a microsyringe.

7. Measure chemiluminescence.

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