A Chemiluminescence Procedure for Determination of the Release of Myeloperoxidase from Activated Human Neutrophils

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Summary: A chemiluminescence (CL) procedure was developed to determine the time course of the release of myeloperoxidase (MPO) from activated human neutrophils using two CL probes, luminol and MCLA. Luminol-dependent CL (LDCL) and MCLA-dependent CL (MDCL) in a hypoxanthine (HX)/xanthine oxidase (XOD) system, both of which were completely inhibited by superoxide dismutase, were a linear function of the XOD concentration, with the relationship formula being LDCL=0.003×MDCL. Under the same conditions, MPO could enhance LDCL in a dose-dependent manner, without influencing MDCL. There was a linear correlation between the MPO concentrations and the values of (LDCL−0.003×MDCL) (coefficient of correlation=0.004). This correlation made it possible to determine the release of MPO in the neutrophil/stimulus system by simultaneously monitoring the generation of LDCL and MDCL. By this CL procedure, it was revealed that there were variations in both neutrophil MPO releasing patterns and levels depending on the stimulating agent used.

Key words: neutrophil — myeloperoxidase — luminol — MCLA — chemiluminescence

Introduction

In neutrophils, the production of a series of reactive oxygen metabolites and the degranulation of myeloperoxidase (MPO) are induced by various soluble or particulate stimuli (Kanofsky et al. 1984). The initial product of oxygen reduction is superoxide radical (O2−) (Babior et al. 1973), which spontaneously or enzymatically dismutates to hydrogen peroxide (H2O2). Following this reaction, the chloride present in tissues and tissue fluids is oxidized to hypochlorous acid (HOCl) by H2O2 and the secreted MPO (Harrison and Schultz, 1976). Several methods for continuously measuring these individual oxidant species have been reported in the literature (Boveris et al. 1972; Ushijima and Nakano, 1980; Suzuki et al. 1982; Nakano, 1990). Although there are some procedures for estimating neutrophil-containing total MPO (Chance and Maehly, 1955; Mashiko et al. 1991), time course determinations of MPO released from activated neutrophils is hampered because of the concurrent MPO-H2O2-halide reaction and MPO autodestruction.

During preliminary experiments, it was observed that luminol-dependent
chemiluminescence (LDCL) specifically monitors only $O_2^-$ production from the hypoxanthine/xanthine (HX/XOD) system, and both $O_2^-$ generation and MPO-mediated reactions from the neutrophil/stimulus system, whereas MCLA-dependent chemiluminescence (MDCL) directly relates to $O_2$ production in both systems. On the basis of this observation, a chemiluminescence method for determining the time course of MPO release from activated neutrophils was developed in the present study.

Materials and Methods

Chemicals and media

The materials were as follows: Hanks' balanced salt solution (HBSS), from Nissui Pharmaceutical Co., Tokyo; 5-[N-2, 3-dihydroxypropylacetamide]-2, 4, 6-tri-iodo N, N' bis [2, 3-dihydroxypropyl] isophthalamide (Nycodenz) from Nyegaad, Oslo, Norway; 5-amino-2, 3-dehydro-1, 4-ophthrazinedione (luminol), phorbol 12-myristate 13-acetate (PMA), formylmethionyl-leucyl-phenylalanine (fMLP), superoxide dismutase (SOD), myeloperoxidase (MPO), catalase, sodium azide, xanthine oxidase (XOD) grade III from Sigma Chemical Co., St. Louis; hypoxanthine (HX) and calcium ionophore A23187 from Wako Kogyo Co., Tokyo; 2-methyl-6-[p-methoxyphenyl]-3, 7-dehydro-imidazo [1,2a]pyridine-3-one (MCLA) from Tokyo Kasei Kogyo Co., Tokyo.

Preparation of reagents for CL assay

HX/XOD $O_2^-$ generation system: One hundred microliters of 100 mM HX sus-
pended in HBSS was transferred to a luminescence counting tube. After adding an aliquot of either 100 µM MCLA (10 µl) or 25 µM luminol (100 µl) and adjusting the final volume to 400 ml with HBSS, the tube was placed in a lumiphotometer (Model L-400, labosience, Tokyo) and allowed to stand until a stable background was obtained. The reactions were initiated by injecting 100 µl of a given concentration of XOD, and light emission was monitored for 30 min at 10-sec intervals. To examine the effects of haemoinhibitors or a quencher on MDCL or LDCL, light emission was also measured in the presence of SOD, MPO, catalase, or sodium azide.

Neutrophil/stimulus system: One hundred microliters of neutrophil suspension (1 × 10⁶) was transferred to a luminescence counting tube previously filled with either 10 µl of 100 µM MCLA and 290 µl of HBSS, or 100 µl of 25 µM luminol and 200 µl of HBSS. The counting tube was placed in the lumiphotometer and allowed to stand until a stable background was obtained. To generate CL, 100 µl of each stimulating agent (10 ng/ml PMA, 20 µg/ml fMLP, 20 µl/ml latex particles, 0.2 µM calcium ionophore A23187, 2 mg/ml IC₃Z, C₂Z, or IgGZ at a final concentration) was added to the counting tube. Haemoinhibitor-added CL assays were also performed. Light emission counts were taken, as described for the HX/XOD system.

Statistical analysis

All data were expressed as mean±SD, and the results were analyzed with the independent sample two-tailed Student’s t-test. The correlations between MDCL and LDCL intensities were examined by least square regression analysis.

Results

MDCL and LDCL in the HX/XOD system, and the relationship between MDCL, LDCL and MPO concentration

When various concentrations of XOD were added to the suspension of a saturating amount of HX, the MDCL abruptly increased. The increase was followed by a slow upstroke to a peak and a subsequent slow decline in the signals (Fig. 1). The LDCL signals had exactly the same pattern with lower

![Fig. 1. Time-courses of MDCL and LDCL intensities in the presence of a saturating amount of HX and various concentrations of XOD. The values represent the mean of four triplicate measurements. r.l.u: relative light unit](image-url)
levels than the MDCL signals. The integral intensities of both MDCL and LDCL were a linear function of the XOD concentration, and the following relationship between the two CLs was obtained; LDCL intensity = 0.003 × MDCL intensity (Fig. 2).

As depicted in Fig. 3, the MDCL was suppressed to the baseline level by adding 0.1 U/ml SOD. MPO (0.05 μg/ml), sodium azide (0.1 mM/ml) and catalase (20 μg/ml) did not influence this MDCL. Under the same conditions, the LDCL was also completely inhibited by 0.1 mU SOD. There was no significant interference by sodium azide or catalase on the measurement of LDCL. In contrast, MPO enhanced the LDCL in a dose-dependent manner. The LDCL in the presence of MPO+HX/XOD was diminished to the MPO-absence LDCL level by adding 0.1 mM/ml sodium azide, and was completely suppressed to the zero level by adding 0.1 mM/ml sodium azide and 0.1 U/ml SOD.

When various concentrations of MPO were added in the O$_2$ generation-fixed HX (10 mM)/XOD (4 mU) system, a linear

\[ y = 0.003x \]

*Fig. 2. Respective relationships between XOD concentrations, MDCL intensities, and LDCL intensities in a HX/XOD system. The values represent the mean of four triplicate measurements.*

*Fig. 3. The effects of haemoinhibitors on MDCL and LDCL intensities in a HX/XOD system and in a MPO+HX/XOD system. The values represent the mean of the integral MDCL and LDCL intensities from three triplicate measurements. Significantly different from control at * p<0.05, ** p<0.01 and *** p<0.001*
relationship between the MPO concentration and MPO-enhanced LDCL intensity (MPO-presence LDCL−MPO-absent LDCL) was observed (Fig. 4). In a MPO+O₂ generation-unknown HX/XOD system, the MDCL and LDCL also yielded a similar relationship between MPO concentration and MPO-enhanced LDCL intensity, as estimated by subtracting 0.003×MDCL from LDCL. The following relationship formula was obtained; MPO concentration=0.004×(LDCL−0.003×MDCL).

**MDCL and LDCL in the neutrophil/stimulus system**

When stimulated with opsonized zymosans, PMA, calcium ionophore A-23187, or fMLP, neutrophils gave typical MDCL and LDCL responses (Fig. 6). These neutrophil MDCLs were completely suppressed by SOD, but not MPO, sodium azide or catalase (Fig. 5). Like LDCL in the MPO+HX/XOD system, the neutrophil LDCLs were completely inhibited by sodium azide and SOD, providing evidence that MDCL and LDCL in the neu-

**Fig. 4.** Relationship between MPO concentrations, MDCL intensities, and LDCL intensities in the HX/XOD system. The values represent the mean of four triplicate measurements.

**Fig. 5.** The effects of haemoinhibitors on MDCL and LDCL measurements in the neutrophil/stimulus system. The values represent the mean of the integral MDCL and LDCL intensities from three triplicate measurements. Significantly different from control at * p<0.05, ** p<0.01 and *** p<0.001
Fig. 6 a

Fig. 6 b
Fig. 6. Time-course of MPO release from stimulus-activated neutrophils estimated by MDCL and LDCL determinations. The values represent the mean of three triplicate measurements.

Table 1.

Quantities of MPO released from stimulus-activated (1x10^5) neutrophils

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>IC₅₀</th>
<th>IgGZ</th>
<th>C₃Z</th>
<th>PMA</th>
<th>Ca²⁺ ionophore</th>
<th>fMLP</th>
</tr>
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<tr>
<td>MPO (ng/min)</td>
<td>10.38±0.36</td>
<td>8.85±0.62</td>
<td>4.63±0.41</td>
<td>2.09±0.23</td>
<td>3.27±0.51</td>
<td>0.84±0.15</td>
</tr>
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trophil/stimulus system are comparable to those in the MPO+HX/XOD system. The MPO formula obtained in the MPO+HX/XOD system is also applicable to the neutrophil/stimulus system.

Time course of MPO release from neutrophils exposed to various stimuli

Fig. 6 shows the time course traces for MDCL, LDCL, and MPO release in human neutrophils when exposed to various stimuli. Upon stimulation with opsonized zymosans, a single peak response was observed for MDCL, LDCL, and MPO release, with the order of the responses being IC₅₀Z>IgGZ>C₃Z. Both PMA and Ca²⁺ ionophore A23187 induced a similar single peak response of MDCL, LDCL and MPO release, while the Ca²⁺ ionophore A23187 induced a fluctuating two-peak MDCL, and single peaks for LDCL and MPO release. As compared with opsonized zymosans, PMA induced low levels of MPO release for the generation of MDCL, while Ca²⁺ ionophore A23187 induced high levels of MPO release for the generation of MDCL. fMLP caused a dual-peak LDCL, an obscure dual-peak MDCL, and a tran-
sitory release of MPO corresponding to the initial peak of the LDCL. The total concentration of MPO released from the stimulus-exposed neutrophils are summarized in Table 1.

Discussion

Of the CL enhancers, luminol has been shown to emit light by the MPO-catalyzed oxidative metabolites (DeChatelet et al. 1982), and the prostaglandin endoperoxide synthase pathway (Marnnett et al. 1982). Using the neutrophil/stimulus system and two haemoinhibitors, SOD as a $O_2^-$ dismutating enzyme and sodium azide as a MPO inhibitor, complete inhibition of neutrophil LDCLs with SOD and sodium azide was observed, providing evidence that LDCL monitors both $O_2^-$ generation and MPO-mediated reactions. This is supported by the LDCL assay using a HX/XOD $O_2^-$ generation system and haemoinhibitors, in which LDCL was completely suppressed by SOD, but was enhanced by MPO in a dose-dependent manner. These results indicate that MPO released from activated neutrophils can be quantitatively determined using an LDCL assay if there are methods for separately measuring MPO-dependent LDCL and $O_2^-$-dependent LDCL, and if a relationship between MPO-dependent LDCL intensity and MPO concentration is established.

With the neutrophil/stimulus system, it is possible to measure MPO-dependent LDCL and $O_2^-$-induced LDCL by the addition of SOD and sodium azide, respectively. However, these procedures are not suitable because i) neutrophil $O_2^-$ generation and MPO release vary with the stimulating agent; ii) a high dose of SOD non-specifically inhibits LDCL (Brestel, 1987), and behaves as an oxidative agent in the presence of high concentrations of $H_2O_2$ (Hodgson and Fridovich, 1975); and iii) sodium azide has pronounced CL-enhancing effects on complex systems (Steele et al. 1988). The present study demonstrated that MCLA specifically emitted light in the presence of $O_2^-$, without interference from biological pigments under normal assay conditions, in agreement with the report of Nishida et al. (1989). Also, the $O_2^-$-dependent light emission of luminol was estimable from that of the MCLA according to the relationship between the two CLs, $O_2^-$-dependent LDCL=0.003×MDCL, obtained from the HX/XOD system. Additionally, by measuring MDCL and LDCL in the MPO+HX/XOD system, a relationship, MPO concentration=0.004×(LDCL−0.003×MDCL), was established.

The reaction leading to the luminol-dependent light emission from neutrophils has been hypothesized to take place both intra- and extracellularly (Dahlgren et al. 1985). If MDCL monitors only extracellular $O_2^-$, it is unreasonable to incorporate the relationship between MPO concentration and the two CLs obtained in the HX/XOD system into the neutrophil/stimulus system. MCLA is a larger molecule (291.74 daltons) than luminol (177.2 daltons), but smaller than transmembrane-impermeable molecules (over 370 daltons)(Dahlgren et al. 1985). When neutrophils were exposed to various stimuli in the presence of MCLA and luminol, the cells produced MDCL and LDCL, respectively. The time-courses of CL emission disclosed that neutrophil MDCL and LDCL had almost the same pattern. It is likely, therefore, that the
mechanisms for neutrophil CL, as recognized by using MCLA and luminol, are nearly identical. This also means that it is possible to apply the formula between MPO, MDCL and LDCL obtained in the HX/XOD system to the neutrophil/stimulus system.

Using the proposed method, the time-course of MPO release in the neutrophil/stimulus system was determined. Upon stimulation with opsonized zymosans, a single peak response for MDCL generation and MPO release was observed, with the characteristic that neutrophil MPO release was in synchrony with MDCL generation. FCγR- and/or CR-mediated neutrophil MDCL generation have been shown to involve a G protein-modulating signal transduction pathway, following the mobilization of intracellular Ca²⁺ and subsequent activation of protein kinase C (PKC) (Shirouzu, 1995). Thus, it can be suggested that a transient rise of intracellular Ca²⁺ and/or activation of PKC are also necessary prerequisites for neutrophil MPO release. To clarify this possibility, neutrophil MPO release was determined after stimulation with a soluble PKC activator, PMA, or the Ca²⁺ ionophore A23187 that induces an influx of extracellular Ca²⁺. As compared with opsonized zymosans, PMA induced a small MPO release for the generation of MDCL, while the Ca²⁺ ionophore A23187 induced a large MPO release for the generation of MDCL, suggesting that intracellular free Ca²⁺ plays an important role in the mechanism of neutrophil MPO degranulation. As expected from previous investigations (Dahlgren and Stendahl, 1983; Dahlgren et al. 1985; Edwards, 1987), stimulation of neutrophils by fMLP resulted in a bimodal LDCL pattern. fMLP also induced a dual-peak MDCL response, but caused a single-peak MPO release corresponding to the initial LDCL peak. Although the mechanism for a bimodal LDCL response is unclear, it is attractive to speculate that the initial response is due to a MPO-mediated reaction (a transient MPO release), and the second response reflects O₂⁻-induced light emission.

In conclusion, a simple method was described to determine the time-course of MPO release from activated neutrophils by continuously measuring MDCL and LDCL. It is now possible to investigate not only oxidative and MPO-degranulating processes within neutrophils, but also the effects of pharmacological agents on both the transmembrane intracellular oxidative signaling pathway and the extracellular oxidative events of these cells.

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