GENERATION OF ACTIVATED OXYGEN SPECIES BY POLYMORPHONUCLEAR LEUKOCYTES

Koichiro TAKANAKA and Peter J. O'BRIEN+

Niigata College of Pharmacy, Kamishingei-cho, Niigata, Japan and +*Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada*

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1. Introduction

Cyanide-resistant respiratory burst during phagocytosis by leukocytes plays an important role in killing bacteria [1,2]. The respiratory burst of leukocytes enhances the release of reduced oxygen molecules such as superoxide anion (O_2^-) [3–5] and hydrogen peroxide (H_2O_2) [6–8]. The possibility that these reduced oxygen species might function as bactericidal agents is thought to be unlikely, since O_2^- or H_2O_2 by itself has only weak bactericidal activity [9,10].

Activated oxygen molecules other than H_2O_2 and O_2^- are also generated during the phagocytosis process. One activated oxygen molecule proposed to account for the bactericidal agent is the hydroxyl radical ($OH^\cdot$) [11–13] which is formed by a reaction between O_2^- and H_2O_2 (a Haber-Weiss reaction) as follows:

$$O_2^- + H_2O_2 \rightarrow OH^\cdot + OH^- + O_2$$

Further reaction might generate singlet oxygen as follows:

$$O_2^- + OH^\cdot \rightarrow OH^- + {}^1O_2$$

However, mechanisms for generating chemiluminescence (which might be due to $^1O_2$) [14–16], as well as hydroxyl radical formation during phagocytosis have not been clarified. Here we study the mechanisms for generating these activated oxygen species by observing the effect of several stimuli of leukocytes and following the time curve for the formation of these activated oxygen species.

2. Materials and methods

Leukocytes were obtained from the intraperitoneal cavity of guinea pig by an injection of casein solution as in [17]. The leukocytes were centrifuged at 500 × *g* for 5 min at 0–4°C, resuspended and washed with Krebs' phosphate solution.

2.1. *Measurement of oxygen consumption*

Oxygen uptake was measured with a Clark oxygen electrode in a 1.8 ml vessel at 37°C. The incubation medium contained Krebs' phosphate solution, 1–1.3 mg protein of leukocytes and heat-killed and opsonized *Escherichia coli* ($\sim 10^8$) or 15 μg digitonin.

2.2. *Measurement of chemiluminescence*

Chemiluminescence (CL) was monitored by a photomultiplication method using a Beckman Liquid Scintillation Counter (Model LD-233) operated in the out-of-coincidence mode [15]. The initial reaction mixture as the assay method for oxygen uptake (mentioned above) was added in the scintillation vial and the reaction was started by the addition of the metabolic stimuli. Scintillation counts (cpm) were estimated every 1–5 min, between which the vial containing the reaction mixture was incubated in a water bath (37°C) with gentle shaking. All operations were carried out in the dark.

2.3. *Measurement of hydroxyl radical production*

Hydroxyl radicals were estimated by the measurement of ethylene found when hydroxyl radicals react with KMBA (α-keto-γ-methiol-butyric acid). The incubation medium contained the identical reaction mixture for the oxygen uptake (see above) with the

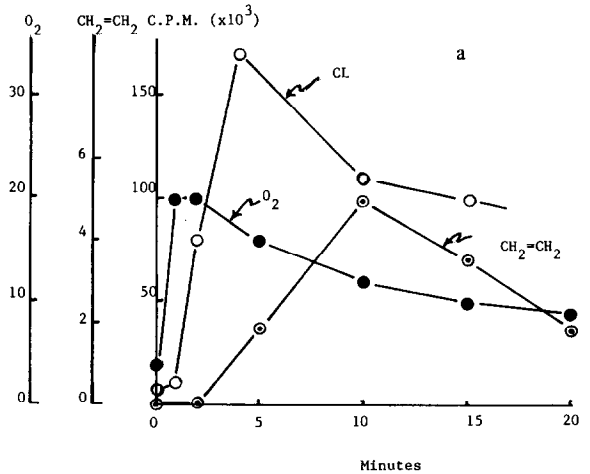
addition of 1 mM KMBA. The reactions took place in plastic test tubes with rubber septa shaking 120 rev./min at 37°C. Every 5 min 0.5 ml aliquots of the vapor phase were analyzed on a Fisher gas chromatograph (series 4800, Fisher Sci., Whitby, Ontario). The chromatograph was equipped with a 200 cm X 2 mm column packed with Parapak type Q (Waters Assoc., Milford, MA).

Chemicals and enzymes such as digitonin, linoleic acid, KMBA, superoxide dismutase (SOD) and catalase (bovine liver) were obtained from Sigma Chem. (St Louis, MO). Purified myeloperoxidase (MPO) was a generous gift from Dr J. Schultz (1.9 units/mg solid). IU of the enzyme is the amount required to decompose 1 μmol H_2O_2/min at 23°C [18]. All other chemicals were analytical grade.

3. Results

The results shown in fig.1(a–c) were typical examples of the relationship between oxygen uptake, chemiluminescence and ethylene formation by variously treated leukocytes. The rates of oxygen consumption (expressed as nmol . min^{-1} . mg $protein^{-1}$) and ethylene production (expressed as pmol . min^{-1} . mg $protein^{-1}$) were compared with the chemiluminescence expressed as cpm. Owing to the limitation of the method for measuring chemiluminescence, it could not be expressed directly as a rate.

The respiratory burst of leukocytes started first of all within 1–2 min incubation with various stimuli



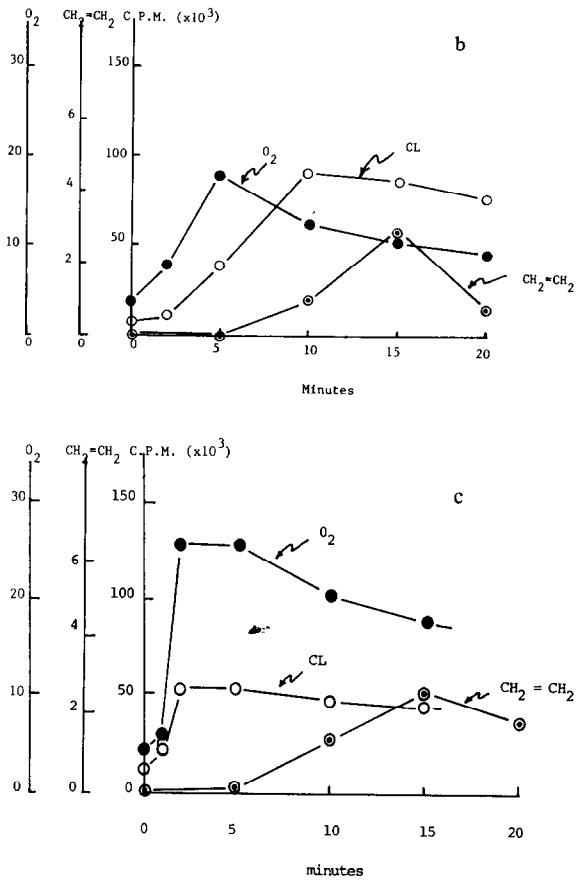


Fig.1. Rates of oxygen consumption, chemiluminescence and ethylene production. (–●–) The rates of oxygen consumption. Reaction mixture contained 1 mg protein of leukocytes in 1.8 ml Krebs' phosphate solution and heat-killed and opsonized bacteria (a), 15 μg digitonin (b) or 0.05 mM linoleic acid (c). Rates were expressed as nmol O_2 consumed . min^{-1} . mg leukocyte $protein^{-1}$. (–○–) The generation of chemiluminescence (CL) was expressed in cpm. The reaction conditions were identical to the oxygen consumption experiments. (–⊗–) The rates of ethylene production ($CH_2 = CH_2$) were expressed in pmol ethylene . min^{-1} . mg leukocyte $protein^{-1}$. Assay conditions were identical to those above with the addition of 1 mM KMBA to the reaction mixture.

and the rate of oxygen consumption remained constant for at least the following 10 min. Then it gradually decreased as a function of time after the maximum rate was observed.

Shortly after the respiratory burst took place, a remarkable increase in chemiluminescence was seen. The increased chemiluminescence was effectively diminished by azide (1 mM), KCN (1 mM) and SOD (superoxide dismutase). The degree of chemilumines-

cence stimulation, however, was not proportional to the degree of the respiratory burst, when the leukocytes stimulated by different means are compared (fig.1a–c)).

Chronologically, the ethylene formation (believed to be due to $OH^{\cdot}$) occurred after the stimulation of oxygen consumption and the chemiluminescence. A typical example was the phagocytizing leukocytes (fig.1a), where an appreciable amount of ethylene was not detected during the first 5 min incubation by the phagocytizing leukocytes, although the respiratory burst and stimulation of chemiluminescence had been initiated within 5 min.

As summarized in table 1, ethylene production by phagocytizing leukocytes was effectively inhibited by mannitol which is known to be a selective hydroxyl radical ($OH^{\cdot}$) scavenger [11]. Similar inhibitory effects were observed with cyanide, catalase and SOD. The digitonin treated leukocytes also demonstrated that the ethylene production could be due to the presence of H_2O_2 and O_2^-. On the contrary however, addition of purified myeloperoxidase to the reaction mixture containing leukocytes and stimuli increased the ethylene production (2–3-fold). This effect was not seen by using the heat-inactivated peroxidase and the peroxidase itself did not show any stimulatory effect on the resting leukocytes. The addition of the purified myeloperoxidase also did not indicate any appreciable changes in the chemiluminescence and the respiratory burst of phagocytizing leukocytes.

Table 1
Ethylene production by leukocytes

Leukocytes	15 min	30 min
+ Bacteria	45 ± 7 (3)[a]	81 ± 12 (3)[a]
+ 50 mM mannitol	17	39
+ 1 mM KCN	25	49
+ 50 μg SOD	18	21
+ 50 μg catalase	23	40
+ 100 μg MPO	152	193
+ 15 μg Digitonin	82 ± 4 (3)[a]	155 ± 10 (3)[a]
+ 1 mM KCN	38	46
+ 50 μg SOD	16	25
+ 50 μg catalase	53	79
+ 100 μg MPO	290	438
+ 100 μg heated MPO	90	150
Leukocytes	<10	<10
+ 100 μg MPO	<10	<10

[a] Average of 3 expt

Ethylene production was assayed with 1 mg protein of leukocytes in 1.8 ml Krebs' phosphate solution containing 1 mM KMBA and various stimuli

4. Discussion

These results show that leukocytes generate active oxygen species in the same chronological order no matter which method was used to stimulate the leukocytes. The respiratory burst as well as O_2^- formation occur in the first stage of the reaction and the increase of chemiluminescence follows the rate of oxygen consumption. After 5–10 min incubation, hydroxyl radicals gradually accumulated in the reaction medium. This order is always identical whether leukocytes are activated by such stimuli as digitonin, linoleic acid or bacteria, although detected amounts differ.

Although the data is not shown here, the time course of oxygen uptake was found to coincide with the production of O_2^- and H_2O_2. Recently, however, doubt has been expressed as to whether or not molecular oxygen is reduced to O_2^- by phagocytizing leukocytes [13,19]. We also observed that cytochrome *c* is reduced by activated leukocytes shortly before the respiratory burst is detected. Nevertheless, we believe that the oxygen uptake is due to the consequence of its reduction to O_2^- and H_2O_2. The cells deplete intracellular oxygen in the first stage and this could explain the delay in oxygen consumption. We also measured H_2O_2 formation using a model system involving horseradish peroxidase [7,8] and the initial rate was stoichiometric with oxygen consumption.

The rates of chemiluminscence production follows the respiratory burst and the maximum rate is always observed within 2–5 min after the oxygen consumption and 5–15 min before the maximum rate of hydroxyl radical formation. Moreover, the chemiluminescence is detected before any hydroxyl radical is generated and addition of purified myeloperoxidase does not increase its intensity. These observations exclude the possibility that the reaction ($O_2^- + OH^{\cdot} \rightarrow OH^- + {}^1O_2$) [20] or peroxidase system (peroxidase + H_2O_2 + Cl^-) [14,15,22] account for the generation of chemiluminescence. Presumably the light emission by the stimulated leukocytes is not due to ${}^1O_2$ [21–22] and is the consequence of the formation of activated carbonyl by O_2^- [23], since O_2

formation and chemiluminescence correlated closely as seen in fig.1.

The mechanisms of hydroxyl radical formation by leukocytes have been discussed [11–13] and most investigations favor a Harber-Weiss reaction ($H_2O_2 + O_2^- \rightarrow OH^{\cdot} + O_2$). The hydroxyl radical is also generated by the Fenton reagent system ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$). Our studies indicate that myeloperoxidase might play an important role for hydroxyl radical formation, since exogenously added peroxidase increases the rate of hydroxyl radical formation by the stimulated leukocytes. Although a mechanism is not postulated, evidence has been provided for the participation of myeloperoxidase in hydroxyl radical formation, as hydroxyl radical formation was decreased in myeloperoxidase deficient leukocytes [26]. Therefore, it seems likely that the enzymic reaction of myeloperoxidase and the accumulated O_2^- and/or H_2O_2 are the source of hydroxyl radical production in the stimulated leukocytes.

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