PEROXIDASE CATALYZED SINGLET OXYGEN FORMATION FROM HYDROGEN PEROXIDE

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1. Introduction

The bactericidal activity of leukocytes has been attributed to H_2O_2 formation following phagocytosis (reviewed by Karnovsky [1]) and recently we reported a plasma membrane located NADPH oxidase in leukocytes which on leukocyte activation forms hydrogen peroxide [2,3]. Klebanoff has described the microbicidal activity of myeloperoxidase or lactoperoxidase, hydrogen peroxide and halides [4–6]. The peroxidase system has also been shown to be cytotoxic for mammalian tumor cells [7,8]. Krinsky has found that a carotenoid-less mutant organism was killed rapidly by polymorphonuclear leukocytes unlike the wild type strain containing the $^1O_2$ quenching carotenoids [9]. Klebanoff has also been able to inhibit the microbicidal activity of the system using a $^1O_2$ trap [10]. In the following, we present unambiguous evidence that $^1O_2$ is generated by the peroxidase, hydrogen peroxide and halide system and suggest that $^1O_2$ is responsible for the cytotoxic and microbicidal properties.

2. Materials and methods

Lactoperoxidase (LPO) (EC 1.11.1.7) A412/A280, spec. act. 62 units/mg protein, was purchased from Sigma Chemical Co., St. Louis. D_2O was obtained from Stohler Isotope Chemicals, NJ. 1,3-Diphenylisobenzofuran (DPIBF) and *o*-dibenzoylbenzene were obtained from Aldrich Chemical Co., Wisconsin. 2,5-Diphenylfuran (DPF) was obtained from Eastman Kodak Co. All other chemicals and reagents used were of the highest grade commercially available.

2.1. *Formation of O_2*

Oxygen evolution from a reaction mixture containing 50 mM acetate buffer, pH 4.5, 0.25 mM KBr^-, 0.25 mM H_2O_2 and 0.031 units of LPO was monitored with a Clark type oxygen electrode at 20°C in a reaction vessel of 1.5 ml capacity. When the reaction was carried out anaerobically, oxygen free nitrogen was bubbled through the medium before addition of LPO.

2.2. *Chemiluminescence*

Chemiluminescence of the above reaction medium was monitored using a Beckman Liquid Scintillation Counter (Model LS-233) operated in the out-of-coincidence mode [11]. The desired concentration of halide and enzyme were added in the dark to the dark-adapted counting vial containing acetate buffer (pH 4.5). After allowing for mixing time, the reaction was initiated by adding H_2O_2. Scintillation counts were recorded every 10 s for the first minute and every 30 s thereafter. The D_2O–acetate buffer was adjusted to a pH of 4.8, which would give an effective p*D* of 4.5.

2.3. *DPIBF oxidation*

DPIBF oxidation was measured spectrophotometrically by recording the decrease in absorbance at 420 nm after initiation of the reaction by the addition of H_2O_2. The reaction medium contained 0.031 units LPO, 0.25 mM KBr, 0.25 mM H_2O_2.05% Triton X-100 and 100 μM DPIBF in 50 mM acetate buffer, pH 4.5, at 20°C.

2.4. *Oxidation products of DPIBF and DPF*

Prior to use, DPIBF was purified by thin-layer

chromatography on Silicagel plates using benzene as the solvent. The reaction mixture, containing 2.5 mM KBr^-, 2.5 mM H_2O_2, 3.1 units LPO and 1 mM DPIBF or DPF after incubation for 10 min at 20°C, was extracted with chloroform and the chloroform extract was spotted on a thin-layer chromatography plate which was eluted in benzene. The analysis of DPF products was carried out by the method of King et al. [12].

3. Results

Studies using the O_2 electrode confirmed the production of O_2 by the $LPO/H_2O_2/Br^-$ system. No O_2 release was observed when the enzyme was inactivated by azide (1 mM) or heat inactivation. The reaction occurred equally well under anaerobic conditions and no difference was observed in the rate or amount of O_2 produced, at pH 4.5 or pH 8.0. The addition of OBr^- to 0.25 mM H_2O_2 also resulted in O_2 production, again with no difference in the rate of O_2 evolution, between pH 4.5 or pH 8.0. Addition of LPO to the OBr^-/H_2O_2 system did not affect the formation of O_2, at either pH.

Examination of the thin-layer chromatography plate of the reaction products under ultraviolet light revealed that the only oxidation products corresponded to *o*-dibenzoylbenzene, the $^1O_2$ oxidation product of DPIBF [13]. Similar results were obtained in a reaction mixture containing OBr^- (generated by adding Br_2 to 0.1 N NaOH) and H_2O_2 (25 mM). The $^1O_2$ oxidation product, *cis*-dibenzoylethylene, was also the only product observed in reaction mixtures containing DPF.

The rate of oxidation of DPIBF by the $LPO/H_2O_2/Br^-$ system was 60 nmol DPIBF oxidized/min under the conditions used. Omission of H_2O_2 or Br^- or LPO resulted in a complete inhibition of the oxidation. The optimal pH found for this reaction was pH 4.5. As seen in table 1, the $LPO/H_2O_2/Br^-$ oxidation of DPIBF was unaffected by anaerobic conditions. The free radical scavengers, 2,6-di-*tert*-butyl phenol and butylated hydroxytoluene and the hydroxyl radical scavengers. Mannitol, benzoate and *t*-butanol had no effect. The rate was however doubled in a D_2O medium in which $^1O_2$ has a longer half-life [14]. Furthermore diazobicyclooctane [15] and the amino acids, methionine, tryptophan and histidine [16] all singlet oxygen quenchers, inhibited DPIBF oxidation at a concentration of 0.15 mM and completely inhibited at higher concentrations.

In fig.1, the temporal traces of chemiluminescence obtained from the $LPO/H_2O_2/Br^-$ system are shown. No chemiluminescence was observed on the omission of H_2O_2 or Br^- or LPO and when the enzyme LPO was inactivated by azide (1 mM) or by heat. To test

Table 1
Effects of inhibitors on the oxidation of diphenylisobenzofuran

Systems	% Control rate
LPO + H_2O_2 + Br^-	100
LPO + H_2O_2 + Br^- + Diazobicyclooctane (10 mM)	56
LPO + H_2O_2 + Br^- + Diazobicyclooctane (0.1 M)	3
LPO + H_2O_2 + Br^- + 2,6-Di-*t*-butyl phenol (0.3 mM)	100
LPO + H_2O_2 + Br^- + Butylated hydroxytoluene (2 mM)	101
LPO + H_2O_2 + Br^- + Mannitol (5 mM)	97
LPO + H_2O_2 + Br^- + Benzoate (2 mM)	98
LPO + H_2O_2 + Br^- + *t*-Butanol (200 mM)	105
LPO + H_2O_2 + Br^- + Methionine (100 M)	15
LPO + H_2O_2 + Br^- + Tryptophan (100 M)	21
LPO + H_2O_2 + Br^- + Histidine (0.5 mM)	3
LPO + H_2O_2 + Br^- + Histidine (0.15 mM)	60

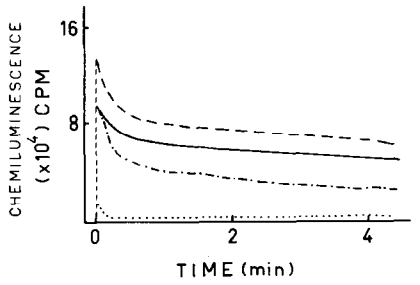

Fig.1. Chemiluminescence traces of the LPO/Br^-/H_2O_2 system, at pH 4.5. (————) The complete system. (. – . – . –) With 0.5 mM diphenylfuran. (.....) With 1 mM methionine. (– – – – – –) In D_2O buffer. The reaction medium contained 5 mM KBr, 5 mM H_2O_2 and 6.2 units LPO.

for $^1O_2$ involvement in the LPO/H_2O_2/Br^- system, chemiluminescence was monitored in the presence of various $^1O_2$ quenchers. Diphenylfuran (0.5 mM), a $^1O_2$ trap [17], inhibited 50% and methionine (1 mM) inhibited 90%. The chemiluminescence was enhanced in a D_2O buffer. The free radical scavenger 2.6 di-*tert*-butyl phenol (0.3 mM) and carbonate [18] also had no effect on the chemiluminescence.

4. Discussion

The proposed mechanism to explain the present results for the peroxidase catalyzed singlet oxygen formation is as follows:

$$H_2O_2 + Br^- \xrightarrow{LPO} OBr^- + H_2O \quad (1)$$

$$OBr^- + H_2O_2 \longrightarrow H_2O + Br^- + {}^1O_2 \quad (2)$$

Both reactions occurred under anaerobic conditions so that the $^1O_2$ was derived from the heterolytic oxidation of H_2O_2. Oxygen release by reaction (2) was found to occur equally well, at pH 4.5 or pH 8.0, but the optimum pH for the overall peroxidase catalyzed reaction was pH 4.5. Reaction (2) has also been demonstrated by others to form $^1O_2$ [19].

Evidence that $^1O_2$ was formed by the peroxidase–halide catalyzed oxidation of H_2O_2 was the total inhibition of the chemiluminescence and DPIBF oxidation by $^1O_2$ quenchers or traps. These substances did not act by inhibiting the peroxidase, as oxygen release was not affected and a similar inhibition was observed for the non enzymic chemiluminescence from OCl^- or $OBr^- + H_2O_2$. Peroxidase also did not enhance the non enzymic chemiluminescence from $OCl^- + H_2O_2$ so that little chemiluminescence resulted from an interaction between $^1O_2$ and peroxidase. Further confirmation of the involvement of $^1O_2$ was the enhancement of chemiluminescence and DPIBF oxygenation in a deuterated medium in which the lifetime of $^1O_2$ is much longer [20,14].

The chemiluminescence found by others for myeloperoxidase, H_2O_2 and chloride [21,22] is also presumably due to singlet oxygen. Furthermore, as $^1O_2$ generated photochemically is microbicidal [10] and highly cytotoxic to tumor cells [23], it is likely that $^1O_2$ is responsible for the microbicidal activity of the polymorphonuclear leukocyte and the cytotoxic properties of the peroxidase/H_2O_2/halide system.

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