FEBS Letters 443 (1999) 154–158 FEBS 21441

Physiological production of singlet molecular oxygen in the myeloperoxidase-H_2O_2-chloride system

Chika Kiryu[a,b], Masao Makiuchi[a], Junji Miyazaki[a], Toru Fujinaga[b], Katsuko Kakinuma[a,*]

[a] *Biophotonics Information Laboratories, Yamagata Advanced Technology Research Center, Matsuei 2-2-1, Yamagata 990-2473, Japan*
[b] *Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, N-18, W-9, Kita-ku, Sapporo 060-0818, Japan*

Received 16 November 1998; received in revised form 15 December 1998

Abstract The putative role of singlet oxygen ($^1O_2$) in the respiratory burst of neutrophils has remained elusive due to the lack of reliable means to study its quantitative production. To measure $^1O_2$ directly from biological or chemical reactions in the near infrared region, we have developed a highly sensitive detection system which employs two InGaAs/InP pin photodiodes incorporated with a dual charge integrating amplifier circuit. Using this detection system, we detected light emission derived from a myeloperoxidase (MPO)-mediated reaction in physiological conditions: pH 7.4, 1–30 nM MPO, 10–100 μM H_2O_2 and 100–130 mM Cl^- in place of Br^- without the use of deuterium oxide. The MPO-H_2O_2-Cl^- system exhibited a single emission peak at 1.27 μm with a spectral distribution identical to that of delta singlet oxygen. Our results suggest physiological production of $^1O_2$ in the MPO-H_2O_2-Cl^- system at an intravacuolar neutral pH. The MPO-mediated generation of $^1O_2$, which may have an important role in host defense mechanisms, is discussed in connection with previous results.

Key words: Singlet oxygen; Myeloperoxidase; Near infrared light emission

1. Introduction

The primary function of neutrophils is phagocytosis, killing and destruction of invading microorganisms. The cytoplasm of neutrophils contains numerous granules such as azurophilic, specific and tertiary granules [1–4]. Myeloperoxidase (MPO) is present in azurophilic granules in an exceptionally high concentration. Following phagocytosis, the membranes of granules fuse with the membrane of the phagocytic vacuole, which phagolysosomes release MPO into the vacuole containing the ingested microorganisms. Early studies on intravacuolar pH reported that appreciable acidification occurred after phagocytosis. However, Segal et al. showed evidence for an apparent rise of intravacuolar pH to 7.75 within the first 2 min, thereafter followed by mild acidification to a neutral pH after 15 min [5]. Other groups also confirmed the initial alkalinization and then mild acidification to maintain a neutral pH in phagosomal vacuoles [6,7]. During phagocytosis, neutrophils exhibit a respiratory burst due to activation of a plasma membrane-bound NADPH oxidase that generates superoxide into phagocytic vacuoles and its dismutation product, hydrogen peroxide, to kill bacteria with a concomitant release of MPO [1–4]. Therefore, the MPO-catalyzing bactericidal reaction may occur at neutral pH for the first 15 min after phagocytosis.

The heme enzyme MPO has the capacity to generate an array of oxidizing species with considerable cytotoxic potential such as hypochlorous acid as a major oxidant [1–4,8]. Electronic excited state oxygen, singlet oxygen ($^1O_2$), was originally proposed as the source of chemiluminescence from neutrophils during the respiratory burst through the MPO-catalyzing reaction [9]. However, no evidence for the production of $^1O_2$ was found in the visible light emission produced by activated neutrophils with various kinds of stimulators and $^1O_2$ inhibitors [10,11]. The chemiluminescence emitted from activated neutrophils was confirmed to be derived from tyrosine and bityrosine in excited states but not from $^1O_2$ [12]. Instead of the visible region, the most convincing evidence for $^1O_2$ formation is to detect the near infrared light emission at 1268 nm, which is a characteristic wavelength derived from the $^1\Delta_gO_2$ to $^3\Sigma_g^-O_2$ transition [13]. With this near infrared spectrometry, Kanofsky et al. demonstrated that $^1O_2$ generation mediated by MPO occurred only at very acidic pH with a high H_2O_2 concentration or at a high bromide ion (Br^-) concentration [14]. Based on these results, they concluded that physiological production of $^1O_2$ by an MPO-mediated reaction appeared unlikely [14]. However, considering the sensitivity of the germanium detector used in their measurement and the low quantum yield of light emission at 1268 nm, detection of $^1O_2$ in physiological conditions may not have been achieved. Recently, we have developed a novel detection system consisting of InGaAs/InP pin photodiodes [15,16] for the study of $^1O_2$ produced from enzymatic reactions. In this paper, we show evidence for the physiological production of $^1O_2$ from the MPO-catalyzing reaction at neutral pH by means of our sensitive detecting system. Our results are in contrast to previous results [4,14].

2. Materials and methods

2.1. Materials

Ficoll-Paque and CM Sepharose CL-6B were obtained from Pharmacia LKB Biotech (Uppsala, Sweden); H_2O_2, *o*-methoxyphenol (guaiacol) was from Wako Pure Chemicals (Osaka, Japan); phenylmethylsulfonyl fluoride (PMSF) was from Nakarai Co. (Kyoto, Japan). All other chemicals were of analytical grade.

2.2. Preparation of myeloperoxidase

Granulocytes (more than 90% neutrophils) were obtained from pig blood and destroyed by sonication as reported previously [17,18]. The sonicated cells were fractionated by centrifugation to obtain an azurophil granule-rich pellet, which was then treated with 1 M NaCl at 0°C, and centrifuged to obtain an MPO-rich supernatant [18]. Ion exchange chromatography was performed with CM Sepharose CL-

*Corresponding author. Fax: (81) (23) 647-3108.

Abbreviations: $^1O_2$, singlet oxygen; $^1\Delta_gO_2$, delta singlet oxygen; $^3\Sigma_g^-O_2$, triplet oxygen; MPO, myeloperoxidase; EPO, eosinophil peroxidase

PII: S0014-5793(98)01700-1

6B equilibrated with 50 mM sodium phosphate buffer, pH 7.4. Salting out proteins was accomplished with NaCl in concentrations from 0.2 to 1.0 M in 50 mM sodium phosphate buffer, pH 7.4. Fractions of MPO and eosinophil peroxidase (EPO) were determined by spectrophotometric analysis as described later. Through the chromatographic procedures, the MPO fraction was completely separated from EPO and then dialyzed four times for 2 h with 50 mM sodium phosphate buffer (pH 7.4) for desalting. All procedures were carried out at 4°C.

2.3. Spectrophotometric measurements

Absorption spectra of the samples were measured in amicrocuvette (10 mm light path, 3 mm width) using a Unisoku single-beam spectrophotometer (model USP-530, Unisoku Co. Ltd., Osaka, Japan) interfaced to a personal computer (NEC-PC9801). Peroxidase concentration was determined spectrophotometrically using the absorbance coefficients of the absolute Soret bands: 89 mM^{-1} cm^{-1} at 428 nm for MPO [19] and 110 mM^{-1} cm^{-1} at 412 nm for EPO [20]. Protein concentration in the samples was measured by the Pierce BCA protein assay reagent (Pierce, Rockford, IL, USA). The purity index (A_{430}/A_{280}) of the MPO was also measured. MPO activity was assayed with the guaiacol test described by Chance and Maehly [21]. All spectrophotometric measurements were performed at room temperature (24°C).

2.4. Detection of near infrared emission derived from singlet oxygen

2.4.1. The singlet oxygen detection system. Near infrared light emission derived from $^1\Delta_g O_2$ was measured using our sensitive detection system, the details of which have been reported previously [15,16]. To remove background emission from the total emission, so that a net signal derived from $^1O_2$ can be obtained, our system takes full advantage of a dual charge integrating amplifier (dual-CIA) circuit that employs two InGaAs/InP pin photodiodes [15] (Fig. 1). The mixing unit had a pair of three tube lines for liquid transfer, linking a pair of three reservoirs to both the sample and reference cells [16]. For the detection of light emission from the MPO-mediated reaction, solutions of MPO, H_2O_2 and halide (Cl^- or Br^-) were poured into signal ($^1O_2$) channel reservoirs 1, 2 and 3, respectively. The reference channel reservoirs 4, 5 and 6 contained H_2O_2, the same buffer and the same halide as in the signal channels, as the sources of background radiation. These solutions were synchronously mixed into the sample and reference cells, by using an actuator driving rapidly with nitrogen gas pressure (Fig. 1). After amplification and filtering, the signal output voltage proportional to the intensity of near infrared light emission was integrated and displayed on a digital oscilloscope. Spectral analysis of the signal light was carried out with a set of 13 bandpass filters, centered at wavelengths of 962, 1009, 1070, 1121, 1184, 1225, 1278, 1313, 1354, 1412, 1455, 1521 and 1566 nm, respectively. To quantify the initial rate of signal output power per integration time (mV/s) in units of photons/s, we calibrated the detection system by measuring a signal light at 1.3 μm, transmitted through a single mode optical fiber that was coupled to a power-stabilized semiconductor laser. The laser power was varied to the appropriate level by means of an optical attenuator.

2.4.2. MPO-catalyzed light emission. The reaction mixture consisted of various concentrations of MPO and H_2O_2 in Krebs-Ringer phosphate buffer (KRP: 122 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 17 mM sodium phosphate buffer, pH 7.4). For the examination of halide effects, the reaction mixture consisted of 10 nM MPO,

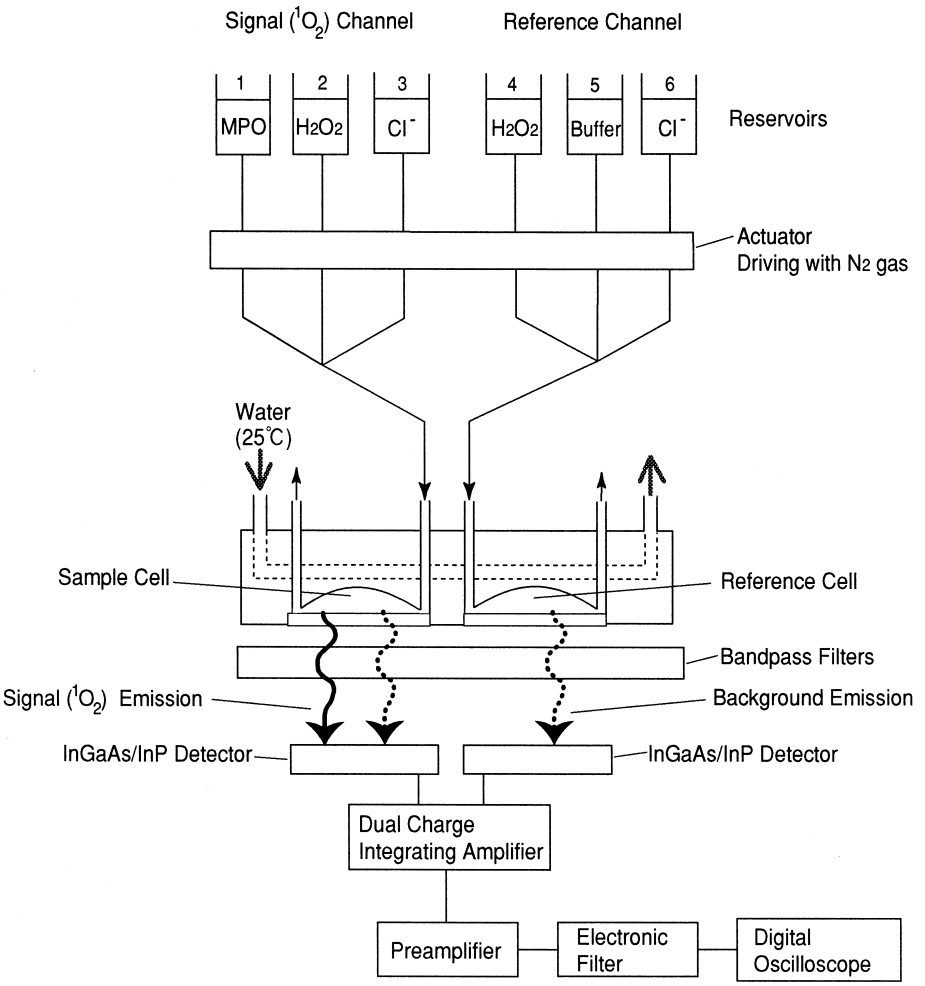

Fig. 1. The $^1O_2$ detection system. The sample mixing unit (upper part) consists of signal and reference channels for light emission from $^1O_2$ in chemical or biological reactions (left) and from the background emission (right). The detection system (lower part) consists of a dual charge integrating amplifier circuit that employs two InGaAs/InP pin photodiodes to detect ultra-weak, near infrared light. For details, see text.

100 μM H_2O_2 and various concentrations of halide ion (Cl^- or Br^-) in 50 mM sodium phosphate buffer (pH 7.4), unless otherwise stated. Prior to the experiment, a fresh H_2O_2 solution was prepared by diluting a commercial 30% solution with H_2O, and the concentration was determined by measuring its absorbance at 240 nm and from the extinction coefficient of 39.4 mM^{-1} cm^{-1} [22]. All MPO-mediated and unmediated reactions were carried out at 25°C in the two rapid mixing cells in the jacketed holder (Fig. 1) connected to a constant temperature water bath.

3. Results and discussion

The $^1O_2$ detecting system (Fig. 1) is so sensitive that a minimal amount of MPO is sufficient to detect the weak, near infrared light emission from MPO-H_2O_2-halide reactions without the use of deuterium oxide. Fig. 2 shows the traces of light emission generated by an MPO-mediated reaction, measured as integrated signal output versus integration time. The signal output depended on the concentration of MPO in the nanomolar range (Fig. 2a–c) under physiological conditions: pH 7.4 and isotonic Cl^- in place of Br^-. Addition of 1 mM histidine as an $^1O_2$ quencher completely inhibited the light emission (Fig. 2d).

Fig. 3A shows the signal output per integration time as a function of MPO concentration in the presence of 100 μM H_2O_2 under the same conditions as in Fig. 2. The light intensity followed a linear function of MPO concentration in the low concentration range of 1.0–30 nM. Next, the signal output per integration time was measured by mixing various concentrations of H_2O_2 with 10 nM MPO under the same physiological conditions as in Fig. 3A (Fig. 3B). The signal output was progressively higher at increasing H_2O_2 concentration, reaching its maximum at 100 μM H_2O_2, before it started to decrease as the H_2O_2 concentration was increased to over 150 μM due to its oxidative inhibition of MPO.

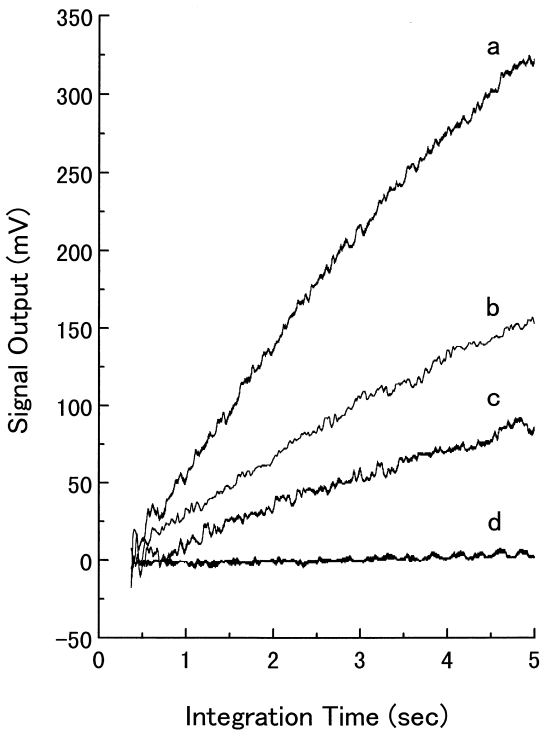

Fig. 2. The time course of near infrared light emission derived from the physiological MPO-H_2O_2-Cl^- system. The signal output voltage proportional to the intensity of near infrared light emission was integrated and displayed on a digital oscilloscope. The reaction was started by rapid mixing of 100 μM H_2O_2, various concentrations of MPO (a; 20 nM, b; 10 nM, c; 5 nM) and KRP buffer (pH 7.4, final 130 mM Cl^-). The bottom trace (d) shows no signal from the reaction mixture of 100 μM H_2O_2, 10 nM MPO and 1 mM histidine in KRP buffer.

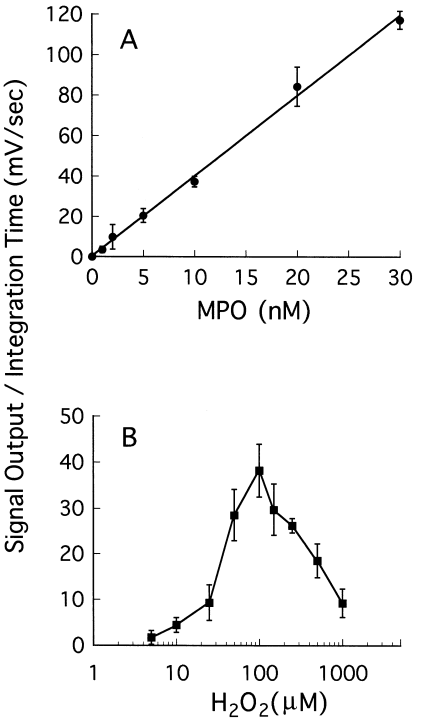

Fig. 3. A: Relationship between integrated signal output and concentration of MPO. The MPO-H_2O_2-Cl^- system under physiological conditions consisted of various concentrations of MPO, 100 μM H_2O_2 and KRP buffer (pH 7.4). B: Relationship between integrated signal output and concentration of H_2O_2. The reaction mixture contained 10 nM MPO, various concentrations of H_2O_2 and KRP buffer (pH 7.4). Values denotes the mean ± S.D. derived from five measurements.

Since the relaxation of $^1\Delta_gO_2$ to the ground state ($^3\Sigma_g^-O_2$) emits light peaking at 1268 nm, possible formation of $^1O_2$ in the MPO-H_2O_2-Cl^- system was examined through spectroscopy measurement in a range of 1000–1500 nm, using bandpass filters. In a parallel experiment, we also analyzed the emission spectrum of $^1\Delta_gO_2$ generated from a chemical model reaction between H_2O_2 and NaOCl [13] (Fig. 4B) to compare with the MPO-mediated reaction. The spectral peaks provide evidence for production of $^1\Delta_gO_2$ from the MPO-H_2O_2-Cl^- system at physiological pH (Fig. 4A).

It has been shown that MPO uses H_2O_2 to oxidize chloride, bromide, iodide and thiocyanate to their respective hypohalous acids [1–4,8]. Therefore, the effects of halide and pseudohalide ions, Cl^-, Br^-, SCN^-, were examined on the light emission from the MPO-H_2O_2-halide system (Table 1). An equivalent light emission (photons/s) occurred in the presence of either 100 mM NaCl or KCl. Br^- is present in plasma at a very low concentration (20–80 μM) [23], in contrast to Cl^-, which is present at a high concentration (100–140 mM) [1]. Therefore, the MPO-H_2O_2-Br^- system was examined in the presence of 100 μM Br^-, a concentration slightly higher than in plasma, which showed no production of $^1O_2$ under physiological conditions. When added at an unphysiological con-

Table 1
Effect of halide and pseudohalide ions on MPO-catalyzed light emission

Halide or pseudohalide ion	Concentration	Signal output/integration time (mV/s)	Photons/s
Cl^-	100 mM	48 ± 3	1.8×10^6
Br^-	100 μM	neg.	neg.
	100 mM	1203 ± 162	3.9×10^7
SCN^-	100 μM	neg.	neg.
	100 mM	neg.	neg.

The reaction mixtures consisted of 10 nM MPO, 100 μM H_2O_2 and various concentrations of halide or pseudohalide ions in 50 mM sodium phosphate buffer, pH 7.4. Data are presented as mean ± S.D. of four measurements. neg., negligible.

centration (100 mM), bromide causes strong light emission in the near infrared region as reported previously [14]. The results indicate that if excess Br^- is present, then the MPO-H_2O_2-Br^- system can produce $^1O_2$ more efficiently than the MPO-H_2O_2-Cl^- system. Next, a pseudohalide, thiocyanate (SCN), was examined on the MPO-mediated light emission because MPO oxidizes SCN^- as well as halide ions, to form hypothiocyanate [3]. Thiocyanate is present at a low concentration (20–120 μM) in plasma [24] and at a relatively high concentration in saliva (1–5 mM) [25]. No emission, however, was detected in either a physiological (100 μM) or an unphysiological (100 mM) concentration under our assay conditions (Table 1). The results suggest that MPO oxidizes Cl^- to OCl^-/HOCl which reacts with H_2O_2 to form $^1O_2$ under physiological conditions.

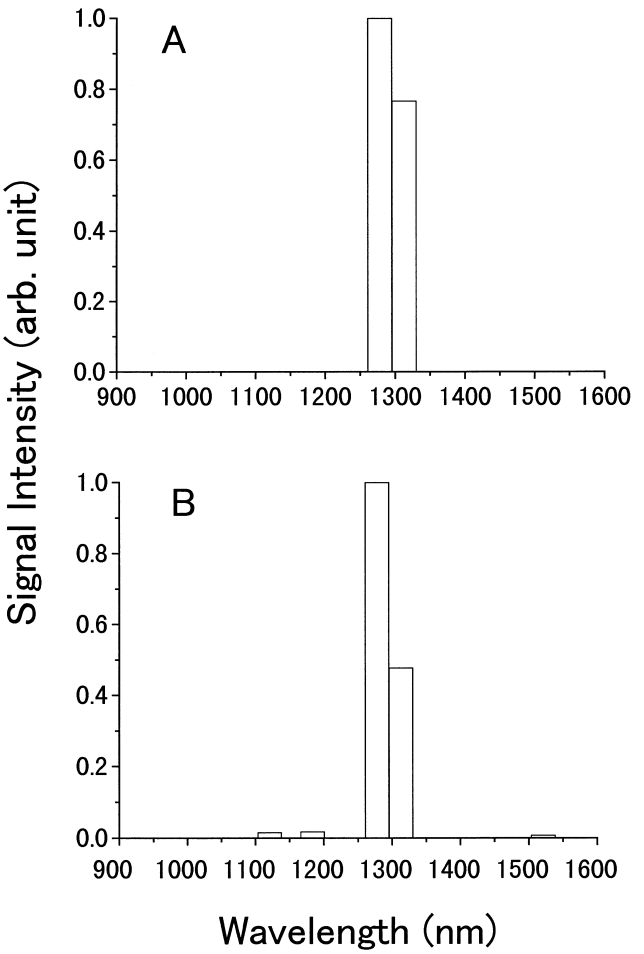

Fig. 4. Spectral analysis of near infrared light emission derived from the MPO-H_2O_2-Cl^- (A) and H_2O_2-NaOCl (B) systems. A: The reaction mixture contained 10 nM MPO, 100 μM H_2O_2 and KRP buffer (pH 7.4). B: The reaction mixture contained 3 μM H_2O_2, 130 μM NaOCl in sodium phosphate buffer (pH 7.4).

Phagocytosing neutrophils consume oxygen about 20–40 nmol/10^7 cells per min and release O_2^- exclusively into phagosomal vacuoles to form H_2O_2 [26]. The H_2O_2 concentration in a vacuole may be more than 100 μM from the space volume of a phagocytic vacuole (10–20 particles per cell in the first 30 s) [26]. Electron micrographs of phagocytosing neutrophils stained for peroxidase showed evidence that ingested bacteria were tightly surrounded by the electron-dense product of MPO on account of the cationic charge of the protein and the anionic charge of the bacterial surface [27]. Therefore in the initial stage of phagocytosis, the MPO-H_2O_2-Cl^- reaction may produce $^1O_2$ extensively at the surface of ingested bacteria to kill them effectively at intravacuolar neutral pH (pH 7.8–7.4). Recently, Nakano's group showed evidence that pure $^1O_2$ has a high ability to kill bacteria despite its very short life time of 3.3 μs, probably due to its direct inhibitory effect on the bacterial electron transport enzyme(s) located in the cell membrane [28,29]. Steinbeck et al. demonstrated intracellular production of $^1O_2$ by phagocytosing neutrophils by using particles coated with a specific chemical trap for $^1O_2$ [30]. Taken together with their reports, the MPO-H_2O_2-Cl^- reaction is probably a source of the $^1O_2$ produced during the process of phagocytosis for a potent bactericidal agent. The consensus has been that if $^1O_2$ is produced in neutrophils, it is only in a very small amount and it may not have the ability to kill bacteria due to its short life time [4,11]. However, from the present results, the role of $^1O_2$ in either neutrophil-mediated host defense mechanism or inflammatory events should be further considered.

Acknowledgements: We are grateful to Dr. M. Nakano of the Japan Immunoresearch Laboratory for personal communications and Dr. K.P. Chan of our institute for the preparation of the manuscript.

References

[1] Klebanoff, S.J. and Clark, R.A. (1978) The Neutrophil: Function and Clinical Disorders, North-Holland, Amsterdam.
[2] Gallin, J.I. and Fauci, A.S. (1982) Advances in Host Defense Mechanisms, Vol. 1, Phagocytic Cells, Raven Press, New York.
[3] Everse, J., Everse, K.E. and Grisham, M.B. (1991) Peroxidases in Chemistry and Biology, Vol. I, CRC Press, Boca Raton, FL.
[4] Kettle, A.J. and Winterbourn, C.C. (1997) Redox Rep. 3, 3–15.
[5] Segal, A.W., Geisow, M., Garcia, R., Harper, A. and Miller, R. (1981) Nature 290, 406–409.
[6] Geisow, M.J., Hart, P.D. and Young, M.R. (1981) J. Cell Biol. 89, 645–652.
[7] Cech, P. and Lehrer, R.I. (1984) Blood 63, 88–95.
[8] Foote, C.S., Goyne, T.E. and Lehrer, R.I. (1983) Nature 301, 715–716.
[9] Allen, R.C., Stjernholm, R.L. and Steele, R.H. (1972) Biochem. Biophys. Res. Commun. 47, 679–684.

[10] Kakinuma, K., Cadenas, E., Boveris, A. and Chance, B. (1979) FEBS Lett. 102, 38–42.
[11] Kanofsky, J.R. (1989) Chem.-Biol. Interact. 70, 1–28.
[12] Ushijima, Y., Totsune, H., Nishida, A. and Nakano, M. (1997) Free Radical Biol. Med. 22, 401–409.
[13] Khan, A.U. (1985) in: Singlet O_2 (Frimer, A.A., Ed.), pp. 39–79, CRC Press, Boca Raton, FL.
[14] Kanofsky, J.R., Wright, J., Miles-Richardson, G.E. and Tauber, A.I. (1984) J. Clin. Invest. 74, 1489–1495.
[15] Makiuchi, M., Miyazaki, J., Shiraishi, T., Kakinuma, K. and Inaba, H. (1997) IEEE Photon. Tech. Lett. 9, 1514–1516.
[16] Makiuchi, M., Kiryu, C., Shiraishi, T., Takeda, M., Miyazaki, J. and Kakinuma, K. (1997) The Proceedings of the 2nd International Conference of Bioradicals, 12–16 October 1997, Yamagata, Yamagata Technopolis Foundation, Yamagata.
[17] Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) J. Biol. Chem. 261, 9426–9432.
[18] Fujii, H. and Kakinuma, K. (1992) Biochim. Biophys. Acta 1136, 239–246.
[19] Odajima, T. and Yamazaki, I. (1970) Biochim. Biophys. Acta 206, 71–77.
[20] Bolscher, B.G.J.M., Plat, H. and Wever, R. (1984) Biochim. Biophys. Acta 784, 177–186.
[21] Chance, B. and Maehly, A.C. (1955) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., Eds.), Vol. 2, pp. 769–773, Academic Press, San Diego, CA.
[22] Nelson, D.P. and Kiesow, L.A. (1972) Anal. Biochem. 49, 474–478.
[23] Holzbecher, J. and Ryan, D.E. (1980) Clin. Biochem. 13, 277–278.
[24] Newman, A.A. (1975) Chemistry and Biochemistry of Thiocyanic Acid and its Derivatives, Academic Press, Orlando, FL.
[25] Grisham, M.B. and Ryan, E.M. (1990) Am. J. Physiol. Cell. Physiol. 258, C115–C121.
[26] Suzuki, H., Kurita, T. and Kakinuma, K. (1982) Blood 60, 446–453.
[27] Klebanoff, S.J. (1970) in: Biochemistry of the Phagocytic Process (Schultz, J., Ed.), pp. 89–110, North-Holland, Amsterdam.
[28] Nakano, M., Kambayashi, Y., Tatsuzawa, H., Komiyama, T. and Fujimori, K. (1998) FEBS Lett. 432, 9–12.
[29] Tatsuzawa, H., Murayama, T., Misawa, N., Fujimori, K., Hori, K., Sano, Y., Kambayashi, Y. and Nakano, M. (1999) FEBS Lett. (in press).
[30] Steinbeck, M.J., Khan, A.U. and Karnovsky, M.J. (1992) J. Biol. Chem. 267, 13425–13433.