Inactivation of bacterial respiratory chain enzymes by singlet oxygen

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Abstract To distinguish the bactericidal action of singlet oxygen ($^1O_2$) from hypohalous acids, wild-type and lycopene transformant *E. coli* strains were exposed to each of the oxidants and then bacterial viability was investigated. $^1O_2$ was generated by chemical and enzymatic systems at pH 4.5. Exposure of wild-type *E. coli* to $^1O_2$ caused a significant loss of *E. coli* viability due to inactivation of membrane respiratory chain enzymes by $^1O_2$. This action of $^1O_2$ could be attenuated by lycopene in the bacterial cell membrane. In the lycopene transformant strain of *E. coli*, inactivation of NADH oxidase and succinate oxidase by hypohalous acids were significantly suppressed, but *E. coli* viability was unaffected. Based on these findings, we suggest that phagocytic leukocytes produce $^1O_2$ as a major bactericidal oxidant in the phagosome.

Key words: Singlet oxygen; 3-(4′-Methyl-1′-naphthyl)propionic acid, 1′,4′-endoperoxide; Bactericidal action; Respiratory chain; Membrane oxidase

1. Introduction

In vivo, the primary role of neutrophils is to destroy pathogenic microorganisms; they respond by producing superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) during a complex process known as the respiratory burst. In 1972, Allen et al. [1] were the first to suggest that neutrophils were likely to produce singlet oxygen ($^1O_2$) during respiratory burst, through reactions catalyzed by myeloperoxidase (MPO). Further, Krinsky suggested that neutrophil derived $^1O_2$ mediates killing of bacteria [2]. Subsequent to this notion, Kanofsky and Tauber [3] reported that neutrophils stimulated under physiological conditions do not produce measurable amounts of $^1O_2$ at pH 7.4.

Until now, the most common technique for detection and measurement of $^1O_2$ has been by near infrared spectrometry using semiconductor based detectors [4,5]. However, because of its low quantum yield, 10^{-6} at best [6], it is not practical to measure small amounts of $^1O_2$ generated in biological systems by near infrared spectrometry. In 1990 Nakano [7], by using a *Cypridina* luciferin analogue (MCLA) as a chemiluminescence probe, reported measuring small amounts of $^1O_2$ generated in an MPO-H_2O_2-halide system. However, even with such a sensitive chemiluminescence method, $^1O_2$ was not detected in the media containing activated leukocytes at pH 7.4 [8]. Since the pH in the phagosomes of activated neutrophils may fall to 4.4–5.7 [9,10], close to optimum pH for MPO activity [11], $^1O_2$ can be efficiently produced in phagosomes, according to the following equations:

$$H_2O_2 + X^- + H^+ \rightarrow HOX + H_2O \quad (1)$$

$$H_2O_2 + HOX \rightarrow {}^1O_2 + HX + H_2O. \quad (2)$$

On the basis of the halide ion concentration in the blood [12], X^- in reaction 1 should predominantly be Cl^- rather than Br^- [13].

In our previous study [14], $^1O_2$ generated by thermal decomposition of a novel water-soluble naphthalene endoperoxide, 3-(4′-methyl-1′-naphthyl)propionic acid, 1′,4′-endoperoxide (NEPO), killed *E. coli* rapidly at pH 4.5 or pH 7.2. In that study, we verified that bacterial killing was via $^1O_2$ and not by a direct action of NEPO. Such $^1O_2$-mediated bacterial killing was significantly attenuated by lycopene in the lycopene-producing strain of *E. coli*.

The present work was undertaken to investigate the mechanism of bactericidal action of $^1O_2$ using a wild-type and a lycopene-producing *E. coli* strain. The $^1O_2$ was generated either in an enzymatic system similar to the neutrophil phagosome or in a chemical system using NEPO.

2. Materials and methods

2.1. Preparation of NEPO

A sample of NEPO was provided to us by Prof. T. Nagano, Tokyo University. Additional amounts of NEPO were synthesized in our laboratory according to the method described by Saito et al. [15]. NEPO was stored at −80°C prior to experiments. Before use, NEPO was dissolved in ice-cold ethanol and kept at 0°C. Thermolytic decomposition of NEPO gives equimolar yields of $^1O_2$ and 3-(4′-methyl-1′-naphthyl)propionic acid (NPA) [15].

2.2. Estimation of total $^1O_2$ generated by enzymatic and chemical systems

The reaction mixture for the enzymatic $^1O_2$ generation contained 0.0125 units/ml MPO, 5 mM KBr, 20 μM desferrioxamine, 0.5 mM H_2O_2, 15 μM MCLA, 0.5 μM SOD and 0.1 M acetate buffer at pH 4.5 in a total volume of 2 ml. MPO activity was assayed and calculated as described by Klebanoff et al. [13]. The reaction was initiated by addition of MPO and MCLA dependent luminescence was measured in a luminescence reader (Type BLR-301; Aloka, Japan) at 25°C.

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Abbreviations: CLI, chemiluminescence intensity; cpm, count/min; ETPs, electron transport particles; H_2O_2, hydrogen peroxide; HOBr, hypobromous acid; HOCl, hypochlorous acid; LT, lycopene transformant; $^1O_2$, singlet oxygen; $O_2^{\bullet-}$, superoxide anion; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one; MPO, myeloperoxidase; NEPO, 3-(4′-methyl-1′-naphthyl)propionic acid, 1′,4′-endoperoxide; NPA, 3-(4′-methyl-1′-naphthyl)propionic acid; SOD, superoxide dismutase; ATP, adenosine triphosphate

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The reaction mixture for chemical $^1O_2$ generating system (NEPO) contained 1 mM NEPO, 20 μM desferrioxamine, 15 μM MCLA, 0.5 μM SOD and 0.1 M acetate buffer at pH 4.5 in a total volume of 2 ml. The reaction was initiated by adding 100 μl ice-cold ethanol solution of NEPO and MCLA-dependent luminescence was measured at 37°C as above. In both MPO and NEPO systems, $^1O_2$ generated during the time indicated was calculated from MCLA-dependent light yield using H_2O_2-HOCl as a standard $^1O_2$ generating system [7]. Almost 100% of $^1O_2$ generated in NEPO system reacts with MCLA to cause photon emission at 465 nm [20].

2.3. Viability test

E. coli transformants carrying the lycopene producing genes (pACCCRT-EIB) [16,17] were used. The amount of lycopene in the transformant was estimated to be about 0.02 fmol/cell. Wild-type *E. coli* and lycopene-bearing *E. coli* (LT-*E. coli*) were cultured in LB medium [18] containing ampicillin (150 μg/ml) and/or chloramphenicol (30 μg/ml) at 27°C with shaking (80 shakes/min) in test tubes. Growth was monitored by measuring the optical density at 600 nm. Cells at an early stationary phase were harvested, washed with and suspended in the minimal medium [19] without vitamin B_{12}. The cell suspensions were dispensed in polypropylene vials (∅ 17×51 mm) and mixed with the $^1O_2$ generating medium in which MCLA was excluded or 10 μM HOBr (or HOCl) in 0.1 M acetate buffer at pH 4.5. The final cell density in the reaction mixture was adjusted to 108 cells/ml. Tests were incubated for 3 min at 25°C in the MPO system, in the HOBr (or HOCl) system or at 37°C in the NEPO system. Then the test specimens were washed and the viable cells were counted by spreading in triplicate on LB agar plate after appropriate dilutions. Colonies were counted after 24 h incubation at 37°C under aerobic incubation.

2.4. Preparation of electron transport particles

Electron transport particles (ETPs) from *E. coli* with or without exposure to $^1O_2$ or other oxidant were prepared as described by Rakita et al. [21]. In brief, *E. coli* were pelleted, washed with 30 mM Tris-HCl buffer at pH 8.0 and used immediately or stored at −70°C. Pellets were suspended in 30 mM Tris-HCl buffer, pH 8.0, and sonicated for 30 min with cooling in an ice bath. Unbroken bacteria were removed by centrifugation at 10 000×*g* for 10 min at 4°C. ETPs were collected by centrifugation at 48 000×*g* for 30 min.

2.5. Estimation of oxidase activity in ETPs

ETPs were suspended in 30 mM Tris-HCl buffer, pH 8.0, at a protein concentration of 0.05 mg/ml, and oxygen consumption was determined with a Clark-type oxygen electrode following addition of 31 mM sodium succinate, 62 mM DL-lactate, 62 mM glycerol-3-phosphate, or 0.94 mM NADH in Tris-HCl buffer, pH 8.0. Results are expressed as nmol O_2 consumed per min per mg protein on the basis of 215 μM O_2 in air-saturated buffer at 37°C.

2.6. Determination of ATP formed from ADP by ETPs

The reaction mixture was essentially the same as previously described (Section 2.5), except that 0.365 mM ADP and a kit of luciferin-luciferase containing Mg^{2+} (Lot 3971017, Kikkoman, Chiba, Japan) were included. The reaction was initiated by the addition of ADP without supplementing inorganic phosphate and continued at 37°C. At five minutes after the initiation of the reaction, the luciferin-luciferase agent (0.1 ml) was injected to the reaction mixture and the brief intense emission was recorded with a luminescence reader. With ATP at known concentration, the intensity of the emission originating from the luciferin-luciferase-ATP reaction was also detected with the luminescence reader and used for the quantification of ATP formed in the experimental systems. Results are expressed as pmol of ATP formed per min per mg protein. With ETPs obtained from wild-type or LT-*E. coli*, ATP formed in the presence of NADH or succinate was detected. O_2 consumption was estimated with the same ETPs as previously described (Section 2.5). The data obtained were used for the calculation of pmol of ATP formed per nmol of O_2 consumed per min per mg protein. This can provide the following values: 4.15 pmol for NADH oxidation and 2.30 pmol for succinate oxidation using ETPs of wild type *E. coli*, 16.65 pmol for NADH oxidation and 4.10 pmol for succinate oxidation using ETPs of lycopene transformant *E. coli*.

2.7. Statistical analysis

Data are expressed as mean ± S.E.M. for *E. coli* viability and the oxidase activities. Mann-Whitney's *U*-test was used to compare viability of wild-type *E. coli* with that of LT-*E. coli* and to compare activities of oxidases with and without exposure to HOBr, HOCl or $^1O_2$. $P < 0.05$ was considered significant.

3. Results and discussion

Fig. 1 shows the time course of $^1O_2$ generation in the MPO-H_2O_2-Br^- system (the MPO system) and NEPO system at pH 4.5. Even though the $^1O_2$ generation profiles were quite different, the total amounts of $^1O_2$ generated in the two systems were essentially the same. Nonetheless, the viability of *E. coli* in the MPO system, which could produce $^1O_2$ at a very rapid rate, was about ten times lower than that in the NEPO system (Fig. 2). The results suggest that MPO, a basic protein, can readily adhere to the bacterial membrane [22], where $^1O_2$ can be efficiently generated and promote membrane damage. Additionally, in the MPO-H_2O_2-halide system, we could assume bacterial killing by oxidants other than $^1O_2$ as well. The results of experimental investigations carried out on the basis of

Table 1
Effect of HOBr or $^1O_2$, generated in the myeloperoxidase-H_2O_2-Br^- system (MPO) or in the NEPO system, on *E. coli* oxidase activities and ATP formation[a]

Type of *E. coli*	Substrate of oxidase	Oxidase activity (% of control)[b] [ATP formed][c]			
		Control	HOBr	MPO	NEPO
Wild type *E. coli*	Succinate	235 ± 4 [540.5]	93 ± 1* (40 ± 0) [234.0]	16 ± 0* (7 ± 0) [36.8]	35 ± 3* (15 ± 1) [80.5]
	NADH	209 ± 1 [867.0]	47 ± 3* (23 ± 1) [195.0]	23 ± 1* (11 ± 0) [95.5]	30 ± 2* (14 ± 1) [124.5]
	DL-Lactate	118 ± 17	28 ± 4* (23 ± 3)	21 ± 1* (18 ± 0)	29 ± 1* (25 ± 1)
	Glycerol-3-phosphate	51 ± 2	36 ± 2* (70 ± 3)	17 ± 1* (33 ± 1)	20 ± 0* (40 ± 0)
Lycopene transformant *E. coli*	Succinate	141 ± 3 [578.0]	148 ± 2 (105 ± 1) [607.0]	41 ± 1* (29 ± 1) [168.0]	24 ± 1* (17 ± 0) [98.5]
	NADH	42 ± 2 [695.0]	41 ± 1 (96 ± 3) [678.5]	24 ± 0* (56 ± 1) [397.0]	21 ± 0* (50 ± 1) [347.5]
	DL-Lactate	69 ± 6	26 ± 4* (38 ± 6)	30 ± 1* (44 ± 1)	17 ± 1* (24 ± 1)
	Glycerol-3-phosphate	29 ± 0	19 ± 1* (67 ± 2)	22 ± 1* (78 ± 2)	20 ± 0* (71 ± 1)

[a]*E. coli* were exposed to oxidants under the experimental condition indicated in the legend to Fig. 2.
[b]The oxidase activity of electron transport particles prepared from *E. coli* untreated or treated with the oxidants were measured with substrates indicated. The oxidase activity was expressed as nmol of O_2 consumed per min per mg protein. All measurements were repeated six times. *$P < 0.005$ vs. control.
[c]The formation of ATP from ADP was expressed as pmol of ATP formed per nmol of O_2 consumed per min per mg protein, described in Section 2.7. This value was multiplied by the oxidase activity to obtain the amount of ATP formed.

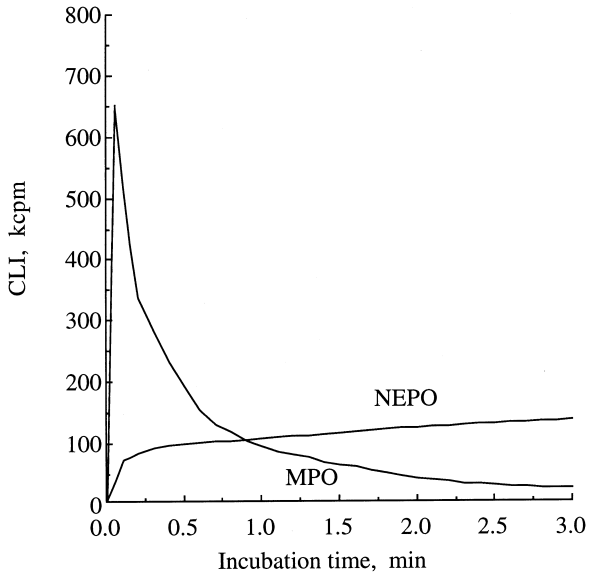

Fig. 1. MCLA-dependent chemiluminescence in different $^1O_2$ generating systems (MPO and NEPO). MPO: MPO-H_2O_2-Br^- system; NEPO: the chemical $^1O_2$ generation system (the naphthalene endoperoxide containing medium); CLI, chemiluminescence intensity; cpm, count/min.

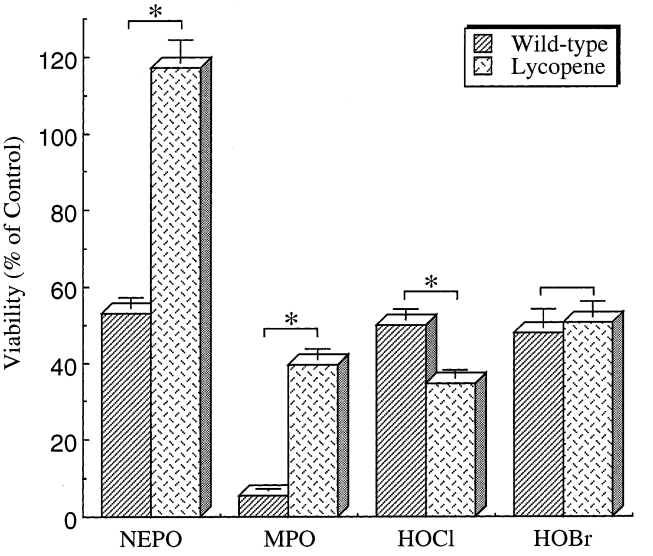

Fig. 2. Viability of wild-type and lycopene-producing strains of *E. coli* exposed to different oxidants at pH 4.5. *E. coli* were exposed to 10 μM $^1O_2$ generated in the MPO-H_2O_2-Br^- system (MPO) or by thermolytical decomposition of a naphthalene endoperoxide (NEPO) for 3 min (see Fig. 1). These *E. coli* strains were also exposed to 10 μM HOCl or HOBr for 3 min. Viability of control corresponds to untreated *E. coli*. Data represent the mean ± S.E.M. ($n = 12$). *$P < 0.05$.

the above assumption are shown in Fig. 2. The viability of *E. coli* in 10 μM HOCl or 10 μM HOBr containing system was about 50%, approximately the same as when exposed to $^1O_2$ in the NEPO system (a pure $^1O_2$ generating system).

In the MPO system excluding KBr, in which compound 1 (ferryl type of MPO), a powerful oxidant for tyrosine and tyrosyl residues in proteins could be formed [23,24], the *E. coli* viability was 80% (data not shown). Similar to the system without KBr, 0.5 mM H_2O_2, 1 mM NPA, or 5% ethanol, did not have any effect on *E. coli* viability during such a short exposure, suggesting that MPO-H_2O_2-Br^- system is highly involved in bacterial killing. In the experiments with LT-*E. coli*, lycopene significantly attenuated the loss of *E. coli* viability in the MPO or NEPO system, but did not affect bactericidal action of HOBr; rather it enhanced the bactericidal action of HOCl (Fig. 2). The results suggest that in the neutrophil phagosome, HOCl or HOBr and $^1O_2$ are powerful bactericidal oxidants and only the action of $^1O_2$ can be attenuated by lycopene in *E. coli*. When *E. coli* at a density of 10^8 cells/ml in a reaction mixture at pH 4.5 were exposed to 10 μM $^1O_2$ in the MPO system for 3 min and their membrane phospholipid hydroperoxides were analyzed by chemiluminescence-based HPLC method [25], no detectable lipid hydroperoxides (< 0.57 pmol/10^9 cells) in the wild-type or in the LT-*E. coli* was observed (data not shown). This indicated that a short time exposure to $^1O_2$ would not be sufficient to cause structural damage to the cell membrane phospholipids.

Respiratory loss in *E. coli* that occurs as a consequence of oxidant insult by HOCl [26,27] or by the MPO-H_2O_2-Cl^- system [28] has been shown to coincide, more or less, with loss of cell viability. The oxidation of bacterial cytochromes by HOCl [29] or both destruction of iron-sulfur centers [30] and inactivation of succinate oxidase [20] by MPO-H_2O_2-Cl^- system is also associated with the *E. coli* killing activity. Therefore, it should be expected that the membrane electron-transport chain associated with the iron-sulfur centers, which require NADH and succinate as electron donors, are very susceptible to HOCl or MPO-mediated inactivation.

Table 2
Effect of HOCl on *E. coli* oxidase activities and ATP formation

E. coli strain	Substrate of oxidase	Oxidase activity (% of control) [ATP formed]	
		Control	HOCl
Wild type	Succinate	335 ± 6 [524]	179 ± 2* (54 ± 1) [411]
	NADH	432 ± 17 [1795]	223 ± 16* (52 ± 4) [925]
	DL-Lactate	112 ± 4	82 ± 3* (73 ± 3)
	Glycerol-3-phosphate	29 ± 3	17 ± 2* (57 ± 8)
Lycopene transformant	Succinate	169 ± 10 [695]	151 ± 3 (90 ± 2) [619]
	NADH	101 ± 11 [1670]	98 ± 16 (96 ± 16) [1589]
	DL-Lactate	62 ± 7	45 ± 3* (72 ± 4)
	Glycerol-3-phosphate	34 ± 3	20 ± 2* (58 ± 5)

HOCl exposed to *E. coli* (a different strain from *E. coli* used for HOBr exposure) under the same conditions as described in the legend of Table 1. Oxidase activity and [ATP formed] were measured as described for Table 1. All experiments for the detection of oxidase activity were repeated three times. Oxidase activity is expressed as nmol O_2/min/mg protein. *$P < 0.05$ vs. control.

Hence, we exposed *E. coli* to HOBr, $^1O_2$ in the NEPO system or MPO system and subcellular electron transport particles (ETPs) were prepared from treated and untreated bacteria to determine oxygen consumption as a measure of oxidase activities with succinate, lactate, NADH and glycerol-3-phosphate. In the same ETPs, synthesis of ATP from ADP was also examined using NADH or succinate as an electron donor to the electron transport system. The electron transport system is known to be located in a phospholipid-rich cytoplasmic membrane of *E. coli* [31]. It should be pointed out that the activities of carotenoid producing enzymes depend on binding to the membrane and therefore the carotenoids which they produce are concentrated in the cell membrane where they can protect the membrane against oxidant insults.

As shown in Table 1, the results seen following exposure of wild-type *E. coli* to HOBr were similar to those in NEPO system, with respect to inactivation of four respiratory enzymes. Inactivation of succinate or NADH oxidase in HOBr containing system was much weaker than in the MPO or NEPO system. Under our experimental conditions, ATP formed from ADP was very low compared with O_2 consumption. However, ATP values can be used as an index of energy production. Sum of ATP formation for succinate oxidation and for NADH oxidation (energy production) in wild-type and LT-*E. coli* were 1407.5 and 1273.0, respectively, which are not significantly different. Thus, one can compare energy production of wild-type *E. coli* with that of LT-*E. coli*, before and after exposure to oxidants. In the HOBr exposure, energy production by wild-type *E. coli* was suppressed by 30.0%, while that of LT-*E. coli* was unaffected. HOCl also produced toxic effects on the membrane electron transport system in *E. coli* (Table 2). In the MPO system, *E. coli* energy production was suppressed by 9.4% which was reversed by up to 44% in the presence of lycopene. Similarly, energy production by wild-type *E. coli* in the NEPO system was suppressed by 14.6% which was also reversed by lycopene by up to 35%. In contrast to the HOBr or HOCl exposure, the protection of bacterial energy production by lycopene, which could be seen in the MPO or NEPO system, directly reflects bacterial viability (Fig. 2). HOBr or HOCl can readily penetrate microbial cytoplasmic membrane and react with lycopene [28]. As a result, the membrane enzymes (NADH oxidase and succinate oxidase) are spared. However, the protective effect of lycopene on energy production calculated by O_2 consumption did not elevate microbial viability, suggesting that HOBr or HOCl also blocked other sequences of events involved in ATP synthesis.

Considering $^1O_2$, this short lived oxidant may also interact with the microbial cytoplasmic membrane and damage the respiratory enzymes together with suppression of ATP formation. Lycopene quenches $^1O_2$ thereby protecting the respiratory enzymes and ATP production.

Since reaction of H_2O_2 with HOBr is faster than its reaction with HOCl [32], the H_2O_2+HOBr reaction is more likely to compete with other reactions consuming HOBr or H_2O_2. Thus, our MPO system can be used as a pure $^1O_2$ generating system. We found that a substantial amount of $^1O_2$ could be generated in an MPO-H_2O_2-Cl^- system in which inactivation of MPO and H_2O_2 generating enzyme used [21] might occur (data not presented).

In this study, we have used an MCLA dependent luminescence method to detect the production of $^1O_2$ by the neutrophil enzyme, MPO, in the presence of Br^- and H_2O_2 at pH 4.5. Nearly 100% of $^1O_2$ generated by thermolytic decomposition of NEPO reacts with MCLA to emit light [20]. Its bactericidal action is attributable to inactivation of bacterial respiratory enzymes. Since MPO and Cl^- are present at sufficiently high concentration within the neutrophil phagosome, activated neutrophils do produce $^1O_2$ and HOCl as efficient bactericidal agents. Albrich et al. [29] have postulated that the protective effect of β-carotene on the bactericidal action of the MPO-H_2O_2-Cl^- system is attributable not to its ability to quench $^1O_2$, but to a rapid scavenging of HOCl in bacterial cytoplasmic membrane, thereby preventing loss of essential bacterial function. Judging from the protective effect of carotenoid on bacterial killing in neutrophils [2], similar to our MPO system or the NEPO system, but not to the HOCl or HOBr containing system, we suggest that $^1O_2$ is generated in the neutrophil phagosome and is a major oxidant in bacterial killing by neutrophils.

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