Mechanism of oxidative DNA damage induction in a strict anaerobe, *Prevotella melaninogenica*

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Abstract

We investigated the mechanism of the oxidative DNA damage induction by exposure to O2 in *Prevotella melaninogenica*, a strict anaerobe. Flow cytometry with hydroethidine and dichlorofluorescein diacetate showed that O2 exposure generated O$_3^-$ and H$_2$O$_2$. Results of electron spin resonance with K-(4-pyridyl-1-oxide)-N-tert-butylnitrone and ethanol showed that O$_2$ exposure also induced OH radical generation in *P. melaninogenica* loaded with FeCl$_2$ but not in samples without FeCl$_2$ loading. In *P. melaninogenica*, O$_2$ exposure increased 8-hydroxydeoxyguanosine (8OHdG), typical of oxidative DNA damage. Catalase inhibited the increase, but the OH radical scavengers did not. Phenanthroline, a membrane-permeable Fe and Cu chelator, increased the 8OHdG induction. In FeCl$_2$-loaded samples, induction of 8OHdG decreased. Addition of H$_2$O$_2$ markedly increased 8OHdG levels. These results indicate that in *P. melaninogenica*, exposure to O$_2$ generated and accumulated O$_3^-$ and H$_2$O$_2$, and that a crypto-OH radical generated through H$_2$O$_2$ was the active species in the 8OHdG induction. ß 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Unable to survive under normal atmospheric conditions, anaerobes are susceptible to oxygen. Several hypotheses have been proposed to account for this susceptibility, such as the generation of reactive oxygen species (ROS) in anaerobes by O$_2$, a lack of ROS scavenging enzymes, and a perturbation of the oxidation–reduction potential in the culture media by O$_2$ [1]. No reports, however, have clarified the mechanism of O$_2$ damage, nor has evidence been presented to show that ROS are generated in anaerobes after exposure to O$_2$. We recently reported for *Prevotella melaninogenica* generation of 8-hydroxydeoxyguanosine (8OHdG) and decreased survival after brief exposure to O$_2$ [2]. Reports indicate that 8OHdG is typical of oxidative DNA damage and that it is induced by ROS [3–5], suggesting that ROS are generated in *P. melaninogenica* after exposure to O$_2$. Oxidative DNA damage, especially 8OHdG, is reported to be highly mutagenic [6]. Study of the mechanism of oxidative DNA damage induction in *P. melaninogenica* may both clarify the response of the anaerobe to O$_2$ and provide useful information about the role that O$_2$ might play in molecular evolution.

Here we report how, using flow cytometry and electron spin resonance spectroscopy (ESR) techniques, we found evidence that exposure to O$_2$ generates ROS in *P. melaninogenica*, and discuss the mechanism of 8OHdG induction in the bacterium.

2. Materials and methods

2.1. Reagents

We obtained α-phenyl-N-tert-butylnitrone (PBN) and α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN) from Labotec, Inc. (Tokyo, Japan); dichlorofluorescein diacetate (DCFH-DA) from Molecular Probes, Inc. (USA); hydroethidine (HE) from Polysciences, Inc. (USA); catalase from Boehringer-Mannheim GmbH (Germany); superoxide dismutase (SOD) from Wako Pure Chemical, Inc. (Japan); phenanthroline and bathocuprine from Do-
jindo Laboratories (Japan); diethylenetriaminepentaacetic acid (DTPA) from Sigma Chemical Co. (USA).

2.2. Strains and growth conditions

For comparative purposes, P. melaninogenica GAI5490 and Bacteroides fragilis ATCC25285 were used as examples of a strict anaerobe and that to some extent aerotolerant; preparation of bacterial cells was carried out as described elsewhere [2].

2.3. Fe loading

At 37°C for 30 min under anaerobic conditions, bacteria were incubated with FeCl₂ (0.1 mM or 1 mM), then similar concentrations of DTPA were added just before O₂ exposure.

2.4. Exposure to oxygen

Exposure to O₂ was carried out as follows: in 1.5-ml tubes [15-ml tubes, conditions in the square brackets were used for 8OHdG evaluation], 0.1-ml [1 ml] samples of bacterial cell suspension were exposed to O₂ by bubbling O₂ gas at 100 ml min⁻¹ for 30 s through a hematocrit capillary [1-ml plastic pipette] with the opening placed at the bottom of the tube. After this, the tubes containing the samples were tightly sealed, and the samples were incubated at 37°C. In the samples with phenanthroline (1 mM), bathocuproine (10 μM), PBN (5 mM), dimethylsulfoxide (0.5%, DMSO), catalase (1000 U ml⁻¹), or SOD (300 U ml⁻¹), each reagent was added just before O₂ exposure.

2.5. Determination of ROS

We used flow cytometry with DCFH-DA or HE to evaluate the intracellular presence of H₂O₂ or O₂⁻⁻. Bacterial cells were incubated with 10 μM DCFH-DA or 10 μM HE at 37°C for 15 min under anaerobic conditions and exposed to O₂ as described above. Exposed cells were incubated at 37°C for 30 min, then diluted 5000 times with Dulbecco’s phosphate-buffered saline (DPBS) and analyzed by FACS Caliber using Cell Quest software (Becton Dickinson, USA).

We investigated the presence of the *OH radical using ESR with POBN and ethanol. POBN (10 mM) and ethanol (1%) were anaerobically added to bacterial cells, then the cells were exposed to O₂ as described above and incubated at 37°C for 1 h. After this, spin adducts were analyzed using an ESP380 ESR spectrometer (Brucker, Germany). ESR conditions were as follows: receiver gain 2.5×10⁵ (POBN), modulation frequency 100 GHz, modulation amplitude 0.8 G, center field 3480 G, microwave power 19 mW.

The rationale for the detection of H₂O₂ and O₂⁻⁻ by flow cytometry [7,8], and the *OH radical by ESR [9] has been described elsewhere.

2.6. Evaluation of 8OHdG

After exposure to O₂, bacterial samples were washed twice with oxygen-free DPBS, and stored as cell pellets at −80°C. The amount of 8OHdG in the cells was evaluated as described elsewhere [10]. The amounts of deoxyguanosine (dG) in these samples were also determined, after which the molar ratio of 8OHdG per 10⁵ dG was calculated. Data are presented as mean ± S.E.M. values from 2–13 independent experiments conducted in duplicate or triplicate.

2.7. Determination of enzyme activities

After extraction of the enzyme from bacterial cells by sonication, catalase and SOD activities were determined as described elsewhere [11]. Data are presented as mean ± S.E.M. values obtained from four samples.

2.8. Other procedures

Using bovine serum albumin to set standard values, we assessed the protein concentration in the sonicates with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Inc., Japan).

3. Results

3.1. Generation of ROS

In P. melaninogenica, exposure to O₂ increased both dichlorofluorescein (DCF, Fig. 1A) and ethidium (Fig. 1B) fluorescence, indicating that both H₂O₂ and O₂⁻⁻ had been generated in the bacterium. Addition of catalase decreased DCF fluorescence (Fig. 1A), but SOD had little effect on ethidium fluorescence (Fig. 1B). In B. fragilis, however, exposure to O₂ had little effect on DCF or ethidium fluorescence (Fig. 1C,D). Addition of up to 0.2 mM H₂O₂ to P. melaninogenica increased DCF fluorescence (Fig. 2).

In samples of P. melaninogenica loaded with FeCl₂, exposure to O₂ generated a POBN-CH(CH₃)OH spin adduct (a² = 15.59 G, d = 2.68 G) (Fig. 3A-3,B-2) indicative of *OH radical formation. This signal derived from the presence of the *OH radical disappeared with the addition of catalase (Fig. 3A-4), but was unaffected by SOD (Fig. 3A-5). By contrast, in samples of P. melaninogenica that were not loaded with FeCl₂, exposure to O₂ did not generate signals indicating the presence of the *OH radical (Fig. 3A-2). Neither were *OH radical-derived signals generated when FeCl₂ and DTPA were added simultaneously to P. melaninogenica (data not shown) nor in samples
containing an equivalent concentration of FeCl₂ and DTPA (Fig. 3A-6) in which *P. melaninogenica* was not present. Similarly, signals were not detected in samples that had not been exposed to O₂ (Fig. 3A-1,B-1). In samples of *B. fragilis* that had been loaded with FeCl₂, exposure to O₂ did not generate an OH radical-derived signal (Fig. 3B-3).

### 3.2. Enzyme activities

Whereas there was no evidence of SOD or catalase activities in *P. melaninogenica*, we did detect SOD (7.1 ± 0.9 U mg⁻¹ protein) and catalase (61.6 ± 5.8 U mg⁻¹ protein) activities in *B. fragilis*.

### 3.3. Induction of 8OHdG

In *P. melaninogenica*, exposure to O₂ increased the amount of 8OHdG (Fig. 4A-2). Addition of catalase inhibited the increase (Fig. 4B-1), but SOD had no effect (Fig. 4B-2). The presence of phenanthroline enhanced 8OHdG induction by O₂ exposure (Fig. 4B-3). In samples that received phenanthroline, the bacterial color changed to red (data not shown), indicating, subsequent to its entry into cells, chelation of the Fe in the cells. Bathocuproine did not affect 8OHdG induction (Fig. 4B-4). Addition of FeCl₂ (data not shown) or loading of FeCl₂ with the subsequent addition of the equivalent concentration of DTPA decreased 8OHdG induction (Fig. 4B-5). DTPA alone did not decrease 8OHdG induction (data not shown). PBN or DMSO (data not shown) had no effects on 8OHdG induction (Fig. 4B-6). Exposure to O₂ had little effect on 8OHdG in *B. fragilis* (Fig. 4A-5).

In *P. melaninogenica*, the addition of H₂O₂ markedly increased 8OHdG induction (Fig. 4A-3).

### 4. Discussion

Exposure to O₂ increased DCF fluorescence in *P. melaninogenica*, which indicates that H₂O₂ was generated in the cells [7,8]. The decrease in this fluorescence in the presence of catalase is also indicative of H₂O₂ generation. Extracellular catalase may be effective because of the high membrane permeability of H₂O₂ [12]. The increase of DCF fluorescence in *P. melaninogenica* was dependent on the concentration of H₂O₂.

We confirmed, with ESR using POBN as a membrane-permeable spin trapper, the generation of H₂O₂ in the bacterium after exposure to O₂ [9]. By itself, H₂O₂ does not generate spin adducts, however, in the presence of FeCl₂ it decomposes to the *OH radical [13] that reacts with ethanol and generates POBN-CH(CH₃)OH [9]. No spin adducts were found in *P. melaninogenica* after exposure to O₂ alone. This finding suggests that O₂ exposure by itself did not generate the *OH radical. After the addition of or loading with FeCl₂, however, exposure to O₂ did generate the spin adduct. Concurrent addition of equivalent concentrations of FeCl₂ and DTPA did not generate the spin adduct. These findings indicate the intracellular generation of H₂O₂, which was decomposed to *OH radical by intracellular unchelated FeCl₂. When cat-
alase is present, non-generation of the spin adduct corroborates the generation of H$_2$O$_2$ in the other samples.

In *P. melaninogenica*, exposure to O$_2$ also increased ethidium fluorescence, which indicates the generation of O$_{2}^{-}$ [7,8]. Because O$_{2}^{-}$ is impermeable to bacterial membranes [14,15], addition of SOD to the medium had little effect on the fluorescence.

These findings confirm that H$_2$O$_2$ and O$_{2}^{-}$ are generated in *P. melaninogenica* after exposure to O$_2$. We believe that this is the first definite evidence that indicates the generation of ROS in anaerobes after exposure to O$_2$. In contrast to *P. melaninogenica*, we could not detect ROS generation in *B. fragilis* after O$_2$ exposure. This may be because *B. fragilis* has both catalase and SOD activities: the generated ROS might be rapidly removed by these enzymes in the conditions under which the experiment was conducted.

Exposure of *P. melaninogenica* solely to O$_2$ presented no evidence of *OH radical generation, but it did increase 8OHdG and this increase was inhibited by a H$_2$O$_2$ scavenger, catalase [2]. Membrane-permeable *OH radical scavengers [16] such as PBN or DMSO did not inhibit the increase. Furthermore, samples to which FeCl$_2$ had been added or had been loaded with it generated the *OH radical with inhibition rather than enhancement of 8OHdG induction. On the other hand, the membrane-permeable Fe and Cu chelator phenanthroline [17,18], which may reduce both the decomposition of H$_2$O$_2$ and the generation of *OH radical [18,19], enhanced 8OHdG induction. Deferoxamine (1 mM) had little effect on the 8OHdG induction due to O$_2$ exposure (data not shown), but we could not tell whether the deferoxamine had entered the bacterium. The anaerobic addition of H$_2$O$_2$ to *P. melaninogenica* markedly induced 8OHdG [2].

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**Fig. 3.** A: ESR spectra of *P. melaninogenica*. Samples of *P. melaninogenica* that had or had not been loaded with FeCl$_2$ (0.1 mM), after addition of DTPA (0.1 mM), were exposed (2–6) or not exposed (1) to O$_2$ for 30 s. Bacteria were incubated at 37°C for 1 h in the presence of POBN and ethanol, and then analyzed by ESR as described in Section 2. (1) Loaded with FeCl$_2$; (2) not loaded with FeCl$_2$; (3) loaded with FeCl$_2$; (4) loaded with FeCl$_2$ in the presence of catalase; (5) in the presence of SOD; (6) same as (3) without *P. melaninogenica*. B: ESR spectra of *P. melaninogenica* (1, 2) or *B. fragilis* (3). Samples of these two bacteria were loaded with FeCl$_2$ (0.1 mM) and, after addition of DTPA (0.1 mM), were exposed to O$_2$ for 30 s. After incubation at 37°C for 1 h in the presence of POBN and ethanol, the samples were analyzed by ESR as described in Section 2. (1) Not exposed to O$_2$; (2, 3) exposed to O$_2$. 

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ica, signals derived from OH radical generation were not detected in samples with phenanthroline or H₂O₂ (data not shown). These findings indicate an intimate relationship between H₂O₂ and 8OHdG induction, but not between *OH radical generation and 8OHdG induction.

On the other hand, bearing in mind reports that H₂O₂ is not reactive enough to induce 8OHdG [11,13,20^22], a crypto-OH radical may be responsible for the 8OHdG induction. We consider that H₂O₂ is the source of this crypto-OH radical; in Fe-loaded cells, H₂O₂ was decreased because of the decomposition by Fe, and in the phenanthroline experiment H₂O₂ was increased as described above. Crypto-OH radicals are *OH radicals that are generated in immediate proximity to molecules, of DNA this case, with which it instantly reacts. Consequently, *OH radical scavengers or spin traps may not be able to access the *OH radical [22–24]. Work still needs to be done to discover the mechanism which generates crypto-OH radicals in anaerobes.

The evidence that we found, in a strict anaerobe exposed to O₂, of ROS generation and the way it acts to induce oxidative DNA damage may be useful not only in clarifying the response of anaerobes to environmental oxygen but also in the study of molecular evolution driven by oxygen [25].

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References


Fig. 4. A: Amounts of 8OHdG detected in *P. melaninogenica* (1–3) and *B. fragilis* (4, 5) after exposure to ROS and subsequent 1 h incubation at 37°C. Amounts of 8OHdG were evaluated as described in Section 2. (1, 4) Not exposed to O₂; (2, 5) exposed to O₂ for 30 s; (3) not exposed to O₂ but incubated with 1 mM H₂O₂ at 37°C for 1 h. Bars represent S.E.M. *Significantly increased induction of 8OHdG (P<0.01). B: Effects of ROS modulators on the induction of 8OHdG in *P. melaninogenica* after 30 s exposure to O₂ and subsequent 1 h incubation at 37°C. Amounts of 8OHdG were determined as described in Section 2. Control (100%) indicates the induction of 8OHdG in the absence of modulators. (1) Catalase (1000 U ml⁻¹); (2) SOD (300 U ml⁻¹); (3) phenanthroline (1 mM); (4) bathocuproine (10 μM); (5) FeCl₂ (1 mM) loaded; (6) PBN (5 mM). *Significantly (1, 5) suppressed or (3) increased induction of 8OHdG (P<0.01). Bars show S.E.M.