FEBS Letters 450 (1999) 178–180 FEBS 21959

Induction of oxidative DNA damage in anaerobes

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Received 1 April 1999

Abstract We compared oxidative DNA damage in strictly anaerobic *Prevotella melaninogenica*, aerotolerant anaerobic *Bacteroides fragilis*, and facultative anaerobic *Salmonella typhimurium* after exposure to O_2 or H_2O_2. Using HPLC with electrochemical detection, we measured 8-hydroxydeoxyguanosine (8OHdG) as a damage marker. O_2 induced 8OHdG in *P. melaninogenica* but not in *B. fragilis*, which shows catalase activity, or in *S. typhimurium*. In *P. melaninogenica*, with catalase, O_2 induced less 8OHdG; superoxide dismutase had no effect; with glucose and glucose oxidase, O_2 induced more 8OHdG. H_2O_2 also markedly increased 8OHdG. O_2 was suggested to induce 8OHdG through H_2O_2. O_2 or H_2O_2 decreased survival only in *P. melaninogenica*. Highly sensitive to oxidative stress, *P. melaninogenica* could prove useful for investigating oxidative DNA damage.

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Key words: Hydrogen peroxide; 8-hydroxydeoxyguanosine; Oxidative DNA damage; Oxygen; *Prevotella melaninogenica*

1. Introduction

Oxygen and reactive oxygen species (ROS) are considered to be factors in carcinogenesis [1], aging [2], and molecular evolution [3]. We have been investigating the effects of ROS on the induction of DNA damage and mutation in mammalian cell systems [4–6]. Mammalian cells, however, are generally well be able to protect themselves against ROS and to repair oxidative DNA damage that may consequently occur [4,6,7]. Even bacteria species, such as *Escherichia coli*, have shown similar tolerance [8,9]. To assess the biological importance of, and investigate the mechanisms that protect against, oxidative DNA damage in biological systems, we need highly sensitive targets for the induction of oxidative DNA damage. Although anaerobes are sensitive to ROS, no reports have yet described oxidative DNA damage in anaerobes. ROS is reported to induce many kinds of DNA damage [10]. For oxidative DNA damage which causes mutation in both in vivo and in vitro systems, 8-hydroxydeoxyguanosine (8OHdG) has been found to be a suitable marker [10–12].

In this study, judging from the evidence provided by 8OHdG, we report how anaerobes respond to oxidative stress. Using an anaerobe, we have established a highly sensitive biological system which clearly shows oxidative DNA damage after exposure to O_2 or H_2O_2.

2. Materials and methods

2.1. Preparation of bacteria

Prevotella melaninogenica GAI5490 and *Bacteroides fragilis* ATCC25285 were used for comparative purposes as examples of a strict anaerobe and an aerotolerant anaerobe; cultures were grown on Brucella HK agar (Kyokuto Seiyaku, Japan) in an anaerobic incubator (EAN-140, Tabai-Espec, Japan) at 37°C. The gas composition in the anaerobic incubator was 90% N_2, 5% H_2 and 5% CO_2. We selected *Salmonella typhimurium* TA98 as a facultative anaerobe; cultures were grown in nutrient broth (NB, Difco, USA) agar with 100 g/ml ampicillin (ABPC) at 37°C, as previously described [13]. Bacteria strains were grown on agar plates for 2 days and then, after oxygen-free Dulbecco's phosphate buffered saline (DPBS, Nikken, Japan) was added to the agar, collected from the agar with plastic loops under oxygen-free conditions. Bacterial cell density was adjusted with oxygen-free DPBS to 2.6 (*B. fragilis* and *S. typhimurium*) or 3.2 (*P. melaninogenica*) at A 660 nm.

2.2. Survival of bacteria

Survival of bacteria was determined by, after dilution of the bacterial cell suspensions with DPBS, inoculating bacteria to Brucella HK agar (*P. melaninogenica* and *B. fragilis*) or NB agar without ABPC (*S. typhimurium*): *P. melaninogenica* and *B. fragilis* were incubated in oxygen-free conditions, and *S. typhimurium* was incubated in ambient conditions. After 2–4 days incubation, the number of colonies was counted and the survival worked out as the proportion of colonies growing from exposed samples compared to samples that had not been exposed. Data were presented as means obtained from 2–5 independent experiments.

2.3. Catalase activity

Catalase activity was determined according to Aebi [14] after extraction of the enzyme from bacteria by sonication.

2.4. 8OHdG determination

Quantities of 8OHdG were determined by HPLC with electrochemical detection after extraction and digestion of DNA under oxygen-free conditions [15]. The amounts of deoxyguanosine (dG) in the same sample were also determined by UV absorption, after which the molar ratio of 8OHdG per 10^5 dG was calculated. Data were presented as means+ or −standard errors (S.E.) obtained from 2–5 independent experiments conducted in duplicate or triplicate. Because S.E. were calculated to be small in *B. fragilis* and *S. typhimurium*, only S.E. in *P. melaninogenica* were presented.

2.5. Exposure to ROS

Exposure to O_2 was carried out as follows: in 15 ml tubes, 1 ml samples of bacterial cell suspension were exposed to O_2 by bubbling O_2 gas at 100 ml/min for 30 s through a 1 ml plastic pipette with the opening placed at the bottom of the 15 ml tube. After this the tubes containing the samples were tightly sealed, and the samples were incubated at room temperature (RT) for 1 or 3 h. In the samples with catalase (1000 U/ml), superoxide dismutase (300 U/ml, SOD), or glucose oxidase (0.2 U/ml), each enzyme or catalase and glucose oxidase were added to *P. melaninogenica* just before O_2 exposure. Glucose (5 mM) was added to the samples with glucose oxidase. Heat-inactivated catalase (HI catalase) was prepared by boiling catalase for 10 min. Exposure to H_2O_2 was carried out as follows: bacteria in oxygen-free DPBS were exposed to H_2O_2 at RT in oxygen-free conditions for 1 h. After exposure to O_2 or H_2O_2, bacteria were washed twice with oxygen-free DPBS in the anaerobic incubator, and then stored at −80°C.

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PII: S0014-5793(99)00497-4

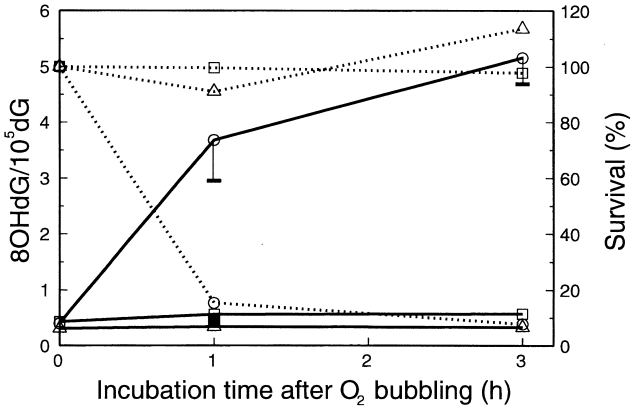

Fig. 1. Amounts of 8OHdG detected (solid lines) and survival (dotted lines) after 30 s exposure to O_2 and subsequent 1 h or 3 h incubation at RT of *P. melaninogenica* (○), *B. fragilis* (□), *S. typhimurium* (△). Amounts of 8OHdG and survival were determined as described in Section 2. Solid square indicates the 8OHdG level in *B. fragilis* with 10 mM NaN_3. Bars represent S.E.

2.6. Other procedures

H_2O_2 was determined as described previously [5]. Protein concentration in the sonicates was determined by a Bio-Rad protein assay kit using bovine serum albumin as a standard.

3. Results

3.1. Catalase activity

Catalase activity was not detected in *P. melaninogenica*. Catalase activity in *B. fragilis* was 52.6 ± 2.2 U/mg protein (mean ± S.E., $n = 4$), and that in *S. typhimurium* was 55.2 ± 1.5 U/mg protein.

3.2. Effects of exposure to O_2

Only for *P. melaninogenica* did exposure to O_2 increase the amounts of 8OHdG detected and decrease the survival (Fig. 1). The increase in 8OHdG was dependent on the incubation time after exposure to O_2. Less 8OHdG was detected in samples that had catalase added, the prior addition of HI catalase or SOD did not show a similar suppressive effect (Fig. 2). HI catalase increased 8OHdG levels significantly. Addition of glucose and glucose oxidase to the samples exposed to O_2 markedly increased 8OHdG, and co-addition of catalase decreased 8OHdG to a similar extent to that with catalase alone. Glucose and glucose oxidase with exposure to O_2 in the absence of *P. melaninogenica* generated 2.3 mM H_2O_2 during the incubation. Catalase inhibited the decrease in survival induced by O_2 exposure both in the presence or absence of glucose and glucose oxidase. Exposure to O_2 changed neither the levels of 8OHdG detected nor survival for either *B. fragilis* or *S. typhimurium*. The addition of 10 mM NaN_3 to *B. fragilis* did not increase the amounts of 8OHdG detected.

3.3. Effects of exposure to H_2O_2

For *P. melaninogenica*, after exposure to H_2O_2 the amounts of 8OHdG detected increased markedly and the survival decreased (Fig. 3). Increased 8OHdG was detected after exposure to concentrations as low as 0.2 mM. H_2O_2 slightly, but significantly increased 8OHdG in *B. fragilis*. Although the addition of 10 mM NaN_3 to *B. fragilis* increased the amounts of 8OHdG detected, at the same concentrations of H_2O_2 the increase was considerably less than with *P. melaninogenica*. H_2O_2 had no detectable effects on *S. typhimurium*.

4. Discussion

This first report investigating oxidative DNA damage in anaerobes shows that *P. melaninogenica* is highly sensitive to O_2 or H_2O_2, exposure to which increased the amounts of 8OHdG detected. The increased 8OHdG levels correlated well with the decreased survival, suggesting that oxidative DNA damage is an important cause of oxygen intolerance in *P. melaninogenica*.

O_2 bubbling of samples of *B. fragilis*, an aerotolerant anaerobe, did not result in higher levels of 8OHdG, probably because *B. fragilis* has catalase activity (our data and [8]), which may protect it from oxidative damage. The addition of catalase to *P. melaninogenica* samples actually resulted in both lower levels of 8OHdG and lower mortality after exposure to O_2. With *B. fragilis* samples, the prior addition of a catalase inhibitor, however, did not result in higher levels of 8OHdG after exposure to O_2, suggesting that catalase is not the only factor providing protection against ROS. This is corroborated by the finding that the inhibition of catalase induced only a small increase in 8OHdG in *B. fragilis* after exposure to H_2O_2. Neither O_2 nor H_2O_2 had any detectable effect on *S. typhimurium*, which suggests that *S. typhimurium* has either or both efficient protective and repair systems for oxidative DNA damage.

With catalase, less 8OHdG was detected after exposure to O_2, however, the prior addition of superoxide dismutase or HI catalase did not show similar results. Addition of HI catalase increased 8OHdG levels, probably due to iron liberated from the enzyme during heat inactivation. Glucose and glucose oxidase, which generate H_2O_2 from dissolved oxygen, markedly increased 8OHdG levels in samples exposed to O_2, and co-addition of catalase decreased 8OHdG levels and mortality. For *P. melaninogenica*, exposure to H_2O_2 results in a

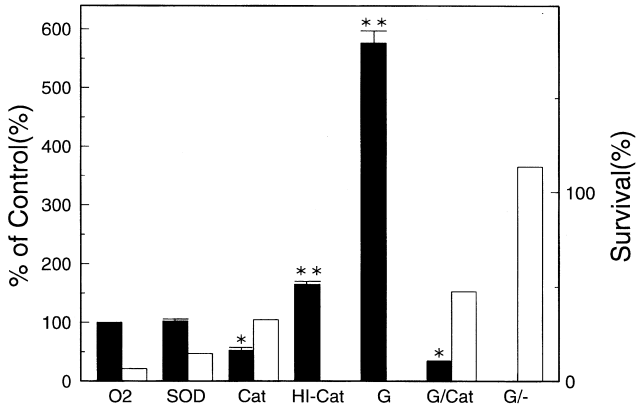

Fig. 2. Effects of ROS scavengers or glucose and glucose oxidase on the induction of 8OHdG in *P. melaninogenica* after 30 s exposure to O_2 and subsequent 3 h incubation at RT. Amounts of 8OHdG were determined as described in Section 2. Control (100%) indicates the induction of 8OHdG in the absence of scavengers. O_2, exposed to O_2, i.e. control; SOD, superoxide dismutase; Cat, catalase; HI-Cat, heat-inactivated catalase; G, glucose and glucose oxidase; G/Cat, glucose and glucose oxidase with catalase; G/−, glucose and glucose oxidase without O_2 exposure. *, significantly suppressed ($P < 0.001$); **, significantly increased ($P < 0.001$) the induction of 8OHdG. Bars represent S.E.

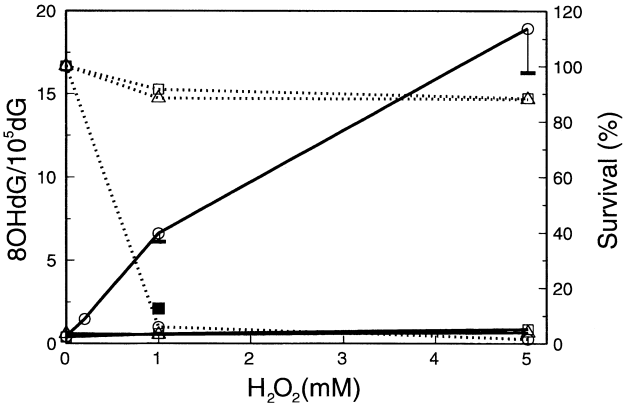

Fig. 3. Amounts of 8OHdG detected (solid lines) and survival (dotted lines) after exposure to H_2O_2 at RT in oxygen-free conditions for 1 h of *P. melaninogenica* (○), *B. fragilis* (□), *S. typhimurium* (△). Amounts of 8OHdG and survival were determined as described in Section 2. Solid square indicates the 8OHdG level in *B. fragilis* with 10 mM NaN_3. Bars represent S.E.

markedly greater induction of 8OHdG. These results suggest that generation of H_2O_2 is a crucial step during 8OHdG induction by O_2 exposure.

Considerable amounts of ROS are required to induce 8OHdG in mammalian cells [4,16]. *S. typhimurium*, *B. fragilis* or even calf thymus DNA dissolved in DPBS (data not shown) do not show increased levels of 8OHdG after exposure to O_2. To our knowledge, *P. melaninogenica* is the first biological system to show measurable evidence of increased levels of 8OHdG after exposure to O_2. *P. melaninogenica* is the most sensitive biological system known to induce ROS-mediated 8OHdG, and could be used to study the ability of other types of ROS to induce oxidative DNA damage. The increase in 8OHdG was considerable, so *P. melaninogenica* is a useful biological system to discover and evaluate materials which suppress the induction of oxidative DNA damage that is shown in Fig. 2. 8OHdG is known to be mutagenic [9,10], so this system should also be useful for investigating the biological implications of the effects of ROS, such as mutagenicity and the molecular evolution that ROS may induce.

Acknowledgements: We thank Miss Makiko Shimizu for her technical assistance to determine catalase activity.

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