Deoxyribonucleic Acid Strand Break Induced by the Hydroxy Radical-Generating Cyclic Peroxide, 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol

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Deoxyribonucleic acid (DNA) strand break caused by a synthetic cyclic peroxide, 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) was studied by both ethidium bromide fluorescence quenching and agarose gel electrophoresis. The Bd-mediated DNA strand break occurred dependently on temperature and also on Bd concentration. The reaction proceeded at a temperature higher than 30 °C (decomposition temperature of Bd), indicating that the reactive species generated by Bd-decomposition are responsible for the reaction. The reaction was protected by 1,4-diazabicyclo[2.2.2]octane and NaN₃, and also moderately by OH radical scavengers such as Na-benzoate and NaBr. Possible involvement of active oxygen radicals including OH radical in the DNA strand break is discussed in relation to the mechanism of Bd decomposition.

Keywords synthetic peroxide; hydroxy radical; DNA strand break; peroxide decomposition; radical generation

Introduction

Cell damaging effects of oxygen radicals are well recognized in many biological systems.¹⁾ The deoxyribonucleic acid (DNA) strand break is one of the critical effects of the oxygen radicals in the cell killing process.^{2–4)} The active oxygen radicals, on the other hand, play a role in tumor cell necrosis by anticancer drugs.^{5,6)} Thus, if one can control the generation of active oxygen radicals at the localized site such as in tumor, new chemotherapeutic methodology could be developed for hypoxic tumor cell necrosis. In this sense, we focused our attention on organic peroxides as the hydroxy radical source and studied the degradation of synthetic cyclic peroxides, 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ols.^{7–10)} We found that the cyclic peroxides oxidatively degraded cytochrome c *via* radical mediated reaction. Further, a water mediated decomposition of the peroxides accompanied OH radical generation which was determined by electron spin resonance using dimethylpyrroline N-oxide as a spin trapping reagent.

Since the OH radicals play a central role in DNA strand break,^{2–4,11,12)} it is of interest to know if DNA break is caused by the synthetic cyclic peroxides. We studied here the DNA breaking reaction caused by 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) *in vitro*.

Materials and Methods

Bd was synthesized by the ozonolysis of naphthalene in ethanol according to the method of Bailey *et al.*¹³⁾ and purified by repeating recrystallization in cold diethylether at 40 °C as described previously.^{8,9)} The purity of Bd was more than 90% when it was determined by nuclear magnetic resonance (NMR) spectroscopy.

Calf thymus DNA and λ phage DNA (*Escherichia coli* GM 119) were purchased from Sigma Co., Ltd. Restriction enzyme, *Hind*III from Toyobo Co., Ltd., ethidium bromide (ETB) from Wako Chemicals Co., Ltd. and agarose (type S) for electrophoresis from Nippon Gene Co., Ltd., respectively. Other reagents were all purchased from Wako Chemicals Co., Ltd., and were all reagent grade. A gas chromatography (GC) contents analysis kit was purchased from Yamasa Sho-yu Co., Ltd.

DNA strand break was determined either by the ETB fluorescence quenching method¹⁴⁾ or agarose gel electrophoresis.¹⁵⁾

For the DNA–ETB intercalation method, an aliquot of Bd solution (10 mM in acetonitrile) was added to 1.0 ml of aqueous solution containing 20 μ g of DNA. For the control, the same volume of acetonitrile was substituted for Bd solution. After 40 min of reaction at 40 °C, the reaction mixture was diluted with 2.0 ml of 1 mM ETB aqueous solution containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) and shaken to mix, then

the fluorescence from the DNA–ETB complex was determined at 590 nm by excitation at 510 nm using Shimadzu Fluorescence spectrophotometer, model RF-502.

For the analysis of DNA strand break by agarose gel electrophoresis, a λ phage DNA was dissolved in a metal free distilled water to make 1 mg/ml solution. To the solution containing 10 μ g of DNA, an aliquot of Bd solution (100 mM methanol) was added and the mixture was incubated in a water bath at 40 °C for a specified time period with or without scavengers (total volume of the reaction mixture was 50 μ l). Aliquots of the reaction mixture were applied on a 0.5% agarose gel and electrophoresis was run under the electrical field of 20 V. Buffers used for gel preparation and running consist of 89 mM Tris/2 mM EDTA/89 mM borate (pH 8.0). DNA and the fragments on the gel were visualized with ETB fluorescence on a transilluminator at 302 nm.

Results

Impairment of DNA is proved by the DNA–ETB intercalation method,¹⁴⁾ which takes advantage of the characteristic fluorescence of the DNA–ETB complex and its inhibition by Bd-mediated alterations of the DNA base pair region. ETB fluorescence excited at 510 nm was markedly enhanced when mixed with DNA. The fluorescence intensity ($E_{m_{max}}$ at 590 nm) was saturated at approximately 20 μ g DNA/ml when 0.3 mM ETB was present. Hence, 20 μ g DNA/ml was used for the following studies. The fluorescence was, however, inhibited when the DNA was pretreated with Bd without changing the spectral shape (not shown), indicating that DNA base-pair region is altered.¹⁴⁾ Thus, the fluorescence change at 590 nm was used as a monitor wavelength for DNA strand break.

Since Bd decomposition is temperature dependent,^{7–10)} DNA was treated with Bd at various temperatures for 40 min and then the DNA–ETB fluorescence intensity was determined (Fig. 1). The fluorescence intensity gradually decreased with increased temperature and became saturated at approximately 30 °C. This temperature range is well consistent with the Bd decomposition temperature determined previously by ESR^{7,8)} and high performance liquid chromatography (HPLC).⁹⁾ It was also shown that the time course of Bd-induced fluorescence inhibition examined at 40 °C was well consistent with that of Bd decomposition in an aqueous medium at the same temperature, that completed by 20 min (not shown). Therefore, it is indicated that the DNA damage is strongly dependent on the Bd decomposition.

Fig. 1. Temperature Dependence of Bd-Induced DNA Strand Break

Calf thymus DNA (20 μ g) was treated with Bd at the final concentration of 0.25 mM for 40 min at the temperatures indicated. The ETB-DNA fluorescence was determined at 590 nm (excitation at 510 nm) after the reaction mixture was diluted with ETB aqueous solution. Relative inhibition % was obtained to the control sample omitting only Bd. Each data points are the average of 2–3 determinations.

Fig. 2. Bd Concentration Dependence of DNA Strand Break

Calf thymus DNA was treated with BD at the concentrations indicated at 40 °C for 40 min, then the ETB-DNA fluorescence intensity was determined as described in Fig. 1.

Fig. 3. Protective Effects of Reactive Oxygen Radical Scavengers on Bd-Induced DNA Strand Break

Calf thymus DNA was incubated with 2.5 mM Bd at 40 °C for 40 min in the presence and absence of increased concentrations of radical scavengers. All the radical scavengers were added as an aqueous solution. ETB-DNA fluorescence was determined as described in Fig. 1.

Bd dependence of the DNA-ETB fluorescence inhibition was examined with the DNA pretreated with increased concentrations of Bd at 40 °C for 40 min. As Fig. 2 clearly shows, the fluorescence was inhibited dependently on Bd concentration. Since Bd is known to mediate cytochrome c degradation *via* the oxygen radicals generated by its

Fig. 4. Time Course of λ Phage DNA Break in the Presence of Bd

DNA cleavage was determined on agarose gel electrophoresis. Cleavage pattern was determined at 302 nm after ETB staining. Lanes: (1) methanol control without Bd (incubation for 180 min), (2) *Hind*III digestion, and λ phage DNA treated with 10 mM Bd at 40 °C for (3) 0, (4) 20, (5) 40, (6) 60, (7) 120, (8) 180 min, respectively.

Fig. 5. Effect of Bd Concentration on λ Phage DNA Break

Lanes: (1) control, (2) *Hind*III digestion, and λ phage DNA treated with (3), 0.5, (4) 1.0, (5) 2.0, (6) 5.0 and (7) 10 mM Bd at 40 °C for 1 h.

decomposition,¹⁰⁾ it is also suggested that the inhibition of DNA-ETB fluorescence is due to the DNA alteration caused by the reactive oxygen radicals generated on Bd decomposition.

Protective effects of reactive oxygen radical scavengers were studied for the Bd-induced quenching of the DNA-ETB fluorescence. All the hydroxy radical scavengers examined protected the Bd-induced fluorescence quenching in their concentration dependent manner (Fig. 3). Na-benzoate and NaBr were active and inhibited the reaction for approximately 70% at 30 mM although mannitol was rather ineffective. On the other hand, 1,4-

diazabicyclo[2.2.2]octane (DABCO) inhibited the reaction almost completely.

Fluorescence quenching is affected not only by DNA strand break or other structural alteration of DNA but also artificial quenching. In order to confirm that Bd actually causes DNA strand break, agarose gel electrophoresis was carried out on Bd-treated λ phage DNA. As is shown in Fig. 4, DNA break occurred after Bd treatment. The ETB fluorescence completely disappeared after 20 to 40 min of reaction, that is, the time range of Bd decomposition.^{8,9)}

The electrophoretic study also showed that the DNA strand break occurs dependently on Bd concentration (Fig. 5). ETB fluorescence at the original DNA mass gradually decreased dependently on Bd concentration and almost completely disappeared at a concentration higher than 5 mM. It is noted that the reaction products which could not penetrate the gel matrix appeared at the initial stage of Bd treatment or with low Bd concentration. It is possible that crosslinking of DNA occurs. The DNA is torn into pieces afterward with a higher Bd concentration or after a long reaction period. Since any characteristic fluorescent fragment with specific molecular weight appeared on the gel, it is expected that the breaks which occurred in the DNA strand are non-specific. Non-specificity of the

Bd-mediated DNA break is also suggested by the GC contents change in the Bd treated λ phage DNA determined by HPLC. The GC contents were not significantly changed after Bd treatment (Table I). However, it is notable that both the relative amounts of d-CMP and d-AMP significantly decreased after the reaction. These results indicate that the major DNA damage occurs in the deoxyribose region rather than in the base region.

Protective effects of radical scavengers on the reaction revealed that DABCO and NaN_3 prevented the DNA scission (Fig. 6). The effects of OH radical scavengers are again variable as in the case of Bd-induced ETB fluorescence protection in Fig. 3. Na-benzoate inhibited the Bd-mediated DNA break but mannitol and dimethyl sulfoxide (DMSO) were ineffective.

Discussion

Present studies revealed that Bd, a synthetic cyclic peroxide, causes DNA strand break. The reactive oxygen species responsible for the DNA break are considered to be the radicals generated on Bd decomposition because the reactions were totally dependent on Bd decomposition conditions previously reported⁷⁻⁹⁾ and were protected by the reactive oxygen radical scavengers, as was consistent with our previous papers dealing with the Bd reactions on cytochrome c.¹⁰⁾

Scavenger studies revealed that singlet oxygen scavengers, DABCO and NaN_3 , and also OH radical scavengers, Na-benzoate and NaBr, significantly prevented the Bd-mediated DNA breaks determined by the EBT intercalation and agarose gel electrophoresis. However, it is unlikely that singlet oxygen is the major species responsible for the DNA strand break because the previous study on the mechanism of Bd decomposition supports direct generation of OH-radical but not singlet oxygen in the Bd aqueous medium.^{9,16)} In addition, we observed that DABCO causes a rapid decrease of Bd absorption at 250 nm, that is, Bd decomposition (unpublished observation), thus the inhibitory action of DABCO may not be due to its singlet oxygen scavenging activity. Further, NaN_3 is reactive not only to singlet oxygen but also to the OH radical.¹⁴⁾ Hence, it is likely that the OH radical is most plausible species involved in the DNA break. Although the effect of OH radical scavengers are divergent such that mannitol and DMSO were ineffective, it is well known that the inhibitory action of the OH scavenger is variable depending on the characteristics of the OH radical generating system.¹⁷⁾

Since the OH radical is extraordinarily reactive, it does not survive more than a few collisions following its formation.¹⁸⁾ Therefore, the site specific DNA strand break by the OH radical occurs when the OH radical generating compounds or systems interact specifically with DNA in such a case as the ternary metal complexes of Cu-phenanthroline-reducing agent- O_2 ¹⁹⁾ and else.²⁰⁾ In the present study, though the DNA strand break occurred as well as in other OH generating systems, Bd did not give rise to any specific fragmentation pattern of DNA on the agarose gel. Moreover, it required rather high concentrations (> 5 mM) for the significant breaking of DNA. These discrepancies could be mainly attributed to the difference in the DNA-chemicals interaction.

TABLE I. GC Contents Change after Bd Treatment

	Relative molar ratio (%)				GC contents (%)
	d-GMP	d-CMP	d-AMP	d-TMP	
Untreated	21.85 $\pm$ 0.41	35.36 $\pm$ 0.16	21.98 $\pm$ 0.16	20.81 $\pm$ 0.28	57.79 $\pm$ 0.74
+ 10 mM Bd	29.16 $\pm$ 0.40	30.07 $\pm$ 0.33	18.84 $\pm$ 0.06	21.93 $\pm$ 0.14	59.23 $\pm$ 0.06

DNA samples were incubated with nuclease P1 (0.1 mg/ml in 1/35 M CH_3COONa /0.2 mM ZnCl_2 , pH 5.3) at 50 °C for 1 h. The liberated mononucleotides were analyzed by HPLC (Nippon Bunko model Twinkle) equipped with reverse phase column (Hitachi #3056) using 5 mM K_2HPO_4 /5 mM KH_2PO_4 as an elution solvent.

Fig. 6. Inhibition of Bd-Induced DNA Break by Reactive Oxygen Radical Scavengers

λ phage DNA was treated with 10 mM Bd at 40 °C in the presence or absence of radical scavengers for 1 h. Lanes: (1) control, (2) with Bd at 4 °C, (3) with Bd at 40 °C, (4) + mannitol (30 mM), (5) + DMSO (30 mM), (6) + Na-benzoate (30 mM), (7) + DABCO (30 mM) and (8) + NaN_3 (30 mM).

Although the involvement of radical species other than the OH radical can not be ruled out, the present studies suggest that further modification of the cyclic peroxide to improve specific interaction with DNA could provide an effective DNA strand breaking chemical system.

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