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Thermally Controlled Degradation of Cytochrome c by Cyclic Peroxides: Possible Approach to the Hyperthermic Sensitizer

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In order to examine the feasibility of using cyclic peroxides as sensitizer for the hyperthermic treatment of cancer, we studied temperature dependent OH radical generation from two synthetic cyclic peroxides, 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol and 4-ethoxy-1,4-dihydro-2,3-benzodioxepin-1-ol. The amounts of 5,5-dimethyl-pyrroline *N*-oxide (DMPO)-OH adduct formed as a result of decomposition of these cyclic peroxides increased markedly at temperatures of above approximately 30 and 40 °C, respectively (degradation temperatures). Peroxide-mediated degradation of cytochrome c was also markedly enhanced above these temperatures. These results suggest that biologically effective OH radical can be produced specifically under thermally controlled conditions from certain cyclic peroxides.

Keywords——hyperthermia; cyclic peroxide; OH radical mediated; cytochrome c degradation; spintrapping

Introduction

Hyperthermia is now undergoing clinical trials as a single agent or in combination with radiation therapy or chemotherapy for the treatment of various malignant tumors.[1] A number of experiments suggest that the membrane system is involved in the thermal cell-damaging process.[2] It is also known that the biological membrane is very sensitive to reactive oxygen radicals.[3] On the other hand, reactive oxygen radicals, typically OH radical, are considered to be central species involved in the cell damaging process, such as deoxyribonucleic acid (DNA) chain breaking by ionizing radiation[4] or by certain carcinostatic agents.[5–7] Therefore, if a compound that decomposes in the vicinity of 42 °C (hyperthermic temperature) and generates reactive oxygen radicals is present at the site of local hyperthermic treatment, such a compound should improve the therapeutic efficiency even in cells which become thermotolerant after repeated hyperthermic treatment.[8]

As candidates for such compounds, we synthesized 6- and 7-membered cyclic peroxides and studied the degradation mechanism of these peroxides. Our previous experiments revealed that the degradation of these peroxides in an aqueous medium produced OH radical.[9,10]

In this work, we studied precisely the temperature profile of the degradation of cyclic peroxides and the OH radical generation. Then, the temperature-dependent reactions of 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol and 4-ethoxy-1,4-dihydro-2,3-benzodioxepin-1-ol with cytochrome c were also studied to evaluate the biological effectiveness of OH radical produced by the thermal degradation of these cyclic peroxides, since cytochrome c is a typical important biological substance whose reactivity with reactive oxygen radicals has been extensively studied.[11]

Materials and Methods

Cyclic peroxides were synthesized according to the methods described previously.[12,13]

OH radical generation was determined by a spin trapping technique using 5,5-dimethyl-pyrroline *N*-oxide (DMPO) as the spin trapping reagent. Electron spin resonance (ESR) conditions were the same as previously described.[9] For studying the temperature dependent formation of spin adduct, 30 μl of DMPO–peroxide mixture consisting of 50 μl of 100 mM DMPO aqueous solution and 10 μl of 10 mM peroxide in acetonitrile was taken in a capillary and heated for 2 min in a water bath at the indicated temperatures. ESR spectra were measured on a Varian model E-4 ESR spectrometer at room temperature. There was no appreciable change of the signal intensity during the ESR measurement at room temperature.

Peroxide-mediated cytochrome c degradation was determined spectrophotometrically using a Hitachi 557 double-wavelength spectrophotometer as follows. One ml of cytochrome c solution in 0.1 M phosphate buffer, pH 6.5 (containing 0.1 mg of protein) was added with 20 μl of 10 mM peroxide in acetonitrile, then, the difference spectra between before and after addition of peroxide were recorded every 6 min in the wavelength range between 200—600 nm.

Heme degradation kinetics were recorded by dual-wavelength spectroscopy. The reactions were carried out in a 1 ml cuvette containing 0.1 mg of cytochrome c in 0.1 M phosphate buffer (pH 6.5) at various temperatures. After addition of 20 μl of 10 mM peroxide in acetonitrile, heme degradation kinetics were recorded at 410—445 nm.

DMPO was purchased from Aldrich Chem. Co., Ltd., and used after purification through a charcoal column as described previously.[9] Cytochrome c (type III) was purchased from Sigma Co., Ltd., and used without further purification. All other reagents were of special reagent grade, and were purchased from Wako Pure Chemical Co., Ltd.

Results and Discussion

When DMPO as a spin trapping agent was mixed with synthetic peroxides, 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) and 4-ethoxy-1,4-dihydro-2,3-benzodioxepin-1-ol (Bdp) (Chart 1) in an aqueous solution, only a signal corresponding to typical DMPO-OH adduct was obtained, consisting of a 1:2:2:1 quartet with a hyperfine coupling constant of $A_N = A_H^\beta = 14.8$.[14] The signal intensity was very small at room temperature but was markedly enhanced when the reaction mixture was heat-treated to facilitate the degradation of peroxide, as shown in our previous paper.[10] Formation of the spin adduct was significantly inhibited by OH radical scavengers such as Na-benzoate or EtOH (Fig. 1). No signals corresponding to hydroxyethyl radical adduct could be detected in the presence of EtOH under our measurement conditions, although the adduct is known to be formed as a result of H-abstraction from EtOH by OH radical.[14]

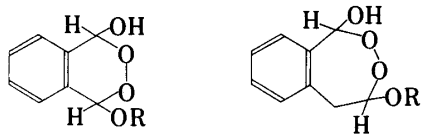

Chart 1. Structures of 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (Bd) and 4-Ethoxy-1,4-dihydro-2,3-benzodioxepin-1-ol (Bdp)
Bd: R=Et Bdp: R=Et

To study the temperature profiles of DMPO-OH formation from the peroxides, reaction mixtures containing DMPO and peroxide were incubated for 2 min at the indicated temperatures, then the ESR spectra were determined. The results are given in Fig. 2. DMPO-OH adduct formation from Bd gradually increased as the temperature increased, even in the lower temperature range below 32 °C, but markedly increased above this temperature. The critical temperature for remarkable increase of DMPO-OH formation was identical with the degradation temperature of Bd as determined by high performance liquid chromatography (HPLC) (not shown). The critical temperatures varied somewhat depending on the water content of the medium. Higher contents of water lowered the degradation temperature. On the other hand, DMPO-OH formation from the 7-membered cyclic peroxide, Bdp, was small and no significant enhancement was observed up to approximately 43 °C. The signal abruptly increased above this temperature. The net amounts of DMPO-OH formed from Bdp were smaller than those from Bd. One reason for the difference in the yield of DMPO-OH might be

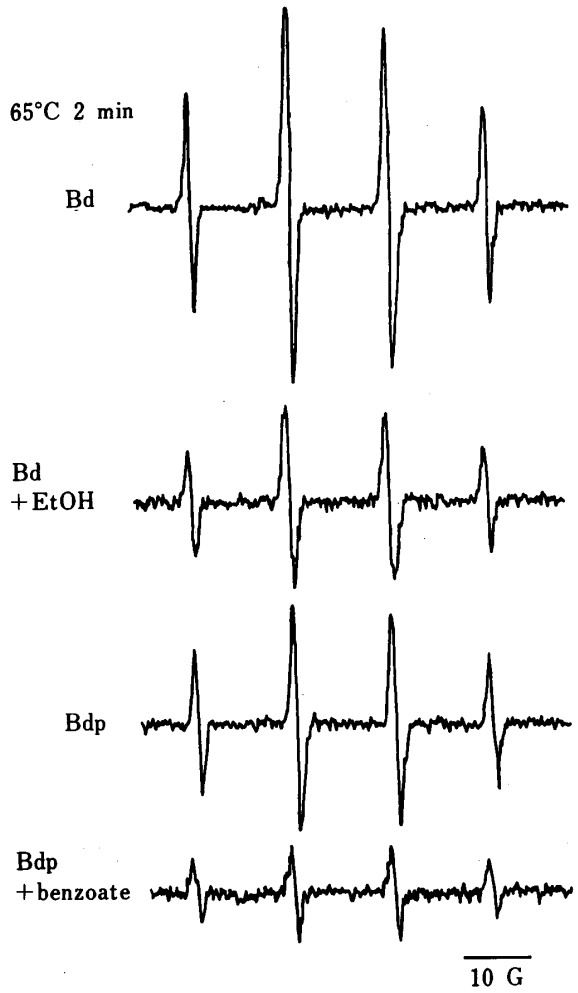

Fig. 1. DMPO-OH Formation from Thermally Degraded Bd and Bdp

A 10 μl aliquot of 10 mM Bd or Bdp in acetonitrile was mixed with 50 μl of 100 mM DMPO aqueous solution and heat-treated at 65 °C for 2 min with or without an OH-radical scavenger, EtOH (1 mM) or Na-benzoate (7.1 mM). The ESR spectra were determined at room temperature. ESR conditions were as described in ref. 10.

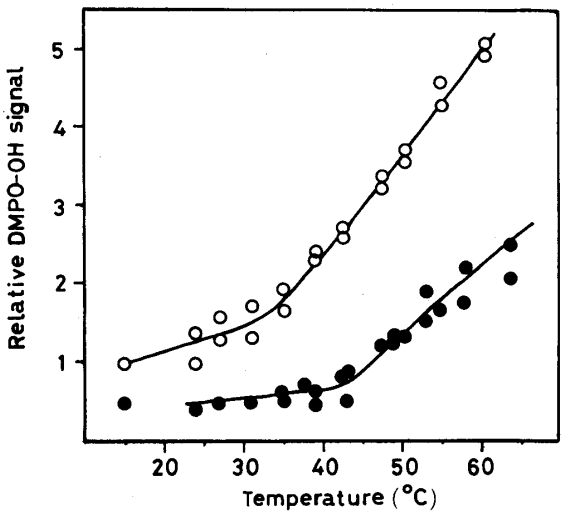

Fig. 2. Temperature-Dependent Profiles of DMPO-OH Formation from Bd and Bdp

Bd- or Bdp–DMPO mixtures were incubated for 2 min at the indicated temperatures, then the ESR was measured at room temperature. Data points are the means of 2 (for Bd) and 3 (for Bdp) different experiments. —○—, Bd; —●—, Bdp.

the difference in the activation energies of decomposition of these cyclic peroxides, but a precise explanation must wait until the mechanisms of their decomposition processes are clarified.

For evaluating the biological effects of these OH radical-releasing peroxides, the reactions with cytochrome c were studied. When Bd was added to cytochrome c solution, the Soret band gradually disappeared as Bd decomposed, indicating that cytochrome heme was degraded (see the difference spectra in Fig. 3). Similar changes in the difference spectra were obtained both in salt-free aqueous solution and under anaerobic conditions. *o*-Phthalaldehydic acid, a major degradation product of Bd,[9] did not show this kind of spectral change. Therefore, the reaction is mediated directly by the degradation of the peroxide. Further, the heme degradation reaction was significantly inhibited by OH-scavengers such as Na-benzoate (Fig. 3 inset), indicating that the OH radical produced by the peroxide degradation is involved in the reaction. The reactions of cytochrome c with reactive oxygen radicals have been extensively studied by pulse radiolysis (for a review, see ref. 11). From these

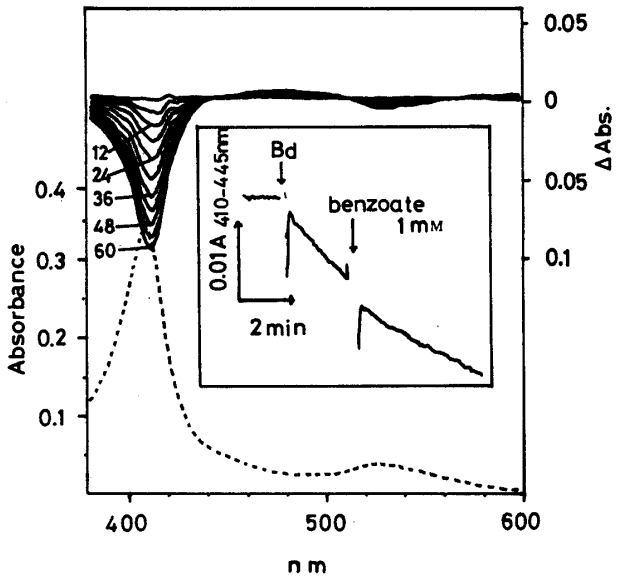

Fig. 3. Bd-Mediated Cytochrome c Degradation

The reactions were carried out in a 1 ml cuvette containing 0.1 mg of cytochrome c in 0.1 M phosphate buffer (pH 6.5). After addition of 20 μl of 10 mM Bd in acetonitrile, difference spectra between before and after addition of peroxide were recorded every 6 min at 40 °C. The spectrum of cytochrome c solution used is given by a dotted line. Inset: Degradation kinetics were recorded by using dual-wavelength spectrometry at 410—445 nm.

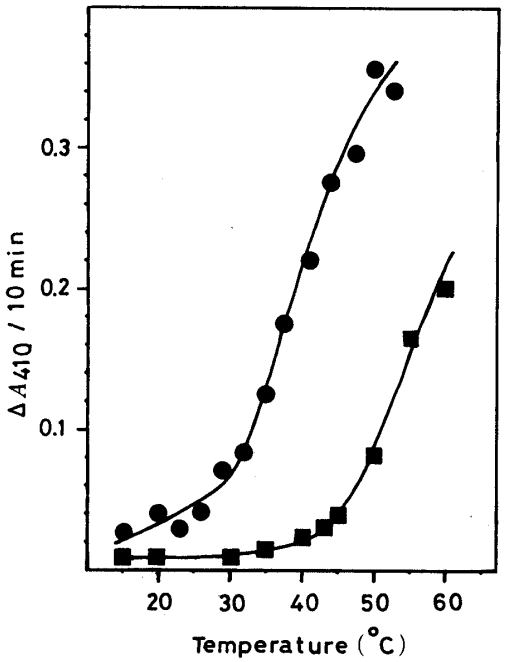

Fig. 4. Temperature-Dependent Profiles of Peroxide-Mediated Degradation of Cytochrome c

Degradation kinetics of cytochrome c were determined by dual-wavelength spectrometry as mentioned in Fig. 4 at the temperatures indicated. —●—, Bd; —■—, Bdp.

studies, it is known that cytochrome c heme is initially reduced by OH radical, and is then degraded.[11] However, in the present studies on the reaction of cytochrome c with Bd, no heme reduction was observed but only degradation. Our preliminary studies[15] on the peroxide-treated cytochrome c revealed that protein cross-linking and the degradation of certain amino acid residues such as tyrosine and lysine occurred after peroxide treatment, as is seen in the radiation damage of proteins, where the major reactive species is known to be OH radical.[16] These results indicate that the disappearance of the Soret band of cytochrome c observed here is attributable to not only heme degradation mediated by the peroxide but also to loss of the globin moiety.

Unusual reactivity of the OH radical produced by Bd was also observed in ESR, where DMPO-OH formation was significantly inhibited by EtOH as an OH scavenger, but no signals corresponding to hydroxyethyl radical adduct could be observed (Fig. 1).

It is possible that the OH radical produced by Bd or Bdp might have the characteristics of a so-called crypto-OH radical, which also has unusual reactivity against OH radical scavengers[17] differing from "free" OH radical generated by pulse radiolysis. Further studies on the precise mechanism of cytochrome c degradation and also on the properties of the OH radical generated by Bd are in progress.

The peroxide-mediated cytochrome c heme degradation reactions were studied with Bd and Bdp at several temperatures ranging from 15 to 60 °C and the results are summarized in Fig. 4. The temperature-dependent profiles of cytochrome c degradation are consistent with those of DMPO-OH formation from Bd and Bdp, respectively (see Fig. 2). Cytochrome c degradation rates were markedly enhanced at temperatures of above approx. 31 °C in the case

of Bd and approx. 43 °C in the case of Bdp. These critical temperatures are the same as those for OH radical formation, *i.e.*, the decomposition temperatures of the peroxides. Here again, the degradation rate of cytochrome c is smaller in the case of Bdp treatment than Bd treatment, in accordance with the temperature profiles of OH radical generation in Fig. 2.

It is likely that OH radical is involved in carcinostatic action of certain antitumor agents such as anthracyclines,[5] platinum complexes[6] and hematoporphyrins[7] or ionizing radiation.[4] From the standpoint of the role of OH radical in tumor cell necrosis, our present studies showing that the synthetic cyclic peroxides, Bd and Bdp, could produce OH radical under thermally controlled conditions and could degrade cytochrome c are quite interesting, because this will provide another modality to produce OH radical specifically at a localized site, that is, a tumor site, by means of an externally applied stimulus such as heat (local hyperthermia).

The present studies also showed that Bdp, a 7-membered cyclic peroxide, is rather stable at physiological temperature as compared with 6-membered Bd, and has a sharper degradation temperature at approx. 43 °C. Since hyperthermic treatment is carried out at 42 °C, a peroxide having sharp degradation temperature in the vicinity of 40 °C should be very useful. Further studies on the effects of alkoxyl substitution and the ring size on the stability and the degradation mechanism of cyclic peroxides are in progress.

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