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DNA BREAKING ACTIVITY OF THE CARBON-CENTERED RADICAL GENERATED FROM 2,2'-AZOBIS(2-AMIDINOPROPANE) HYDROCHLORIDE (AAPH)

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When supercoiled plasmid DNA was incubated with 2,2'-azobis (2-amidinopropane)hydrochloride (AAPH) at pH 7.4 in the presence and absence of oxygen, the DNA single strands were effectively cleaved. The breaking in the presence of oxygen was not inhibited by superoxide dismutase and catalase, but inhibited by mannitol, ethanol, butyl hydroxyanisole, thiol compounds, tertiary amines and spin trapping agents N-*tert*-butyl-α-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline N-oxide (DMPO). The breaking in the absence of oxygen was inhibited by ethanol, a tertiary amine and PBN. By electron spin resonance spin-trapping with PBN, the carbon-centered radical was detected both in the presence and the absence of oxygen. Hydroxyl radical was detected by use of DMPO only in the presence of oxygen. The DNA breaking activity of AAPH was found to be due primarily to the aliphatic carbon-centered radical. While the reactivity of carbon-centered radicals have received little attention, the aliphatic carbon-centered radical generated from AAPH was found to be highly reactive to break the DNA strands.

KEY WORDS: DNA breaking, 2,2'-azobis(2-amidinopropane)hydrochloride (AAPH), carbon-centered radical, hydroxyl radical, spin trapping.

INTRODUCTION

DNA strand breaks induced by active oxygen radicals have received much attention in relation to the development and the prevention of cancer. Hydroxyl radical generated by interaction of metal ions with thiol compounds,[1] ascorbic acid,[2] polyphenols,[3] sugars,[4] hydrogen peroxide,[5] lipid hydroperoxides[6] and bleomycin,[7] and singlet oxygen[8-11] break the DNA single strands. In contrast, recent studies have suggested the importance of carbon-centered radicals in the DNA breaking. Thus the carbon-centered radicals generated from enediyne antibiotics including neocarzinostatin,[12] hydrazines[13-15] and aromatic diazonium salts[16,17] may directly cleave the DNA strands. In order to clarify the participation of carbon-centered radicals in the DNA strand breaking, further studies may be necessary.

2,2'-Azobis(2-amidinopropane)hydrochloride (AAPH) is a well-known water soluble radical initiator, which has been successfully introduced as an initiator of

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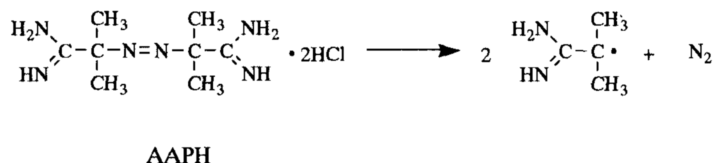

AAPH

SCHEME 1

lipid peroxidation by Niki *et al.*[18] It is considered that the agent initially forms the aliphatic carbon-centered radical at the tertiary carbon atom (Scheme 1), which may be transformed into more reactive peroxy radical in the presence of oxygen. The agent may be a good model for the investigation of the DNA breaking by aliphatic carbon-centered radicals. In the present study, we examined the DNA strand breaking by the agent, and investigated what radical species participated in the breaking. It was found that the aliphatic carbon-centered radical could effectively cleave the the DNA single strands.

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MATERIALS AND METHODS

2,2′-Azobis(2-amidinopropane)hydrochloride (AAPH), triethylenediamine, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA) and N, N, N′, N′-tetramethylethylenediamine (TEMED) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Butyl hydroxyanisole (BHA) was a product of Nikki Universal Company (Tokyo, Japan). 5,5-Dimethyl-1-pyrroline N-oxide(DMPO) was from Labotec Company (Tokyo, Japan). N-*tert*-butyl-α-phenylnitrone (PBN) and 2,2,6,6-tetramethylpiperidine (TEMP) were from Aldrich Chemical Company (Milwaukee, WI, USA).

Plasmid pBR 322 DNA[19] (1 mg/ml in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) was obtained from Takara-shuzo Company (Kyoto, Japan). Catalase [EC 1.15. 1.6] and superoxide dismutase (SOD) [EC 1.15.1.1] were from Sigma Chemical Company (St. Louis, MO, USA).

DNA Breaking by AAPH

A mixture of pBR 322 DNA (10 μg/ml) and 1 mM AAPH in 0.1 M phosphate buffer (pH 7.4) with or without the scavengers at the indicated concentration was incubated at 37°C for 3 hr. An aliquot of 10 μl of the reaction mixture was subjected to agarose gel electrophoresis after addition of 1 μl of a solution composed of 0.5% bromophenol blue, 0.5% xylene cyanol and 50% glycerol.

For the reaction in the absence of oxygen, the DNA solution (40 μg/ml) in 0.4 M phosphate buffer (pH 7.4), 4 mM AAPH solution in water, and a solution of the scavenger in water were separately purged with nitrogen gas for 15 min, and they were combined using a microsyringe at a ratio of 1:1:2 into a tube with a silicon cap which had been substituted by nitrogen gas. The mixture was incubated and subjected to the electrophoresis.

Agarose gel for electrophoresis was prepared by dissolving 0.7% Seakem® ME agarose (FMC BioProducts, Rockland, ME, USA) in 45 mM Tris-borate buffer

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(pH 8.3), 1 mM EDTA and 0.5 μg/ml ethidium bromide (Wako Pure Chemical Industries). Electrophoresis was run at 4 V/cm for 2 hr using a Mupid-2 submarine electrophoretic apparatus (Advance Company, Tokyo, Japan). The DNA band was visualized by irradiation at 300 nm, and was photographed through an orange filter.

Electron Spin Resonance (ESR) Spectra of Spin Adducts of the Radicals Generated from AAPH

AAPH (50 mM) was incubated with 0.1 M PBN or DMPO in 0.1 M phosphate buffer (pH 7.4) at 37°C for 3 hr. For examination of the effect of ethanol, triethylenediamine, TEMED, EDTA and DTPA, the agents at the indicated concentrations were added before the incubation. For investigation of the adduct formation in the absence of oxygen, a solution of 0.2 M PBN or DMPO in 0.2 M phosphate buffer (pH 7.4) and a solution of 0.1 M AAPH in water were separately purged with nitrogen gas for 15 min, and they were mixed at a ratio of 1:1 under nitrogen atmosphere. The mixture was incubated at 37°C for 3 hr.

For detection of singlet oxygen, a mixture of 2.4 mM AAPH and 0.1 M TEMP in ethanol was incubated at 37°C for 3 hr.

The ESR spectra were obtained on a Varian E-4 EPR spectrometer. The instrumental conditions were: field setting at 338.5 mT, scan range of 10 mT, microwave power of 10 mW and modulation amplitude of 0.1 mT.

RESULTS

DNA breaking activity of AAPH was examined by using a supercoiled plasmid pBR 322 DNA. It is known that the supercoiled (form I) DNA is converted into a nicked open circular form (form II) and/or into a linear form (form III) when the single strands are cleaved, and they can be separated by agarose gel electrophoresis.[20] The supercoiled DNA was incubated with AAPH at the concentrations between 10 μM and 10 mM at pH 7.4 and 37°C for 3 hr (Figure 1,A). The gel electrophoresis showed that form I DNA was converted into form II by 1 mM AAPH, and into form II and form III by 10 mM AAPH. Time course studies of the DNA breaking by 1 mM AAPH indicated that the breaking was extensive in incubation for more than 1 hr (Figure 1,B).

Effects of several radical scavengers[21] on the DNA breaking were investigated (Figure 2). SOD that decomposes superoxide anion, and catalase that destroys hydrogen peroxide showed no inhibitory effect (lane 3 and 4) indicating that superoxide anion and hydrogen peroxide did not participate in the breaking. Mannitol (lane 5) and ethanol (lane 6) inhibited the breaking. Azide ion (lane 7) was slightly inhibitory, but thiol compounds cysteine (lane 8) and 2-mercaptoethanol (lane 9), and antioxidant BHA (lane 10) were effective. While triethylamine was little inhibitory (lane 11), tertiary amines including triethylenediamine (lane 12), TEMED (lane 13), EDTA (lane 14) and DTPA (lane 15) inhibited the breaking. Spin trapping agents PBN (lane 16) and DMPO (lane 17), which traps radicals as stable spin adducts.[22] were also inhibitory. The results suggested that radical species other than superoxide anion and hydrogen peroxide participated in the breaking.

When the supercoiled DNA was incubated with AAPH in the absence of oxygen, the DNA was similarly transformed into form II (Figure 3, lane 1), indicating that the breaking was induced without the aids of molecular oxygen. The aliphatic

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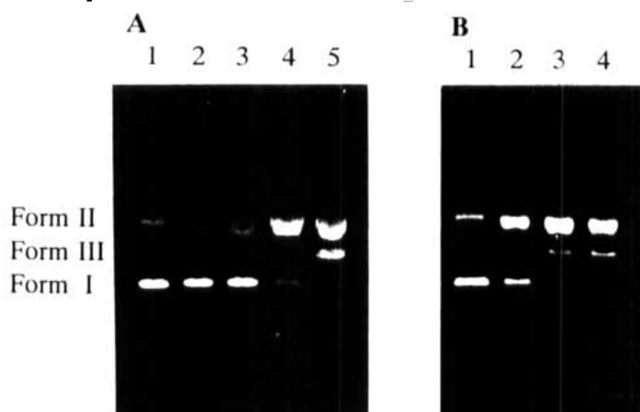

FIGURE 1 Agarose gel electrophoresis of supercoiled pBR 322 DNA treated with AAPH. A. Dose dependence. Supercoiled pBR 322 DNA (10 μg/ml) was incubated with 0 (lane 1), 0.01 (lane 2), 0.1 (lane 3), 1 (lane 4) and 10 mM (lane 5) of AAPH at pH 7.4 and 37°C for 3 hr. B. Time course. The supercoiled DNA (10 μg/ml) was incubated with 1 mM AAPH at pH 7.4 and 37°C for 0 (lane 1), 1 (lane 2), 3 (lane 3) and 5 hr (lane 4). Positions of a supercoiled form (form I), a nicked open circular form (form II) and a linear form (form III) are indicated.

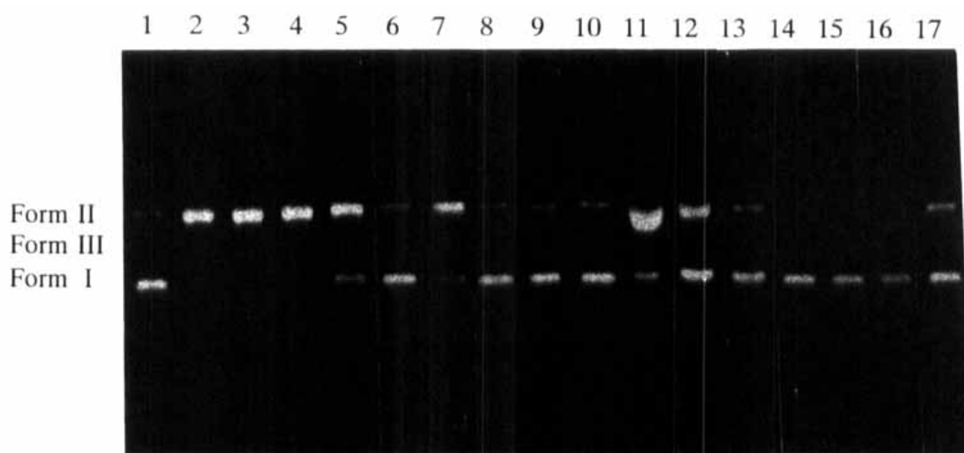

FIGURE 2 Agarose gel electrophoresis of supercoiled pBR 322 DNA treated with AAPH in the presence of free radical scavengers. Supercoiled pBR 322 DNA (10 μg/ml) (lane 1) was incubated with 1 mM AAPH (lane 2) at PH 7.4 and 37°C for 3 hr, in the presence of 0.1 mg/ml SOD (lane 3), 0.1 mg/ml catalase (lane 4), 50 mM mannitol (lane 5), 10% ethanol (lane 6), 50 mM sodium azide (lane 7), 10 mM cysteine (lane 8), 10 mM 2-mercaptoethanol (lane 9), 10 mM BHA (10% acetonitrile) (lane 10), 50 mM triethylamine (lane 11), 50 mM triethylenediamine (lane 12), 50 mM TEMED (lane 13), 50 mM EDTA (lane 14), 50 mM DTPA (lane 15), 50 mM PBN (lane 16) and 50 mM DMPO (lane 17).

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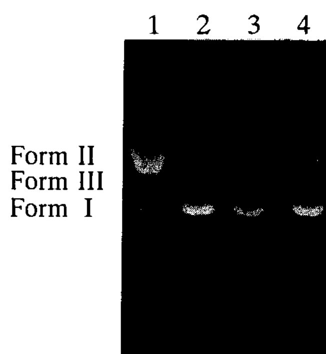

FIGURE 3 Agarose gel electrophoresis of supercoiled pBR 322 DNA treated with AAPH in the absence of oxygen. Supercoiled pBR 322 DNA (10 μg/ml) was incubated with 1 mM AAPH in the absence of oxygen and in the presence of none (lane 1), 10% ethanol (lane 2), 50 mM TEMED (lane 3) and 50 mM PBN (lane 4) at PH 7.4 and 37°C for 3 hr.

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carbon-centered radical initially generated may effectively cleave the DNA strands. Ethanol (lane 2), TEMED (lane 3) and PBN (lane 4) effectively inhibited the breaking. The carbon-centered radical may be converted into the non-reactive species by these scavenging agents. The result indicates that the carbon-centered radical was more reactive than oxygen-derived radicals.

ESR spin-trapping technique was employed in order to know what radical species were generated in the AAPH solution. When AAPH was incubated with PBN, spin signals as doublets of a triplet with hyperfine splitting constants of $a_N = 1.54$ mT and $a_H = 0.40$ mT appeared (Figure 4,A). The a_H value was similar to that of PBN-aliphatic hydrocarbon adduct,[22,23] and larger than that of PBN-OH[22,24,25] and PBN-OO*tert*Bu adducts.[22,26] The signals were slightly reduced but not greatly altered in incubation in the absence of oxygen (B), indicating no great contribution of oxygen in the radical generation. They were greatly reduced in incubation in the presence of ethanol (C) and TEMED (D). The results indicate that the carbon-centered radical was generated in the presence and absence of oxygen, which was scavenged by ethanol and TEMED, and the DNA breaking activity of AAPH was due to the carbon-centered radical. As control experiments for the spin-trapping experiment, hydroxyl radical generated by combination of hydrogen peroxide and ferrous ion was incubated with PBN,[25] to obtain signals of PBN-OH adduct as doublets of a triplet with $a_N = 1.63$ mT and $a_H = 0.25$ mT (E). The signals were transformed into those of PBN-hydroxyethyl adduct[22-24] with $a_N = 1.58$ mT and $a_H = 0.40$ mT by addition of ethanol (F), and into those characteristic to PBN-carbon adduct[22-24] with $a_N = 1.58$ mT and $a_N = 0.32$ mT by addition of TEMED (G).

When AAPH was incubated with DMPO, spin signals as a quartet with a relative intensity of 1:2:2:1 and with $a_N = a_H = 1.48$ mT (Figure 5,A) characteristic to DMPO-OH adduct[22,24] appeared. The signals were distinguishable from those of DMPO-hydrocarbon adduct[22,24] and from those of DMPO-OO*tert*Bu that should show doublets of a triplet with $a_N = 1.45$ mT and $a_H = 1.05$ mT.[22,27] The signals disappeared almost completely when the mixture was incubated in the absence of oxygen (B), indicating that oxygen was involved in the generation of the radical.

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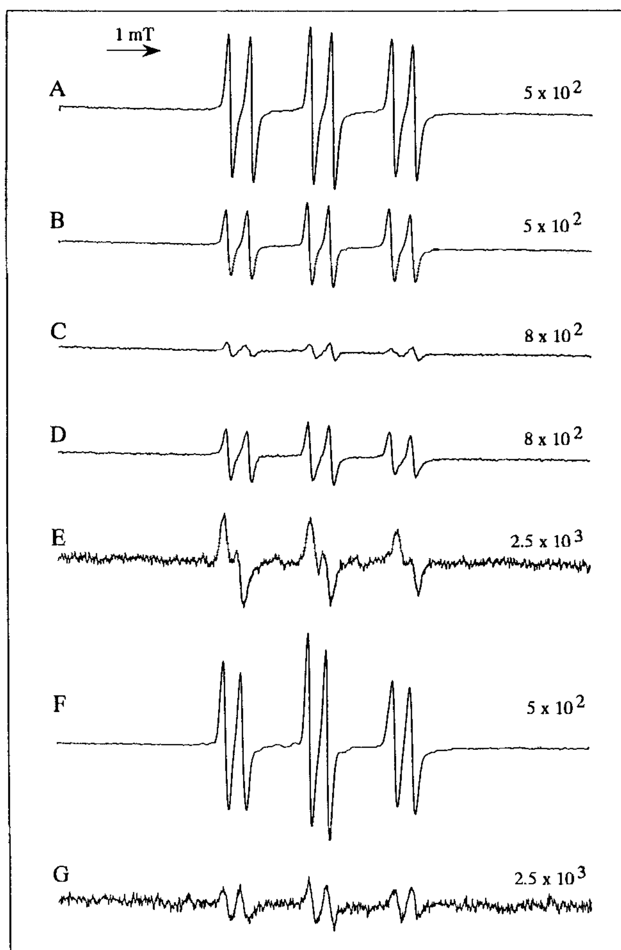

FIGURE 4 ESR spectra of the spin adducts of PBN and AAPH. A mixture of 0.1 M PBN and 50 mM AAPH in 0.1 M phosphate buffer (pH 7.4) + none (A), + N_2 (B), + 10% ethanol (C), and + 0.1 M TEMED (D) was incubated at 37°C for 3 hr. As a control experiment, a mixture of 0.1 M PBN and 1 mM H_2O_2-1 mM $FeSO_4$ in water + none (E), + 10% ethanol (F), and + 0.1 M TEMED (G) was incubated at room temperature for 5 min. Receiver gains of the recording are indicated in the figure. No ESR signals were observed for 0.1 M PBN in the buffer incubated similarly.

Addition of ethanol reduced the signals and gave new signals due to DMPO-hydroxyethyl adduct as doublets of a triplet with $a_N = 1.59$ mT and $a_H = 2.28$ mT (C).[22-24] The results clearly demonstrated that hydroxyl radical was generated in the AAPH solution only in the presence of oxygen. Addition of tertiary amines such as triethylenediamine (D), TEMED (E), EDTA (F) and DTPA (G) decreased the signals, indicating that these amines scavenged hydroxyl radical and/or the radical species generating hydroxyl radical. As control experiments for the spin trapping, hydroxyl radical generated from combination of hydrogen peroxide and ferrous ion was incubated with DMPO.[28] Signals due to DMPO-OH adduct (H)

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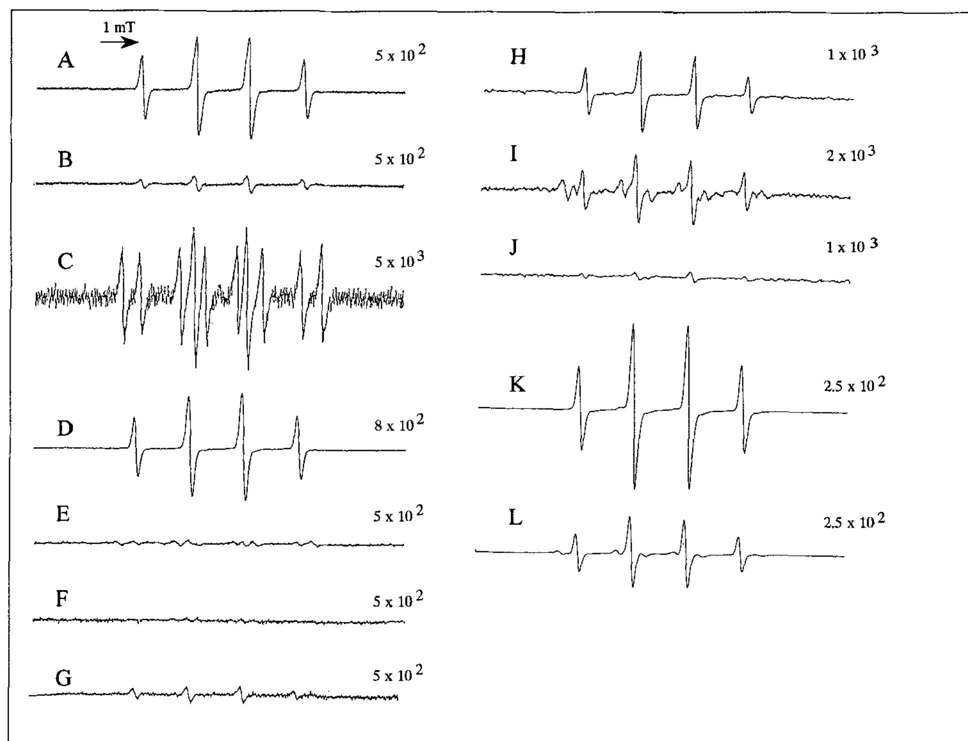

FIGURE 5 ESR spectra of the spin adducts of DMPO and AAPH. A mixture of 0.1 M DMPO and 50 mM AAPH in 0.1 M phosphate buffer (pH 7.4) + none (A), + N_2 (B), + 10% ethanol (C), +1 mM triethylenediamine (D), + 0.1 M TEMED (E), + 0.1 M EDTA (F), and + 0.1 M DTPA (G) was incubated at 37°C for 3 hr. As a control experiment, a mixture of 0.1 M DMPO and 1 mM H_2O_2-1 mM $FeSO_4$ in water + none (H), + 10% ethanol (I), + 1 mM triethylenediamine (J), + 1 mM EDTA (K), and + 0.2 M EDTA (L) was incubated at room temperature for 5 min. Receiver gains of the recording are indicated in the figure. No ESR signals were observed for 0.1 M DMPO in the buffer incubated similarly.

were reduced and new signals due to DMPO-hydroxyethyl adduct[22-24] with $a_N = 1.57\,mT$ and $a_H = 2.30\,mT$ appeared by addition of ethanol (I), and the signals were reduced by addition of triethylenediamine (J). The signals were enhanced at the low concentration of EDTA (K) by activating ferrous ion,[28] but reduced at the higher concentration of EDTA (L). The higher concentration of EDTA may act as a scavenger of hydroxyl radical as well as a masking agent of ferrous ion.

To detect singlet oxygen, AAPH was incubated with TEMP. TEMP can be converted by singlet oxygen into 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) detectable by ESR.[29,30] No ESR signals of TEMPO were observed in the incubation mixture.

By ESR spin-trapping with PBN, the carbon-centered radical was detected in the AAPH solution both in the presence and the absence of oxygen. Hydroxyl radical was detected by use of DMPO only in the presence of oxygen. Because AAPH effectively cleaved the DNA strands even in the absence of oxygen, the breaking

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activity may be primarily due to the aliphatic carbon-centered radical. The agents that scavenged the radical effectively prevented the DNA breaking.

DISCUSSION

It is considered that carbon-centered radicals react swiftly with oxygen and the peroxy radicals formed are more detererious to biomolecules than the parent carbon-centered radicals.[31,32] However, the formation of carbon-centered radicals from xenobiotics under physiological conditions and their importance have become recognized recently by application of the ESR spin-trapping technique. Cleavage of DNA strands by enediyne antitumor agents including neocarzinostatin has been extensively studied, and proposed to occur through a highly reactive diradical intermediates.[12] Generation of the carbon-centered radicals by the hemoglobin-catalyzed oxidation of 2-phenylethylhydrazine and peroxidase-catalyzed oxidation of methylhydrazine, and participation of the corresponding 2-phenylethyl and methyl radicals in the DNA damage have been shown.[13-15] On the other hand, there is a contradictory evidence showing that the carbon-centered radicals generated from hydrazines are less active than oxygen-derived radicals.[33] During the decomposition of diazoquinones or benzenediazonium salt, phenyl radicals which induce DNA strand breaking are formed.[16,17] In the present experiments, it was found that AAPH effectively cleaved the DNA strands by generating the aliphatic carbon-centered radical, and the reactivity of the carbon-centered radical may be higher than oxygen-derived hydroxyl radical because the carbon-centered radical cleaved the strands in the absence of oxygen. The carbon-centered radical can cleave the DNA strand without conversion into oxygen-derived radicals.

ESR spin-trapping technique demonstrated that the neutral aqueous solution of AAPH generated the aliphatic carbon-centered radical both in the presence and absence of oxygen. The carbon-centered radical could be trapped by PBN. However, by use of DMPO hydroxyl radical was detected only in the AAPH solution in the presence of oxygen. AAPH may initially decompose into the aliphatic carbon-centered radical as shown in Scheme 1, which may be rapidly converted into the peroxy radical in the presence of oxygen, although the peroxy radical could not be trapped in the present experiments. During breakdown process of the peroxy radical, hydroxyl radical may be generated. Since there are no evidences for the hydroxyl radical production during the decomposition of diazoquinones,[16] AAPH may be differently decomposed to generate hydroxyl radical.

Scavenging agents for hydroxyl radical and the carbon-centered radical were characterized. Ethanol is known to scavenge hydroxyl radical by donating hydrogen and converted into hydroxyethyl radical[24] as shown in Figure 4,F and 5,I. Ethanol was found effective to scavenge the carbon-centered radical (Figure 4,C), whereas similar scavenging effect for phenyl radicals has been shown.[16] Triethylenediamine is known to scavenge hydroxyl radical[21] as shown in Figure 5,J. Similar tertiary amines such as TEMED, EDTA and DTPA scavenged both the carbon-centered radical (Figure 4,D) and hydroxyl radical (Figure 4,G and 5,L). TEMED may scavenge hydroxyl radical by conversion into certain carbon-centered radical (Figure 4,G). It is interesting to note that, as well as triethylenediamine and TEMED, EDTA can act as a scavenger of hydroxyl radical.

In conclusion, AAPH effectively cleaved the DNA strands both in the presence and absence of oxygen. The aliphatic carbon-centered radical initially generated

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may be reactive to cleave the DNA strands, since the breaking activity was high in the absence of oxygen, and the trappers of the carbon-centered radicals effectively inhibited the breaking.

Acknowledgements

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Accepted by Professor E. Niki

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