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Radical production and DNA damage induced by carcinogenic 4-hydrazinobenzoic acid, an ingredient of mushroom *Agaricus bisporus*

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Abstract

4-Hydrazinobenzoic acid, an ingredient of mushroom *Agaricus bisporus*, is carcinogenic to rodents. To clarify the mechanism of carcinogenesis, we investigated DNA damage by 4-hydrazinobenzoic acid using ^{32}P-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes. 4-Hydrazinobenzoic acid induced Cu(II)-dependent DNA damage especially piperidine-labile formation at thymine and cytosine residues. Typical hydroxyl radical scavengers showed no inhibitory effects on Cu(II)-mediated DNA damage by 4-hydrazinobenzoic acid. Bathocuproine and catalase inhibited the DNA damage, indicating the participation of Cu(I) and H_2O_2 in the DNA damage. These findings suggest that H_2O_2 generated by the autoxidation of 4-hydrazinobenzoic acid reacts with Cu(I) to form reactive oxygen species, capable of causing DNA damage. Interestingly, catalase did not completely inhibit DNA damage caused by a high concentration of 4-hydrazinobenzoic acid (over 50 μM) in the presence of Cu(II). 4-Hydrazinobenzoic acid induced piperidine-labile sites frequently at adenine and guanine residues in the presence of catalase. 4-Hydrazinobenzoic acid increased formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), a characteristic oxidative DNA lesion, in calf thymus DNA, whereas 4-hydrazinobenzoic acid did not increase the formation of 8-oxodG in the presence of catalase. ESR spin-trapping experiments showed that the phenyl radical was formed during the reaction of 4-hydrazinobenzoic acid in the presence of Cu(II) and catalase. Matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF/mass) spectrometry analysis showed that phenyl radical formed adduct with adenosine and guanosine. These results suggested that 4-hydrazinobenzoic acid induced DNA damage via not only H_2O_2 production but also phenyl radical production. This study suggests that both oxidative DNA damage and DNA adduct formation play important roles in the expression of carcinogenesis of 4-hydrazinobenzoic acid.

Keywords: *DNA damage, 4-hydrazinobenzoic acid, 8-oxo-7, 8-dihydro-2′-deoxyguanosine, reactive oxygen species, copper, adduct formation*

Abbreviations: *8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DTPA, diethylenetriamine-N,N,N′,N″,N″-*pentaacetic acid; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; HPLC-ECD, electrochemical detector coupled to HPLC; $^{\cdot}$OH, hydroxyl free radical; DMSO, dimetyl sulfate; ESR, electron spin resonance; MALDI-TOF/mass, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Introduction

The cultivated mushroom *Agaricus bisporus* diet gave rise to a statistically significant incidence of tumors in the lung, blood vesseles, cecum and colon in mice [1–5]. *A. bisporus* is known to contain several hydrazines and a diazonium ion [4,6]. Among these compounds the most notable are β-*N*-[γ-L(+)-glutamyl] hydroxymethylphenylhydrazine

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(agaritine), its breakdown product 4-hydroxymethylphenylhydrazine, β-*N*-[γ-L(+)-glutamyl]-4-carboxyphenylhydrazine, 4-(hydroxymethyl) benzenediazonium ion. It is reported that 4-hydroxymethylphenylhydrazine and 4-(hydroxymethyl)benzenediazonium ion induce cancers in mice [7,8]. 4-Hydroxymethylphenylhydrazine showed a distinct direct-acting mutagenicity with the strain TA1537 and with TA97 [9]. 4-(hydroxymethyl) Benzenediazonium ion also showed the highest mutagenic activity with TA1537 [9]. In addition, Hiramoto et al. demonstrated that the carbon-centered radical generated from 4-(hydroxymethyl) benzenediazonium salt caused DNA base and deoxyribose modification [10,11].

It was reported that *A. bisporus* contains 4-hydrazinobenzoic acid, another nitrogen–nitrogen bonded chemical, at a level of 10 μg/g of wet mushroom weight [12]. 4-Hydrazinobenzoic acid is a precursor of the biosynthesis of agaritine in mushroom and induces aortic smooth muscle tumors [13]. It has been shown that 4-hydrazinobenzoic acid is mutagenic in the Salmonella assay (Ames test) [14]. Thus, 4-hydrazinobenzoic acid is an carcinogenic ingradient of the widely consumed mushroom *A. bisporus*. However, the mechanism of DNA damage to elicit carcinogenicity by 4-hydrazinobenzoic acid has not been clarified.

To clarify the mechanism of the carcinogenesis, we examined DNA damage induced by 4-hydrazinobenzoic acid using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* and *p16* tumor suppressor genes. We analyzed the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation in calf thymus DNA by 4-hydrazinobenzoic acid in the presence of Cu(II) using an electrochemical detector coupled to an HPLC (HPLC-ECD). A characteristic oxidative DNA lesion, 8-oxodG, has attracted much attention in relation to mutagenesis and carcinogenesis [15,16]. We studied the participation of free radicals in the reaction of 4-hydrazinobenzoic acid and Cu(II) using electron spin resonance (ESR) spin-trapping technique. We also analyzed adduct formation by using matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF/mass) spectrometry.

Materials and methods

Materials

T4 polynucleotide kinase was purchased from New England Biolabs (Beverly, MA). Restriction enzymes and alkaline phosphatase from calf intestine were from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]-ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). Calf thymus DNA, catalase (45,000 units/mg from bovine liver) and superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) were from Sigma Chemical Co (St. Louis, MO). Acrylamide, bisacrylamide, and piperidine were from Wako Chemical Industries, Ltd. (Osaka, Japan). Sodium formate, ethanol, D-mannitol and $CuCl_2$ were from Nacalai Tesque, Inc., (Kyoto, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co., (Kumamoto, Japan). 4-Hydrazinobenzoic acid was from Research Chemicals (Toronto, Canada).

Preparation of ^{32}P-labeled DNA fragments obtained from the p16 *and* p53 *tumor suppressor genes*

Two fragments containing exon 1 or 2 of the human *p16* tumor suppressor gene [17] were obtained as described previously [18]. The ^{32}P-5′-end-labeled 490-base pair fragment (*Eco* R I⋆5841-*Eco* R I⋆ 6330) containing exon 1 was further digested with *Mro* I to obtain the singly labeled 328-base pair fragment (*Eco* R I⋆ 5841-*Mro* I 6168) and the 158-base pair fragment (*Mro* I 6173-*Eco* R I⋆ 6330). The 5′ end-labeled 460-base pair fragment (*Eco* R I⋆ 9481-*Eco* R I⋆ 9940) containing exon 2 was also further digested with *Bss* H II to obtain the singly labeled 309-base pair fragment (*Eco* R I⋆ 9481-*Bss* H II 9789) and the 147-base pair fragment (*Bss* H II 9794-*Eco* R I⋆ 9940).

DNA fragments were also obtained from the human *p53* tumor suppressor gene [19]. The ^{32}P-5′-end-labeled 650-base pair (*Hind* III⋆ 13972-*Eco* R I⋆ 14621) and 460-base pair (*Hind* III⋆ 13038-*Eco* R I⋆ 13507) fragments were obtained as described previously [20]. The 650-base pair fragment was digested with *Apa* I to obtain the singly labeled 211-base pair (*Hind* III⋆ 13972-*Apa* I 14182) and the 443-base pair (*Apa* I 14179-*Eco* R I⋆ 14621) DNA fragments. The 460-base pair fragment was digested with *Sty* I to obtain the singly labeled 118-base pair (*Hind* III⋆ 13038-*Sty* I 13155) and the 348-base pair (*Sty* I 13160-*Eco* R I⋆ 13507) fragments. An asterisk indicates ^{32}P-labeling.

Detection of damage to isolated ^{32}P-labeled DNA fragments induced by 4-hydrazinobenzoic acid in the presence of Cu(II)

The standard reaction mixtures in microtubes (1.5-ml Eppendorf) containing ^{32}P-labeled DNA fragment, 20 μM/base of calf thymus DNA, 4-hydrazinobenzoic acid and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated for 60 min at 37°C. The DNA fragments were heated in 1 M piperidine for 20 min at 90°C. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel as described previously [21,22]. The autoradiogram was obtained by exposing an X-ray film to the gel. The preferred cleavage sites were determined by directly comparing the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure [23], using a DNA

sequencing system (LKB 2010 Macrophor). The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL).

Analysis of 8-oxodG formation in calf thymus DNA induced by 4-hydrazinobenzoic acid in the presence of Cu(II)

Measurement of 8-oxodG in calf thymus DNA was performed as described previously [24]. The reaction mixtures containing 100 μM/base of calf thymus DNA, 4-hydrazinobenzoic acid and 20 μM $CuCl_2$ in 4 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA were incubated at 37°C. After ethanol precipitation, DNA was digested into nucleosides with nuclease P_1 and calf intestine phosphatase. The amount of 8-oxodG was measured with an HPLC-ECD [25].

ESR spectra measurement

ESR spectra were measured at room temperature using a JES-TE100 (JEOL, Tokyo, Japan) spectrometer with 300-kHz field modulation according to the method described previously [26]. Spectra were recorded with a microwave power of 16 mW and a modulation amplitude of 0.1 mT. The magnetic fields were calculated by the splitting of Mn(II) in MgO ($\Delta H_{3-4} = 8.69$ mT). DMPO was used as a radical trapping reagent. Reaction mixtures, containing 500 μM 4-hydrazinobenzoic acid, 20 μM $CuCl_2$, 50 units of catalase and 50 mM nucleotide (dGMP, dAMP, TMP or CMP), were incubated for 20 min at 37°C.

MALDI-TOF/mass analysis

All MALDI analyses were performed with a Voyager B-RP (PerSeptive Biosystems, Framingham, MA). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. All spectra were acquired in reflector mode at 20 kV for ion source. A single acquisition run was the sum of at least 5 series with 100 total added shots. Mass spectra were analyzed using Data Explorer (PerSeptive Biosystems). Reaction mixture, containing 500 μM 4-hydrazinobenzoic acid, 20 μM $CuCl_2$ and 5 mM nucleosides (adenosine, guanosine, cytidine or thymidine), were incubated for 24 h at 37°C, and then air-dried on a stainless-steel probe tip. α-Cyano-4-hydroxycinnamic acid solution as a matrix was added to the sample.

Results

Damage to ^{32}P-labeled DNA fragments by 4-hydrazinobenzoic acid in the presence of Cu(II)

Figure 1A shows the autoradiogram of DNA fragments treated with 4-hydrazinobenzoic acid in the presence of Cu(II). 4-Hydrazinobenzoic acid caused DNA damage in the presence of Cu(II). The intensity of the DNA damage increased with the 4-hydrazinobenzoic acid concentration. Furthermore, in the presence of catalase, a high concentration of 4-hydrazinobenzoic acid caused Cu(II)-mediated DNA damage (Figure 1B). DNA damage was enhanced by piperidine treatment, suggesting that 4-hydrazinobenzoic acid caused not only DNA strand breakage but also base modification (data not shown). Neither 4-hydrazinobenzoic acid nor Cu(II) alone caused DNA damage. In addition, 4-hydrazinobenzoic acid did not cause DNA damage in the presence of Fe(III), Fe(III)EDTA or Fe(III) citrate (date not shown).

Effects of scavengers on DNA damage induced by 4-hydrazinobenzoic acid in the presence of Cu(II)

Figure 2 shows the effects of scavengers and a Cu(I)-specific chelator, bathocuproine, on DNA damage by 4-hydrazinobenzoic acid in the presence of Cu(II). Bathocuproine and catalase inhibited the DNA damage caused by 20 μM 4-hydrazinobenzoic acid, suggesting the involvement of H_2O_2 and Cu(I). Typical hydroxyl free radical (˙OH) scavengers, such as ethanol, mannitol and sodium formate did not inhibit DNA damage. DMSO showed little effect on DNA damage. However, the DNA damage was

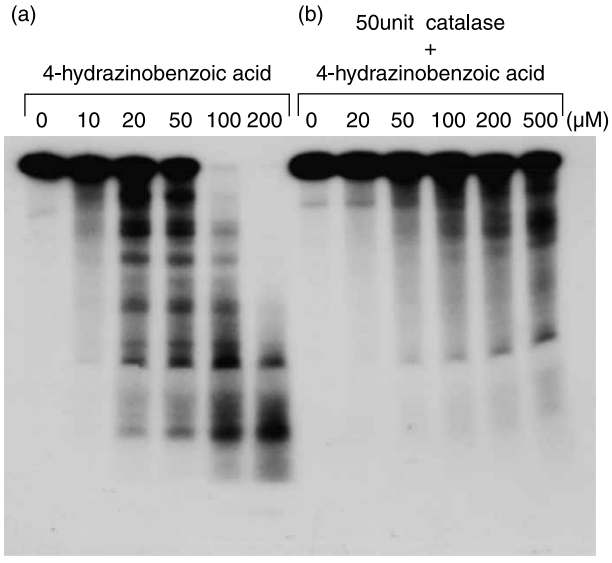

Figure 1. Autoradiogram of ^{32}P-labeled DNA fragment incubated with 4-hydrazinobenzoic acid plus Cu(II) in the presence or absence of catalase. The reaction mixture contained the ^{32}P-5′-end-labeled 211-base pair fragment, 20 μM/base of calf thymus DNA, the indicated concentrations of 4-hydrazinobenzoic acid and 20 μM $CuCl_2$ in 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated for 60 min at 37°C. The DNA fragments were treated with piperidine and electrophoresed on a polyacrylamide gel.

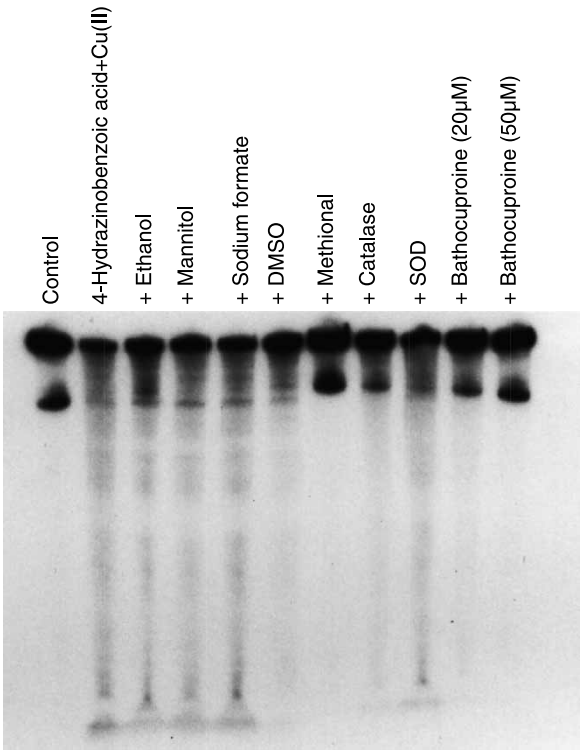

Figure 2. Effects of ˙OH scavengers, catalase and bathocuproine on DNA damage induced by 4-hydrazinobenzoic acid in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 211-bp fragment, 20 μM/base of calf thymus DNA, 20 μM 4-hydrazinobenzoic acid, 20 μM $CuCl_2$, and scavenger in 200 μl of 10 mM sodium phosphate buffer at pH 7.8 containing 5 μM DTPA. Scavenger was added where indicated. After the incubation at 37°C for 60 min, followed by the piperidine treatment, the treated DNA fragments were analyzed by the method described in Figure 1. Lane 1, control; lane 2, no scavenger; lane 3, 0.8 M ethanol; lane 4, 0.1 M mannitol; lane 5, 0.1 M sodium formate; lane 6, 0.8 M DMSO; lane 7, 1.0 M methional; lane 8, 30 units of catalase; lane 9, 30 units of SOD; lane 10, 20 μM bathocuproine; lane 11, 50 μM bathocuproine.

inhibited by methional, which can scavenge not only ˙OH but also other radicals like crypto-hydroxyl radicals [27]. SOD did not affect the DNA damage.

In the presence of catalase, bathocuproine inhibited Cu(II)-mediated DNA damage caused by a high concentration (200 μM) of 4-hydrazinobenzoic acid (data not shown). ˙OH scavengers and methional did not inhibit DNA damage. DMSO partially inhibited DNA damage. These results indicate that Cu(I) is necessary for DNA damage induced by a high concentration of 4-hydrazinobenzoic acid and Cu(II) in the presence of catalase.

Site specificity of DNA damage induced by 4-hydrazinobenzoic acid

The patterns of DNA cleavage induced by 4-hydrazinobenzoic acid in the presence of Cu(II) were determined by DNA sequencing using the Maxam-Gilbert procedure [24]. The relative intensity of DNA cleavage obtained by scanning autoradiogram with a laser densitometer is shown in Figures 3 and 4. 4-Hydrazinobenzoic acid induced piperidine-labile sites frequently at thymine and cytosine residues in double-stranded DNA fragments obtained from the human *p53* (Figure 3A) and *p16* (Figure 4A) tumor suppressor genes. Catalase inhibited site-specific DNA damage at thymine and cytosine residues induced by 4-hydrazinobenzoic acid and Cu(II) via H_2O_2 formation. When denatured single-stranded DNA was used, DNA cleavage increased and occurred at guanine and adenine residues (Figure 3B). Interestingly, when catalase was added, a high concentration of 4-hydrazinobenzoic acid with Cu(II) induced piperidine-labile sites frequently at adenine, guanine and cytosine residues in double-stranded DNA fragments (Figure 4B).

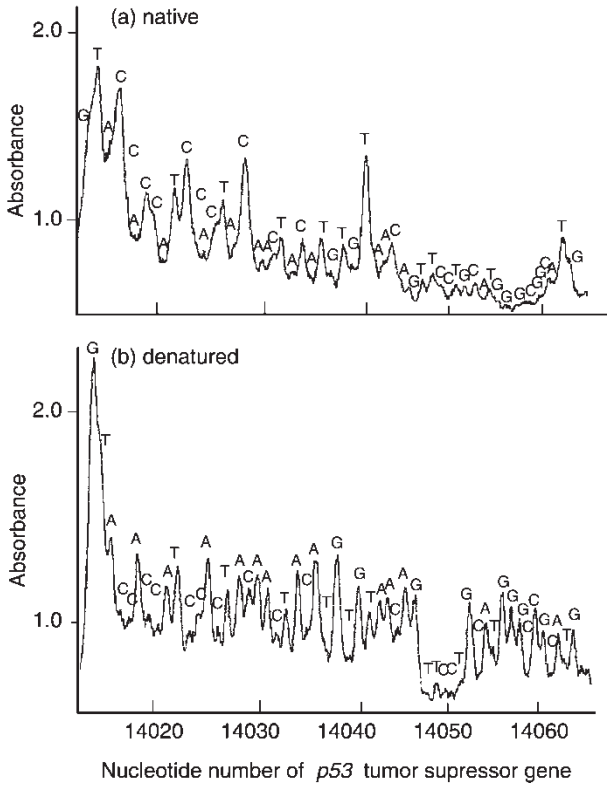

Figure 3. Site specificity of DNA cleavage induced by 4-hydrazinobenzoic acid in the presence of Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 211-base pair (HindIII* 13972-ApaI 14182) DNA fragment, 20 μM/base of calf thymus DNA, 30 μM 4-hydrazinobenzoic acid and 20 μM $CuCl_2$ in 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated for 60 min at 37°C. For the experiment with denatured DNA (b), DNA was treated at 90°C for 10 min and quickly chilled before the addition of 4-hydrazinobenzoic acid and Cu(II). After the piperidine treatment, the DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing film to the gel.

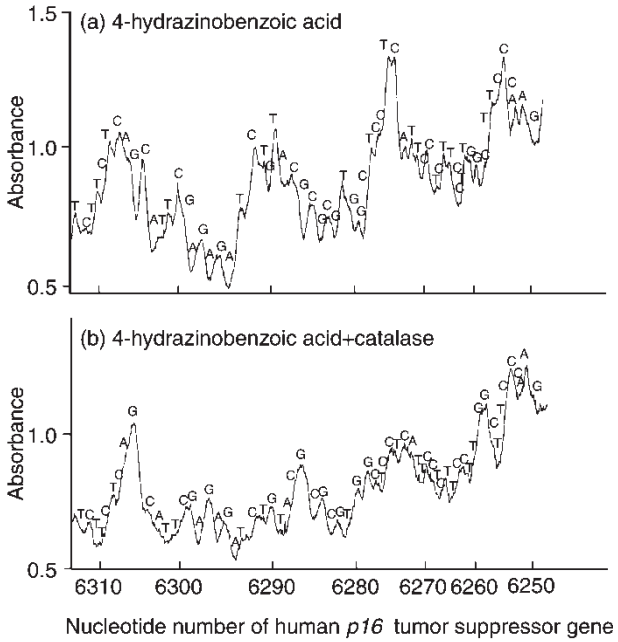

Figure 4. Site specificity of DNA cleavage induced by 4-hydrazinobenzoic acid and Cu(II) in the presence or absence of catalase. The reaction mixture contained the ^{32}P-5′-end-labeled 158-base pair fragment (Mro I 6173-EcoR I⋆ 6330), 20 μM/base of calf thymus DNA, 30 μM (a) or 500 μM (b) 4-hydrazinobenzoic acid, 50 units of catalase (b) and 20 μM $CuCl_2$ in 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixtures were incubated for 60 min at 37°C. After the piperidine treatment, the DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing film to the gel.

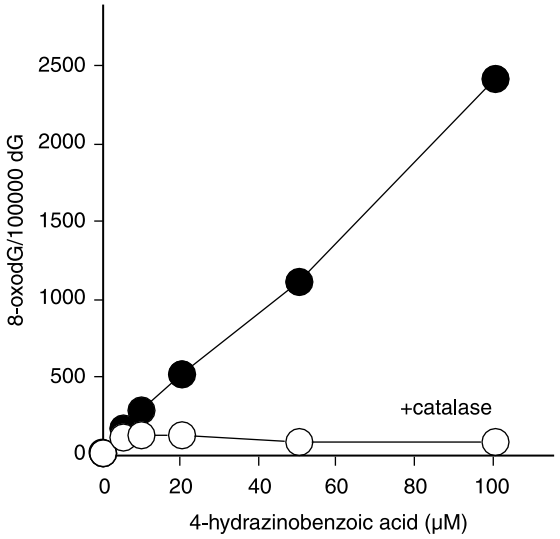

Figure 5. Formation of 8-oxodG in calf thymus DNA induced by 4-hydrazinobenzoic acid and Cu(II) in the presence or absence of catalase. The reaction mixture containing 100 μM/base of calf thymus DNA, the indicated concentrations of 4-hydrazinobenzoic acid, 20 μM $CuCl_2$ and 5 μM DTPA with or without 50 units of catalase in 400 μl of 4 mM sodium phosphate buffer (pH 7.8) was incubated for 60 min at 37°C. After ethanol precipitation, the DNA was subjected to enzyme digestion and analyzed by HPLC-ECD as described under "Materials and Methods".

Formation of 8-oxodG in calf thymus DNA by 4-hydrazinobenzoic acid in the presence of Cu(II)

Figure 5 shows the 8-oxodG formation in calf thymus DNA treated with 4-hydrazinobenzoic acid in the presence of Cu(II). The formation of 8-oxodG increased with the concentration of 4-hydrazinobenzoic acid. The yield of 8-oxodG was enhanced by DNA denaturation (data not shown). In the presence of catalase, 4-hydrazinobenzoic acid did not increase 8-oxodG formation.

Production of phenyl radical during the reaction of 4-hydrazinobenzoic acid with Cu(II) in the presence of catalse

ESR spin-trapping method was used to detect free radicals produced during the reaction of 4-hydrazinobenzoic acid with Cu(II) in the presence of catalase. Figure 6A shows an ESR spectrum of a spin adduct observed when 4-hydrazinobenzoic acid was added to a buffer solution containing DMPO, Cu(II) and catalase. The signals ($a_N = 1.600$ mT, $a_H = 2.205$ mT) can be assigned to phenyl radical (benzoic acid ring C radical) adduct of DMPO by the reference to the hyperfine splitting constants previously reported [28]. To examine the reactivity of the radical to DNA, effects of mononucleotides on the radical formation were observed. Addition of dGMP (Figure 6B), dAMP (Figure 6C) or CMP (Figure 6D) induced a decrease in the signal, whereas TMP (Figure 6E) had no significant effects on the intensity of the signal. This result suggests that phenyl radical can react with the adenine, guanine and cytosine.

Adduct formation of phenyl radical with adenosine and guanosine

A MALDI-TOF/mass spectra of the products was observed when adenosine or guanosine was added to a mixture solution of 4-hydrazinobenzoic acid, Cu(II) and catalase (Figure 7). The product obtained from the reaction mixture of 4-hydrazinobenzoic acid and adenosine showed the mass spectrum with molecular ion (M + 1) at m/z 388.41, which is assigned that phenyl radical formed adduct with adenosine (Figure 7A). The peak at m/z 404.26 in Figure 7B can be assigned to phenyl radical formed adduct with guanosine. However, adduct formation of phenyl radical with cytidine or thymidine was not observed.

Discussion

In this study, we demonstrated that 4-hydrazinobenzoic acid caused DNA damage in the presence of

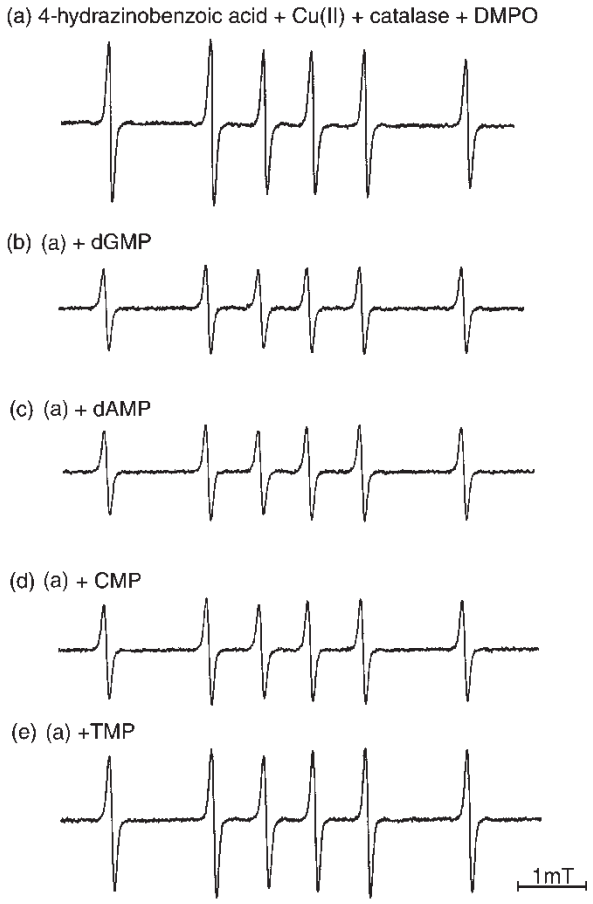

Figure 6. ESR spectra of the spin adduct of DMPO produced by 4-hydrazinobenzoic acid in the presence of Cu(II) and catalase. Reaction mixture containing 500 μM 4-hydrazinobenzoic acid, 10 mM DMPO, 100 unit of catalase, 50 mM nucleotide, 20 μM $CuCl_2$ and 5 μM DTPA in 100 μl of 10 mM sodium phosphate buffer at pH 7.8 was incubated for 20 min at 37°C. After incubation, ESR spectra were measured at room temperature as described under "Materials and Methods" section. (a) 4-hydrazinobenzoic acid + $CuCl_2$ + catalase + DMPO; (b) dAMP was added in (a); (c) dGMP was added in (a); (d) CMP was added in (a); (e) TMP was added in (a).

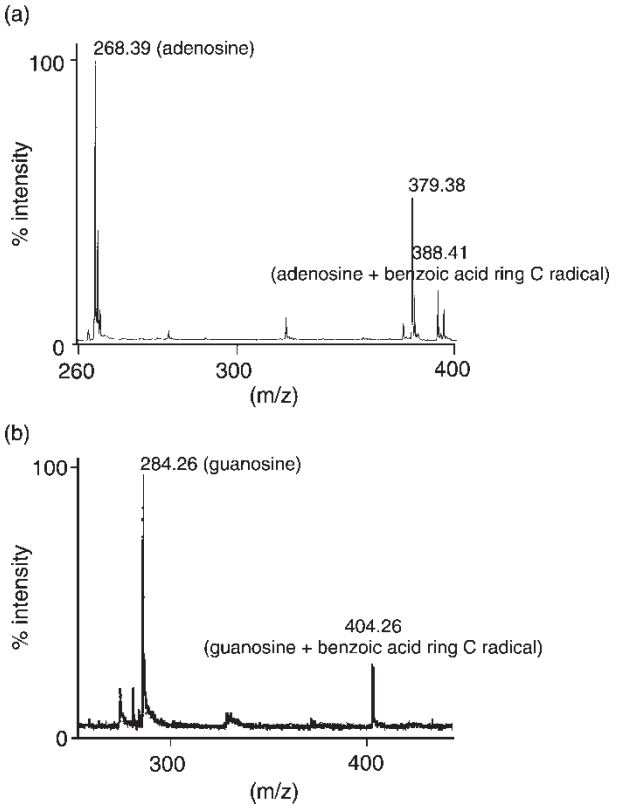

Figure 7. Adduct formation of phenyl radical with adenosine and guanosine. Reaction mixture, containing 500 μM 4-hydrazinobenzoic acid, 5 mM nucleoside (adenosine (a) or guanosine (b)), 20 μM $CuCl_2$ and 100 unit of catalase, was incubated for 24 h at 37°C, and then air-dried on a stainless-steel probe tip. α-Cyano-4-hydroxycinnamic acid solution was added to the sample. TOF/mass analysis was performed on a Voyager B-RP (PerSeptive Biosystems) equipped with a nitrogen laser (337 nm, 3 ns pulse).

Cu(II). The effect of piperidine treatment suggested that 4-hydrazinobenzoic acid plus Cu(II) induced not only strand breakage but also base damage. 4-Hydrazinobenzoic acid plus Cu(II) caused site-specific DNA damage at thymine and cytosine residues. We also observed the increase of 8-oxodG, which is piperidine-inert site, in calf thymus DNA treated with 4-hydrazinobenzoic acid plus Cu(II). In order to clarify what kinds of reactive oxygen species cause oxidative DNA damage, the effects of various scavengers on the DNA damage were examined. Inhibitory effects of catalase and bathocuproine on the DNA damage indicated that H_2O_2 and Cu(I) were involved in the DNA damage process. Typical $^{\cdot}OH$ scavengers showed little or no inhibitory effect on the DNA damage, whereas methional inhibited it. Methional scavenges not only $^{\cdot}OH$ but also a variety of reactive species other than $^{\cdot}OH$ [27]. From these results, we considered that reactive species such as Cu(I)-hydroperoxo complex participate in the DNA damage by 4-hydrazinobenzoic acid (Figure 8).

Interestingly, a high concentration of 4-hydrazinobenzoic acid induced Cu(II)-mediated DNA damage even in the presence of catalase. Catalase is an antioxidant enzyme that plays a very important role in the protection against oxidative damage by breaking down H_2O_2. A high concentration of 4-hydrazinobenzoic acid caused site-specific DNA cleavage at adenine and guanine residues in the presence of catalase. Typical $^{\cdot}OH$ scavengers and methional showed little or no inhibitory effect on the DNA damage. A high concentration of 4-hydrazinobenzoic acid with Cu(II) did not increase the formation of 8-oxodG in the presence of catalase. Therefore, it is considered that DNA damage induced by a high concentration of 4-hydrazinobenzoic acid in the presence of catalase is not oxidative damage. To clarify the mechanism of the DNA damage induced by a high concentration of 4-hydrazinobenzoic acid plus Cu(II) in the presence of

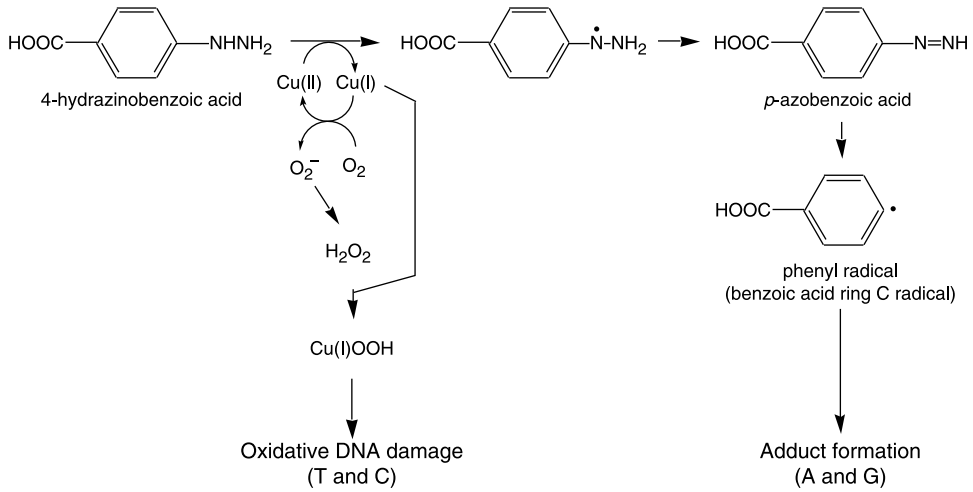

Figure 8. Possible mechanisms of DNA damage induced by 4-hydrazinobenzoic acid in the presence of Cu(II).

catalase, we examined ESR spin-trapping experiments and MALDI-TOF/ mass analysis. ESR spin-trapping experiments showed that autoxidation of 4-hydrazinobenzoic acid produced the phenyl radical in the presence of Cu(II) and catalase. The spin-signals obtained by the reaction of 4-hydrazinobenzoic acid with Cu(II) and catalase was decreased by addition of dAMP, dGMP and CMP. These results suggested that the phenyl radical reacted with dAMP, dGMP and CMP. Furthermore, we demonstrated that phenyl radical formed adducts with adenine and guanosine. Relevantly, Hiramoto et al. [10] reported that carbon-centered 4-(hydroxymethyl)phenyl radical generated by removal of nitrogen molecule of 4-(hydroxymethyl)benzenediazonium salt can directly attack at the 8-positions of the purine base moieties of DNA. On the basis of this literature and our results, we propose possible mechanism of adduct formation by 4-hydrazinobenzoic acid as shown in Figure 8. 4-Hydrazinobenzoic acid is oxidized into the *p*-azobenzoic acid in the presence of Cu(II). It is considered that phenyl radical (benzoic acid ring C radical) generated by removal of nitrogen molecule of *p*-azobenzoic acid reacts with adenosine or guanosine to form the adduct.

Copper is present in the nuclei of mammalian cells and may participate in the association of DNA with histones to form high order chromatin structures [29]. Several studies have demonstrated that the metal-binding histone reacts with H_2O_2, leading to oxidative damage to a nucleobase [30–32]. In addition, Midorikawa et al. revealed that histone peptide enhanced DNA damage induced by H_2O_2 and Cu(II) [33]. Furthermore, it is reported that endogenous metals, copper and iron, catalyze ROS generation from various organic carcinogens, resulting in oxidative DNA damage [34]. Therefore, it is speculated that the copper-mediated DNA damage induced by 4-hydrazinobenzoic acid occurs in human body.

Hydrazine derivatives have been implicated as mutagens and carcinogens [35–37]. It is reported that the levels of 8-oxodG were significantly higher in liver DNA from phenylhydrazine-treated rats as compared to untreated controls [38]. It has also been reported that 1,2-dimethylhydrazine (DMH), a specific colon carcinogen with DNA-methylating activity, is also an inducer of DNA oxidative damage [39,40]. Since we are much interested in the property of hydrazine derivatives to generate a variety of free radicals and reactive oxygen species in relation to mutational hotspots, we have studied the mechanism of sequence-specific DNA damage induced by various hydrazine derivatives [21,22,26,35,41,42]. In this study, we demonstrated that 4-hydrazinobenzoic acid, a hydrazine found in the mushroom *A. bisporus*, caused both oxidative DNA damage via H_2O_2 production and DNA adduct formation via phenyl radical production in the presence of catalase. These results suggest that both oxidative DNA damage and DNA adduct formation play important roles in the expression of carcinogenesis of 4-hydrazinobenzoic acid. It is considered that phenyl radical are involved in tumor initiation by causing adduct formation and oxidative DNA damage induced by 4-hydrazinobenzoic acid plus Cu(II) participates in tumor promotion and/or progression. Further examination is required to investigate the correlation between 4-hydrazinobenzoic acid and carcinogenesis.

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