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Copper-Dependent DNA Damage Induced by Hydrazobenzene, an Azobenzene Metabolite

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Hydrazobenzene is carcinogenic to rats and mice and azobenzene is carcinogenic to rats. Hydrazobenzene is a metabolic intermediate of azobenzene. To clarify the mechanism of carcinogenesis by azobenzene and hydrazobenzene, we investigated DNA damage induced by hydrazobenzene, using ^{32}P-5′-end-labeled DNA fragments obtained from the c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. Hydrazobenzene caused DNA damage in the presence of Cu(II). Piperidine treatment enhanced the DNA damage greatly, suggesting that hydrazobenzene caused base modification and liberation. However, azobenzene did not cause DNA damage even in the presence of Cu(II). Hydrazobenzene plus Cu(II) caused DNA damage frequently at thymine residues. Catalase and a Cu(I)-specific chelator inhibited Cu(II)-mediated DNA damage by hydrazobenzene. Typical $^{\bullet}$OH scavengers did not inhibit the DNA damage. The main active species is probably a metal oxygen complex, such as Cu(I)–OOH. Formation of 8-oxo-7, 8-dihydro-2′-deoxyguanosine was increased by hydrazobenzene in the presence of Cu(II). Oxygen consumption and UV–Visible spectroscopic measurements have shown that hydrazobenzene is autoxidized to azobenzene with H_2O_2 formation. It is considered that the metal-mediated DNA damage by hydrazobenzene through H_2O_2 generation may be relevant for the expression of carcinogenicity of azobenzene and hydrazobenzene.

Keywords: Hydrazobenzene, azobenzene, DNA damage, copper, hydrogen peroxide, carcinogenicity

Abbreviations: 8-oxodG, 8-oxo-7, 8-dihydro-2′-deoxyguanosine; HPLC–ECD, an electrochemical detector coupled to a high performance liquid chromatography; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; SOD, superoxide dismutase

INTRODUCTION

Azo dyes are widely used in the textile, printing, and food-processing industries and in laboratories. There are often results showing their mutagenicity and carcinogenicity.[1] It is well known that amino azo dyes such as dimethylaminoazobenzene (butter yellow) and *o*-aminoazotoluene are carcinogenic to rodents and dogs. In these amino azo dyes, an exocyclic amino group is the key to any carcinogenicity, because this group undergoes biochemical N-oxidation, further

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conversion to reactive electrophiles to form DNA adducts.[2] On the other hand, azobenzene is a potent rat carcinogen,[3] although it has no exocyclic amino group that covalently reacts with DNA. The exocyclic amino group depending DNA adducts formation[2] cannot explain the carcinogenicity of azobenzene.

Hydrazobenzene is a metabolite of azobenzene.[4] Hydrazobenzene used in dye manufacturing industries is carcinogenic to rats and mice. Bioassay program by National Cancer Institute revealed that hydrazobenzene caused hepatocellular carcinoma and Zymbal's gland squamous-cell neoplasms in male Fisher 344 rats and neoplastic nodules of the liver and mammary adenocarcinomas in female Fisher 344 rats.[5] In female $B6C3F_1$ mice, hepatocellular carcinomas were induced.[5]

To clarify the mechanism of carcinogenesis by azobenzene and hydrazobenzene, we examined DNA damage induced by azobenzene and hydrazobenzene, using ^{32}P-5′-end-labeled DNA fragments obtained from the c-Ha-*ras*-1 proto-oncogene and the *p53* tumor suppressor gene. In addition, we measured the content of 8-oxodG, a marker of oxidative DNA damage, in calf thymus DNA with an electrochemical detector coupled to a high performance liquid chromatography (HPLC–ECD). It has been reported that 8-oxodG formation can lead to DNA misreplication resulting in mutation and cancer.[6,7]

MATERIALS AND METHODS

Materials

Restriction enzymes (*Bam*HI, *Apa*I, *Hin*dIII, *Eco*RI, *Pst*I and *Ava*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear. Alkaline phosphatase from calf intestine was purchased from Boehringer Mannheim GmbH. Hydrazobenzene, aniline, dimethyl sulfoxide and piperidine were from Wako Chemical Industries Ltd. (Osaka, Japan). Sodium formate, ethanol, D-mannitol and copper(II) chloride dihydrate 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). Calf thymus DNA, superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co. (St. Louis, MO, USA). Nuclease P_1 (400 units/mg) was from Yamasa Shoyu Co. (Chiba, Japan). Azobenzene and methional were from Tokyo Kasei Co. (Tokyo, Japan).

Preparation of ^{32}P-5′-End-Labeled DNA Fragments Obtained from the *p53* and the c-Ha-*ras*-1 Genes

DNA fragments were obtained from the human *p53* tumor suppressor gene containing exons.[8] DNA fragments from the *p53* gene containing exons (exon 5, 13055–13238; exon 6, 13320–13432; exon 7, 14000–14109; exon 8, 14452–14588) were amplified by the PCR method using an Omnigene Temperature Cycling System. The PCR products were digested with *Sma*I and ligated into *Sma*I-cleaved pUC18 plasmid, and then transferred to *Escherichia coli* JM109. The plasmid pUC18 was digested with *Eco*RI and *Hin*dIII, and the resulting DNA fragments were fractionated by electrophoresis on a 2% agarose gel. The 5′-end-labeled 650-bp (*Hin*dIII* 13972-*Eco*RI* 14621) fragment was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The asterisk indicates ^{32}P-labeling. The 650-bp fragment was digested with *Apa*I into a singly labeled 443-bp (*Apa*I 14179-*Eco*RI* 14621) fragment and a 211-bp fragment (*Hin*dIII* 13972-*Apa*I 14182).[9] DNA fragment was also obtained from the human c-Ha-*ras*-1 protooncogene.[10] DNA fragment was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal

DNA segment containing the human c-Ha-*ras*-1 protooncogene.[11,12] A singly labeled 337-bp (*Pst*I 2345-*Ava*I* 2681) fragment was obtained according to the method described previously.[11] The nucleotide numbering starts with the *Bam*HI site.[10]

Detection of DNA Damage

The standard reaction mixture (in a microtube; 1.5 ml; Eppendorf) contained metal ions, ethanol solution of indicated concentration of hydrazobenzene, azobenzene or aniline, the ^{32}P-labeled DNA fragment and 25 μM/base of sonicated calf thymus DNA in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C for indicated duration, the DNA fragment was heated at 90°C in 1 M piperidine for 20 min and treated as described previously.[13] The preferred cleavage sites were determined by direct comparison of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam–Gilbert procedure[14] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used for the measurement of the relative amounts of oligonucleotides from the treated DNA fragments.

Measurement of 8-oxodG Formation

Calf thymus DNA fragments (100 μM/base) were incubated with ethanol solution of indicated concentration of hydrazobenzene or azobenzene in the presence of $CuCl_2$ in 400 μl of 4 mM sodium phosphate buffer (pH 7.8), containing 2.5 μM DTPA, at 37°C for indicated duration. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed with a HPLC–ECD, as described previously.[15]

UV–Visible Spectra Measurement

UV–Visible spectra of hydrazobenzene were measured with a spectrometer (UV-2500PC Shimadzu, Kyoto, Japan). The samples contained 100 μM of hydrazobenzene or azobenzene, in 10 mM sodium phosphate buffer (pH 7.8) containing 10% (v/v) ethanol and 2.5 μM DTPA. The reaction was started by the addition of 20 μM $CuCl_2$. Spectral tracings of the reaction mixture were recorded every 9 min at 37°C.

Measurement of Oxygen Consumption

Oxygen consumption by the reactions of hydrazobenzene with Cu(II) was measured using a Clarke oxygen electrode (Electronic Stirrer Model 300, Rank Brothers LTD. Bottisham Cambridge, England). The reactions were performed in the mixture containing hydrazobenzene and 10% (v/v) of ethanol in 2 ml of 10 mM sodium phosphate buffer (pH 7.8), containing 2.5 μM DTPA, and were started by the addition of $CuCl_2$ into the chamber of the oxygen electrode. Catalase was added to detect H_2O_2 generation resulting from oxygen consumption.

RESULTS

Damage to ^{32}P-Labeled DNA Fragments Induced by Azobenzene and Hydrazobenzene in the Presence of Cu(II)

Figure 1A shows the autoradiogram of DNA damage by hydrazobenzene. Hydrazobenzene caused DNA damage in the presence of Cu(II). Hydrazobenzene caused no DNA damage in the presence of Fe(II), Fe(III), Mn(II) or Co(II) (data not shown). The intensity of DNA damage increased depending on concentrations of hydrazobenzene. Azobenzene and aniline did not cause DNA damage in the presence of Cu(II) (data not shown).

Effects of Scavengers and Bathocuproine on DNA Damage

The effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage by

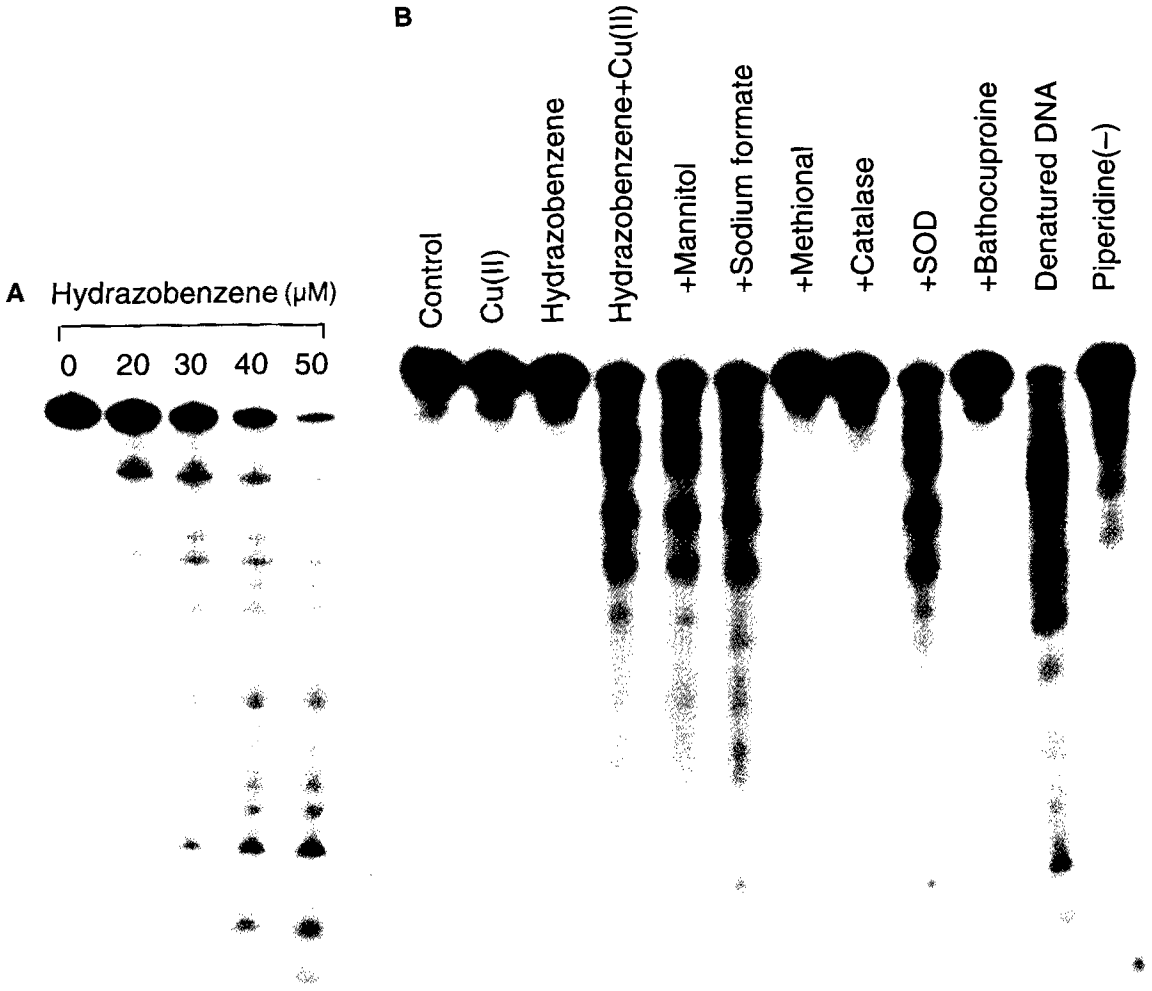

FIGURE 1 DNA damage by hydrazobenzene and effects of scavengers. The reaction mixture of (A) contained the ^{32}P-5′-end-labeled 211-bp fragment, 25 μM/base of sonicated calf thymus DNA, indicated concentration of hydrazobenzene, 10% (v/v) of ethanol and 20 μM $CuCl_2$ in 200 μl of 10 mM phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The reaction mixture of (B) contained the ^{32}P-5′-end-labeled 443-bp DNA fragment, 25 μM/base of sonicated calf thymus DNA, 20 μM hydrazobenzene, 10% (v/v) of ethanol and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated for 60 min at 37°C. The DNA fragments were heated at 90°C with 1 M piperidine for 20 min and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing an X-ray film to the gel. Scavenger or bathocuproine was added as follows; mannitol, 0.1 M; sodium formate, 0.1 M; methional, 0.1 M; catalase, 150 units/ml; SOD, 150 units/ml; bathocuproine, 50 μM. Denatured DNA; DNA was denatured at 90°C for 5 min and quickly chilled on ice before incubation. Piperidine (−); without piperidine treatment.

hydrazobenzene were investigated (Figure 1B). Typical $^{\bullet}$OH scavengers, mannitol and sodium formate did not inhibit DNA damage induced by hydrazobenzene in the presence of Cu(II), whereas methional inhibited the DNA damage. Bathocuproine inhibited the DNA damage, suggesting the involvement of Cu(I). DNA damage induced by hydrazobenzene was inhibited by catalase. SOD showed no inhibitory effect on DNA damage. When denatured DNA was used,

formation of oligonucleotides increased. The piperidine treatment enhanced the DNA damage greatly, suggesting that hydrazobenzene with Cu(II) caused mainly base modification, rather than DNA strand breakage.

Site Specificity of DNA Damage by Hydrazobenzene

An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA damage in the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene. Figure 2 shows the site specificity of DNA damage. Hydrazobenzene plus Cu(II) induced piperidine-labile site at thymine residues in both fragments from the human *p53* tumor suppressor gene and the human c-Ha-*ras*-1 protooncogene, although there remains a possibility that base damage might depend on their sensitivity to piperidine treatment.

Formation of 8-oxodG in Calf Thymus DNA by Hydrazobenzene Plus Cu(II)

We measured 8-oxodG content in calf thymus DNA treated with hydrazobenzene or azobenzene plus Cu(II) using an HPLC–ECD (Figure 3). The amount of 8-oxodG increased dose-dependently in DNA treated with hydrazobenzene in the presence of Cu(II). However, the amount of 8-oxodG did not increase in DNA treated with azobenzene plus Cu(II).

Cu(II)-Mediated Autoxidation of Hydrazobenzene

Figure 4A shows spectral changes during the autoxidation of hydrazobenzene with Cu(II). Without Cu(II), only little change in the spectra of hydrazobenzene was observed (data not shown). When Cu(II) was added, the absorbance maximum at 241 nm rapidly decreased

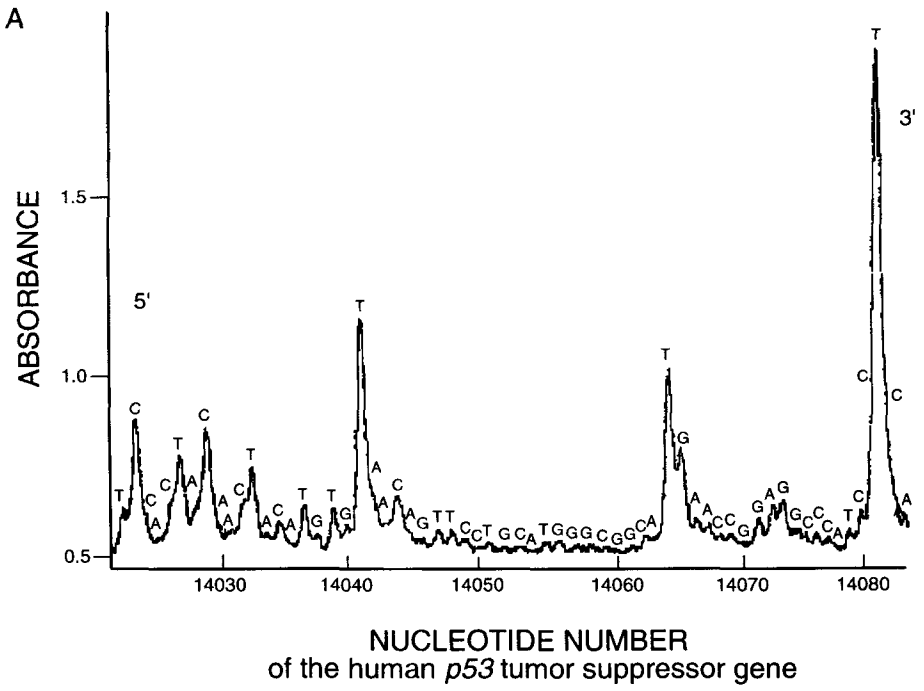

FIGURE 2A

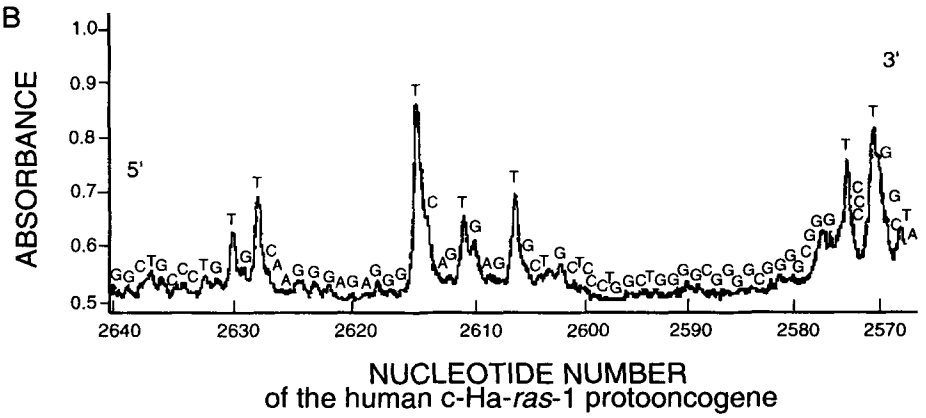

FIGURE 2B

FIGURE 2 Site specificity of DNA cleavage induced by hydrazobenzene in the presence of Cu(II). The reaction mixture contained the ^{32}P-5'-end labeled 211-bp fragment (*Hin*dIII* 13972-*Apa*I 14182) of the human *p53* tumor suppressor gene (A) or 337-bp fragment (*Pst*I 2345-*Ava*I* 2681) of the human c-Ha-*ras*-1 protooncogene (B), 25 μM/base of sonicated calf thymus DNA, 30 μM hydrazobenzene, 10% (v/v) of ethanol and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer at pH 7.8. After incubation at 37°C for 60 min, the DNA fragments were heated at 90°C with 1 M piperidine for 20 min, and then electrophoresed on an 8% polyacrylamide/8 M urea gel using a DNA-sequencing system. The autoradiogram was obtained by exposing an X-ray film to the gel. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer. The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene or c-Ha-*ras*-1 protooncogene.

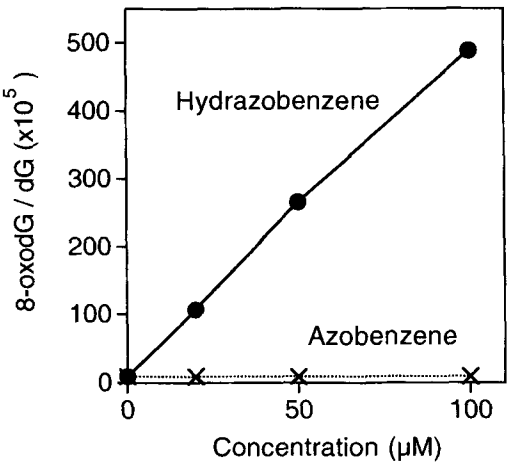

FIGURE 3 Formation of 8-oxodG by hydrazobenzene or azobenzene plus Cu(II). The reaction mixture contained calf thymus DNA (100 μM/base), indicated concentrations of hydrazobenzene or azobenzene and 10% (v/v) of ethanol, 20 μM $CuCl_2$ in 400 μl of 4 mM phosphate buffer (pH 7.8), containing 2.5 μM DTPA. After incubation at 37°C for 60 min, 0.2 mM DTPA was added to stop the reaction, and then the DNA was precipitated in ethanol. The DNA fragment was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC–ECD as described in Materials and Methods.

concomitantly with the increase in the absorbance maxima at 320 and 430 nm. Figure 4B shows the spectra of azobenzene and hydrazobenzene. Azobenzene has the absorbance maxima at 320 and 430 nm, and hydrazobenzene has the absorbance maxima at 241 and 286 nm. This result shows that Cu(II) can mediate autoxidation of hydrazobenzene to azobenzene.

H_2O_2 Generation by the Autoxidation of Hydrazobenzene in the Presence of Cu(II)

Figure 5 shows oxygen consumption during the autoxidation of hydrazobenzene with Cu(II). When Cu(II) was added, hydrazobenzene consumed oxygen. The addition of catalase increased dissolved oxygen, suggesting that in the presence of Cu(II) hydrazobenzene generated H_2O_2, which was decomposed by catalase to generate oxygen. In the absence of Cu(II), hydrazobenzene did not consume oxygen.

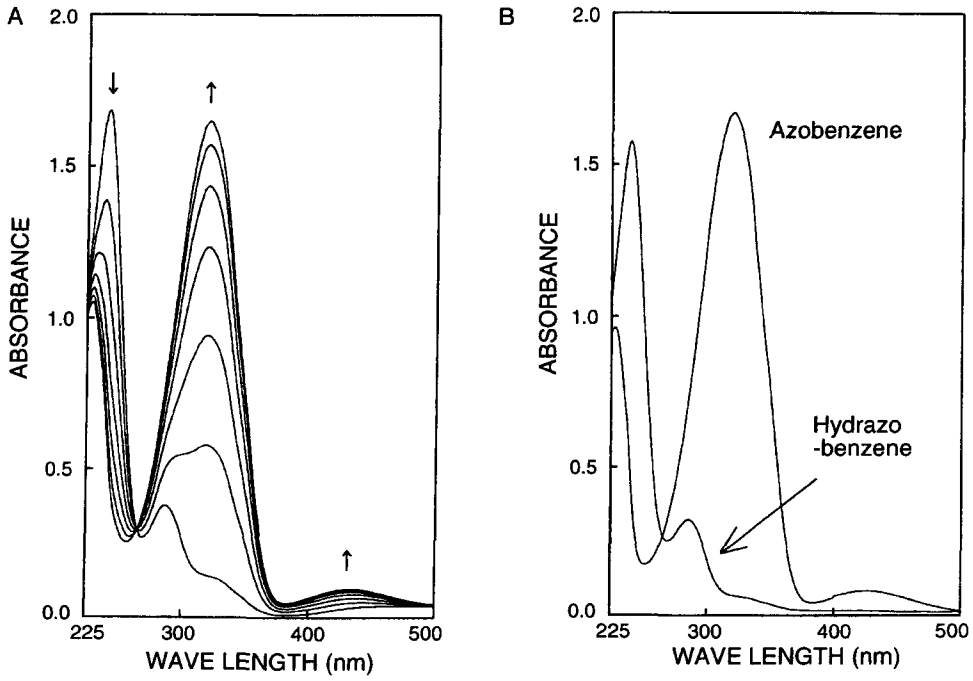

FIGURE 4 Changes in UV–Visible spectra of hydrazobenzene. A: The reaction mixture contained 100 μM hydrazobenzene, 20 μM $CuCl_2$, 10% (v/v) of ethanol and 2.5 μM DTPA in 10 mM phosphate buffer (pH 7.8). The spectrum of the mixture was measured every 9 min for 54 min at 37°C. B: The sample contained 100 μM azobenzene or 100 μM hydrazobenzene, 10% (v/v) of ethanol and 2.5 μM DTPA in 10 mM phosphate buffer (pH 7.8).

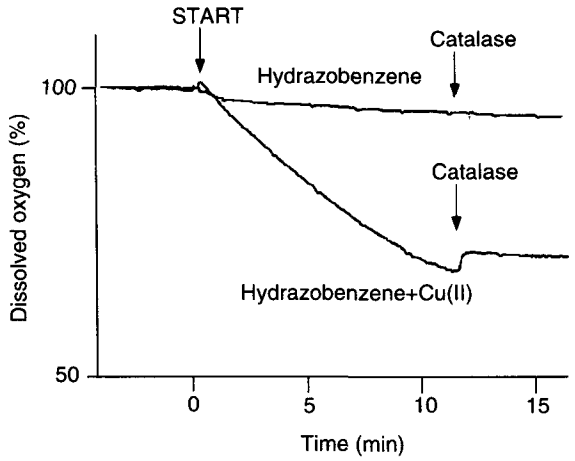

FIGURE 5 Oxygen consumption by the interaction of hydrazobenzene with Cu(II). The reactions were performed in the reaction mixtures in 2 ml of 10 mM phosphate buffer (pH 7.8) containing 10% (v/v) of ethanol and 2.5 μM DTPA at 37°C. The reaction was started by the addition of 200 μM hydrazobenzene and 20 μM $CuCl_2$ into the mixture (indicated by an arrow). To detect H_2O_2 generation due to oxygen consumption, 300 units of catalase were added at 12 min (indicated by arrows).

DISCUSSION

The present study showed that hydrazobenzene, a metabolic intermediate of azobenzene, caused DNA damage including 8-oxodG in the presence of Cu(II). However, azobenzene did not cause DNA damage under the condition used. To clarify the kinds of active species that participated in DNA damage induced by hydrazobenzene, the effects of scavengers on the DNA damage were examined. Both catalase and a Cu(I)-specific chelator, bathocuproine inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). Typical •OH scavengers showed only weak inhibitory effects on the DNA damage, suggesting that free •OH does not play an important role. The inhibitory effect of methional on the DNA damage can be explained by assuming that sulfur compounds are reactive with •OH and less reactive species.[16] It has been reported that chemical

repair of DNA radicals by cationic thiols becomes a significant factor in their DNA protection.[17] However, chemical repair by methional in the DNA damage is negligible, because methional is not cationic thiol but neutral thioether. The crypto-hydroxyl radical was reported to be reactive to sulfur compounds like methionine and methional.[16,18] Thus probably, scavenging effect of radical species such as Cu(I)–OOH contributes protection of DNA damage by methional. UV–Visible spectroscopic study showed that hydrazobenzene is autoxidized to azobenzene in the presence of Cu(II). Measurement of oxygen consumption revealed that H_2O_2 was generated by the autoxidation of hydrazobenzene. Based on these results, a possible mechanism of DNA damage induced by hydrazobenzene could be explained as follows (Figure 6): Hydrazobenzene undergoes Cu(II)-mediated autoxidation to generate radicals and Cu(I). The radicals and Cu(I) react with oxygen to generate O_2^-, which is dismutated to H_2O_2. H_2O_2 interacts with Cu(I) to form the metal–oxygen complex, such as Cu(I)–OOH, causing DNA damage.

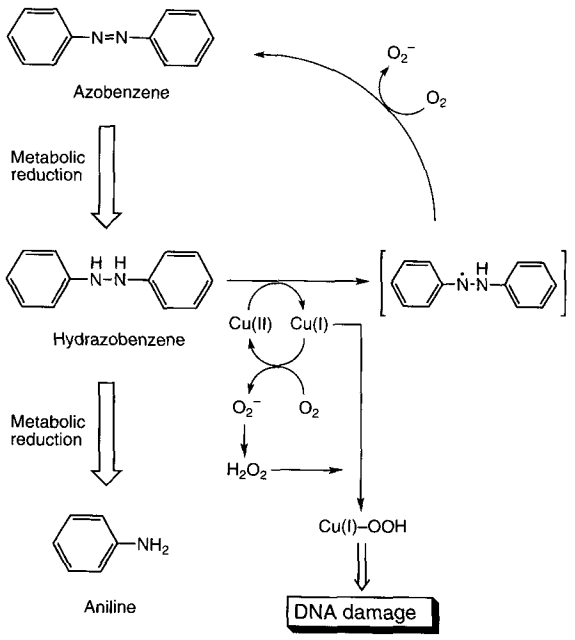

FIGURE 6 Proposed mechanism of DNA damage by hydrazobenzene, an azobenzene metabolite.

Aruoma *et al.* reported that 8-hydroxyguanine was the major product in the Cu(II)/H_2O_2 and the Cu(II)/H_2O_2/ascorbic acid systems, analyzing by GC-MS.[19] Furthermore, Rodriguez *et al.* reported that guanine bases were most frequently modified by Cu(II)/ascorbate/H_2O_2, using ligation-mediated polymerase chain reaction.[20] In this study, hydrazobenzene induced piperidine-labile sites frequently at thymine residues in the presence of Cu(II). In addition, using an HPLC–ECD, we also observed the increase of 8-oxodG, which is piperidine-inert site. Thus, hydrazobenzene with Cu(II) should cause oxidative damage at guanine. Site-specific and characteristic mutations were found in human cancers as molecular mutational fingerprints associated with chemical carcinogens.[21] To our knowledge, there are no reports concerning the mutational hotspots of azobenzene/hydrazobenzene *in vivo*. Further researches may reveal the correlation between the site specificity and the carcinogenic process.

Copper occurs in the mammalian cell nuclei, and may contribute to high order chromatin structures.[22] Dizdaroglu *et al.* reported that H_2O_2 in the presence of Cu(II) caused more DNA damage in chromatin than in the presence of Fe(III).[23] Therefore, the copper-dependent DNA damage by hydrazobenzene is possible *in vivo*. It seems to be reasonable that the copper-mediated oxidative DNA damage by hydrazobenzene is relevant for the expression of the carcinogenicity of hydrazobenzene.

Carcinogenicity of azo compounds with exocyclic amino group have been explained by DNA adducts through activated nitrogen of the amino group.[2] On the other hand, azo compounds undergo metabolic reduction to hydrazobenzene derivatives, followed by the cleavage of azo linkage to yield aniline derivatives. Chung has proposed that the reductive cleavage of azo linkage is the most important reaction in mutagenesis and carcinogenesis caused by azo compounds. For example, dimethylaminoazobenzene (butter

yellow) is metabolized to aniline and dimethylaniline through the cleavage of azo linkage. The mutagenicity of this compound is due to the formation of dimethylaniline, but aniline is not involved in mutagenesis.[1] In this study, aniline did not cause DNA damage under the condition used. Therefore, it is unlikely that aniline plays an important role in carcinogenesis caused by azobenzene and hydrazobenzene.

The present study revealed that hydrazobenzene has DNA-damaging ability. Hydrazobenzene is a hydrazine derivative. We have previously reported that carcinogenic hydrazine and its derivatives[24] caused oxidative damage to DNA through the interaction with copper ion to generate reactive species.[25] Therefore, on the basis of our results, it is concluded that azobenzene would be metabolized to hydrazobenzene, which causes metal ion-mediated oxidative DNA damage, leads to carcinogenicity.

Acknowledgments

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