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Oxidative DNA Damage Induced by an *N*-Hydroxy Metabolite of Carcinogenic 4-Dimethylaminoazobenzene

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Formation of adducts has been considered to be a major causal factor of DNA damage by carcinogenic aminoazo dyes. We investigated whether a metabolite of hepatocarcinogenic 4-dimethylaminoazobenzene (DAB) can cause oxidative DNA damage or not, using ^{32}P-5′-end-labeled DNA fragments. The DAB metabolite *N*-hydroxy-4-aminoazobenzene (N-OH-AAB) was found to cause Cu(II)-mediated DNA damage, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation. When an endogenous reductant, β-nicotinamide adenine dinucleotide (NADH) was added, the DNA damage was greatly enhanced. Very low concentrations of N-OH-AAB could induce DNA damage via redox reactions. Catalase and a Cu(I)-specific chelator inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). A typical •OH scavenger did not inhibit the DNA damage. The main reactive species are probably DNA-copper-hydroperoxo complexes. We conclude that oxidative DNA damage may play an important role in the carcinogenic processes of DAB, in addition to DNA adduct formation.

Key words: Azobenzene — DNA damage — Copper — Hydrogen peroxide

There is ample evidence for the carcinogenicity of 4-dimethylaminoazobenzene (DAB) in experimental animals. DAB induced lung tumors and hepatomas in mice and liver tumors in rats. In dogs it produced bladder tumors following oral administration. DAB has also been tested by s.c. injection in mice, and the results are suggestive of local and hepatic carcinogenicity.[1)] The International Agency for Research on Cancer (IARC) has assessed that DAB is possibly carcinogenic to humans (group 2B).[2)]

DAB is metabolized to *N*-methyl-4-aminoazobenzene (MAB) through *N*-demethylation. MAB is metabolized to 4-aminoazobenzene (AAB) through demethylation or to *N*-hydroxy-*N*-methyl-4-aminoazobenzene (N-OH-MAB) through *N*-hydroxylation, followed by further transformation to *N*-hydroxy-4-aminoazobenzene (N-OH-AAB).[3–5)] *N*-Hydroxylation is believed to be a step leading aminoazo dyes to proximate carcinogenic or mutagenic metabolites. Watanabe and Hashimoto reported that N-OH-AAB elicited higher levels of unscheduled DNA synthesis (UDS) than AAB, suggesting higher DNA damaging activity of the *N*-hydroxy derivative than that of the corresponding mother aminoazo dye.[6)] It was also reported that N-OH-AAB dyes showed greater mutagenicity than the mother AAB dyes, without S-9 treatment.[7)] It is generally accepted that covalent binding of these metabolites with DNA is a major carcinogenic factor.[8)] *N*-(Deoxyguanosin-8-yl)-4-aminoazobenzene was also obtained from mice or rats given an i.p. dose of AAB.[4)] It was regarded as an adduct formed by reaction of deoxyguanosine with a metabolite of AAB after *N*-hydroxylation and esterification.[4)]

On the other hand, N-OH-AAB and N-OH-MAB are reported to generate H_2O_2 and $O_2^{\cdot-}$.[9)] Administration of 3′-methyl-4-dimethylaminoazobenzene (3′-MeDAB) which is a stronger carcinogenic derivative of DAB, increased the levels of 8-hydroxyguanine and its repair activity in rodent liver DNA.[10)] These reports suggested that oxidative DNA damage plays a part in carcinogenesis by aminoazo dyes.

To clarify the mechanism of carcinogenesis by DAB, we examined oxidative DNA damage induced by N-OH-AAB, using ^{32}P-5′-end-labeled DNA fragments obtained from the c-Ha-*ras*-1 protooncogene and the *p53* tumor suppressor gene. In addition, we measured the content of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in calf thymus DNA by high-performance liquid chromatography with an electrochemical detector (HPLC-ECD). It has been reported that 8-oxodG is a marker of oxidative DNA damage and that its formation can lead to DNA misreplication, resulting in mutation and cancer.[11, 12)]

MATERIALS AND METHODS

Materials N-OH-AAB was prepared from 4-nitroazobenzene, according to the references.[13, 14)] Restriction enzymes (*Sma*I, *Eco*RI, *Hin*dIII, *Apa*I, *Sty*I and *Xba*I) and T_4 polynucleotide kinase were purchased from New England

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Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear (Boston, MA). Alkaline phosphatase from calf intestine was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Piperidine was purchased from Wako Chemical Industries Ltd. (Osaka). Copper (II) chloride dihydrate was purchased from Nacalai Tesque, Inc. (Kyoto). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemicals Co. (Kumamoto). Calf thymus DNA, superoxide dismutase (SOD) (3000 units/mg from bovine erythrocytes) and catalase (45 000 units/mg from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba). Methional (3-(methylthio)propionaldehyde) was purchased from Tokyo Kasei Co. (Tokyo).

Preparation of ^{32}P-5′-end-labeled DNA fragments obtained from the *p53* gene and the c-Ha-*ras*-1 gene DNA fragments were obtained from the human *p53* tumor suppressor gene.[15] Two fragments containing exons from the *p53* gene 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 pUC 18 plasmid, and then transferred to *Escherichia coli* JM 109. The plasmid pUC 18 was digested with *Eco*RI and *Hin*dIII, and the resulting DNA fragments were fractionated by electrophoresis on 2% agarose gels. The 5′-end-labeled 650-bp fragment (*Hin*dIII*13972-*Eco*RI*14621) and 460-bp fragment (*Hin*dIII*13038-*Eco*RI*13507) were obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The 650-bp fragment was further digested with *Apa*I to obtain a singly labeled double-stranded 211-bp fragment (*Hin*dIII*13972-*Apa*I 14182). The 460-bp fragment was further digested with *Sty*I to obtain a singly labeled double-stranded 343-bp fragment (*Sty*I 13160-*Eco*RI*13507) and a 118-bp fragment (*Hin*dIII*13038-*Sty*I 13155). A DNA fragment was also obtained from the human c-Ha-*ras*-1 protooncogene.[16] A 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. A singly labeled double-stranded 341-bp fragment (*Xba*I 1906-*Ava*I*2246) was obtained according to the method described previously.[17] The nucleotide numbering starts with the *Bam*HI site.[16]

Detection of DNA damage The standard reaction mixture (in a microtube; 1.5 ml) contained the indicated metal ions, NADH as necessary, an ethanol solution of the indicated concentrations of N-OH-AAB, the ^{32}P-labeled double-stranded DNA fragments and 25 μ*M*/base of sonicated calf thymus DNA in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 2.5 μ*M* DTPA. After incubation at 37°C for 30 min, the DNA fragments were precipitated and dried, followed by heating at 90°C in 1 *M* piperidine for 20 min. By piperidine treatment, we can detect not only strand breakage, but also base damage. The DNA fragments recovered by ethanol precipitation were dissolved in formamide dye. The DNA fragments were denatured at 90°C for 2 min and immediately chilled on ice. The denatured DNA was electrophoresed on an 8% polyacrylamide/8 *M* urea gel in Tris borate/EDTA buffer. The autoradiogram was obtained by exposing X-ray film to the gel.[18] The extent of DNA damage was roughly estimated by using a laser densitometer (LKB 2222 UltroScan XL).

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[19] 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 fragment was incubated with N-OH-AAB and $CuCl_2$, in the presence and absence of NADH. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by means of HPLC-ECD, as described previously.[20]

Measurement of oxygen consumption Oxygen consumption by the reaction of N-OH-AAB with Cu(II) and NADH was measured using a Clarke oxygen electrode (Electronic Stirrer Model 300, Rank Brothers Ltd., Bottisham, Cambridge, England). The reaction was performed in phosphate buffer containing 10% (v/v) ethanol, and was started by the addition of NADH, $CuCl_2$, or N-OH-AAB to the chamber of the oxygen electrode. Catalase was added to detect H_2O_2 generation resulting from oxygen consumption, 5 min after starting the reaction (data not shown).

RESULTS

Damage to ^{32}P-labeled DNA fragment induced by N-OH-AAB in the presence of various concentrations of Cu(II) and NADH Fig. 1 shows an autoradiogram of a DNA fragment treated with N-OH-AAB plus Cu(II) in the presence and absence of NADH. Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. In the absence of N-OH-AAB, DNA damage was not observed with NADH and Cu(II) under the conditions used. N-OH-AAB alone or N-OH-AAB plus NADH did not cause DNA damage. In the presence of Cu(II), N-OH-AAB induced DNA damage. The intensity of DNA damage increased with the concentration of N-OH-AAB. When NADH was added, low concentrations of N-OH-AAB efficiently induced Cu(II)-mediated DNA damage.

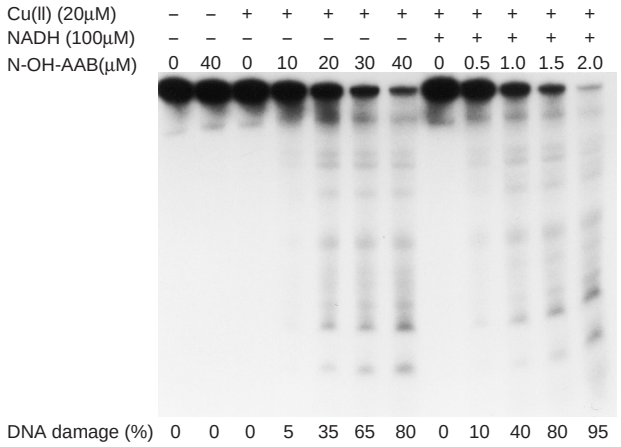

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragment incubated with N-OH-AAB in the presence of NADH and Cu(II). The reaction mixture contained a ^{32}P-5′-end-labeled 211-bp DNA fragment (singly labeled double-stranded DNA), 25 μM per base of sonicated calf thymus DNA, the indicated concentrations of N-OH-AAB, 100 μM NADH, 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 at 37°C for 30 min, followed by a piperidine treatment, as described in "Materials and Methods." After denaturation, the DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing X-ray film to the gel.

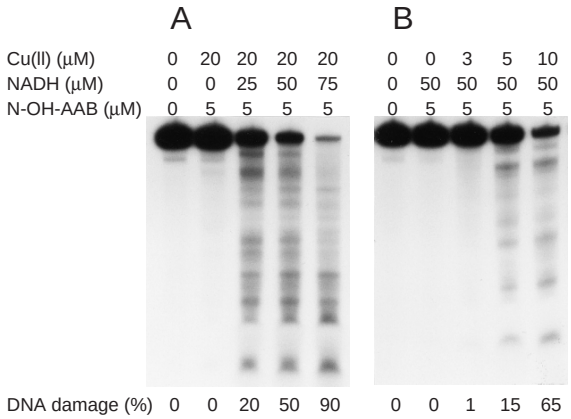

Fig. 2. DNA damage induced by N-OH-AAB in the presence of NADH and Cu(II). The reaction mixture contained a ^{32}P-5′-end-labeled 118-bp DNA fragment (singly labeled double-stranded DNA), 25 μM per base of sonicated calf thymus DNA, 5 μM N-OH-AAB, various concentrations of NADH and $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 30 min. After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. (A) Effect of NADH concentrations on DNA damage. (B) Effect of Cu(II) concentrations on DNA damage.

The extent of DNA damage was dependent on the concentrations of both NADH and Cu(II) when the same dose of N-OH-AAB was used (Fig. 2). The increase of oligonucleotides with piperidine, treatment compared with no treatment (data not shown), suggested that N-OH-AAB induced not only strand breakage but also base modification and/or liberation.

N-OH-AAB caused no DNA damage in the presence of Mn(II), Fe(II), Co(II) or Ni(II) (data not shown).

Effects of scavengers and bathocuproine on DNA damage The effects of scavengers and bathocuproine on DNA damage by N-OH-AAB were investigated (Fig. 3). A typical •OH scavenger, mannitol, did not inhibit DNA damage induced by N-OH-AAB in the presence of Cu(II), whereas methional inhibited the DNA damage. Bathocuproine, a Cu(I)-specific chelator, also inhibited the DNA damage, suggesting the involvement of Cu(I). DNA damage induced by N-OH-AAB was inhibited by catalase, but not by heated catalase. SOD showed little inhibitory effect on DNA damage. In the presence of NADH, these effects were similar (data not shown).

Site specificity of DNA damage by N-OH-AAB An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensity of DNA cleavage in the human *p53* tumor suppressor gene (Fig. 4A)

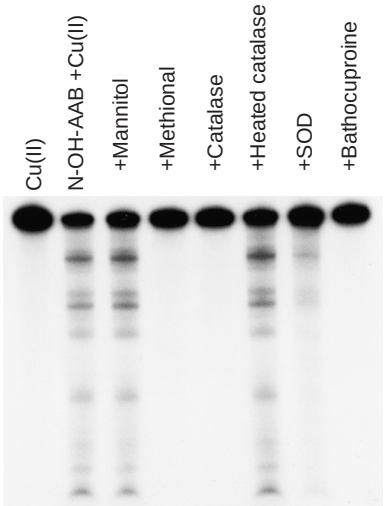

Fig. 3. Effects of scavengers and bathocuproine on DNA damage induced by N-OH-AAB in the presence of Cu(II). The reaction mixture contained a ^{32}P-5′-end-labeled 211-bp DNA fragment, 25 μM per base of sonicated calf thymus DNA, 30 μM N-OH-AAB and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 30 min, followed by a piperidine treatment. The DNA fragment was analyzed as described in the legend to Fig. 1. The concentrations of scavengers and bathocuproine were as follows: 0.1 M mannitol; 0.1 M methional; 100 units of catalase; 100 units of catalase heated at 90°C for 10 min; 30 units of SOD; 50 μM bathocuproine.

and the c-Ha-*ras*-1 protooncogene (Fig. 4B). N-OH-AAB plus Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues. When NADH was added, a similar cleavage pattern was observed.

Formation of 8-oxodG in calf thymus DNA by N-OH-AAB in the presence of Cu(II) and the effect of NADH Using HPLC-ECD, we measured 8-oxodG content in calf thymus DNA treated with N-OH-AAB in the presence of Cu(II) (Fig. 5A). The amount of 8-oxodG increased with the concentration of N-OH-AAB in the presence of Cu(II). The formation of 8-oxodG increased after DNA denaturation. When NADH was added, 8-oxodG formation was observed at very low concentrations of N-OH-AAB (Fig. 5B).

H_2O_2 generation by the autoxidation of N-OH-AAB in the presence of Cu(II) and NADH Oxygen consumption

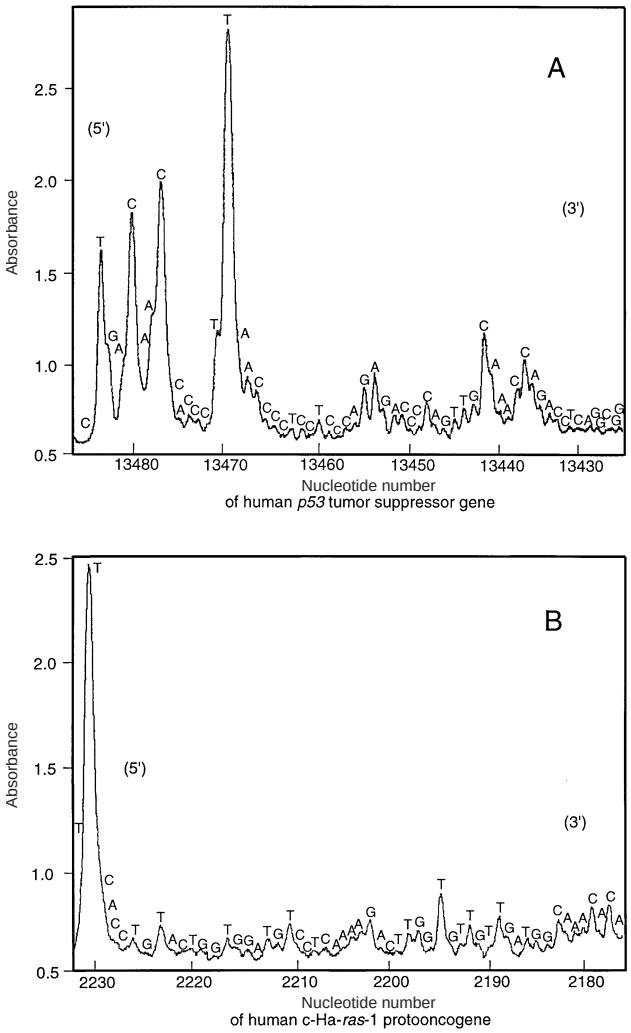

Fig. 4. Site specificity of DNA cleavage induced by N-OH-AAB in the presence of Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled DNA, 25 μM per base of sonicated calf thymus DNA, 30 μM N-OH-AAB and 20 μM $CuCl_2$, in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The reaction mixture was incubated at 37°C for 30 min. After piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The horizontal axis shows the nucleotide number.[15, 16] (A) 343-bp fragment (*Sty*I 13160-*Eco*RI*13507) of the *p53* tumor suppressor gene. (B) 341-bp fragment (*Xba*I 1906-*Ava*I*2246) of the c-Ha-*ras*-1 protooncogene.

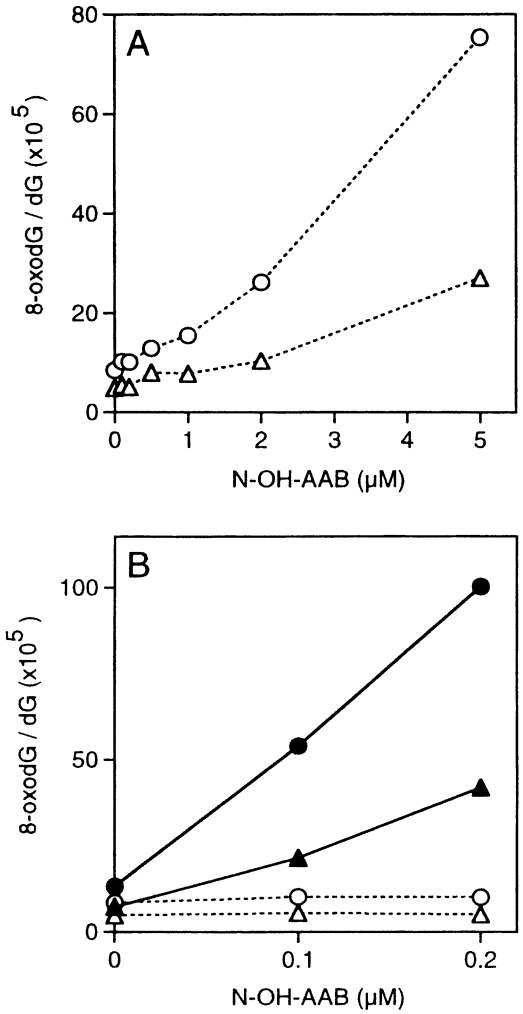

Fig. 5. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by N-OH-AAB (A), and the effect of NADH (B). Calf thymus DNA (100 μM per base) was incubated with the indicated concentrations of N-OH-AAB and 20 μM $CuCl_2$ in the presence and absence of 100 μM NADH for 30 min at 37°C. After ethanol precipitation, DNA was enzymatically digested into nucleosides, and 8-oxodG formation was measured by means of HPLC-ECD as described in "Materials and Methods." Open symbols indicate the reaction without NADH, and closed symbols indicate that with NADH. Triangles indicate that native DNA was used, and circles indicate denatured DNA.

occurred during the autoxidation of N-OH-AAB in the presence of NADH and Cu(II). The addition of catalase increased dissolved oxygen, suggesting that N-OH-AAB generated H_2O_2, which was decomposed by catalase to generate molecular oxygen. When NADH was omitted, N-OH-AAB plus Cu(II) induced a little oxygen consumption. Cu(II) plus NADH did not consume molecular oxygen under the conditions used (data not shown).

DISCUSSION

The present study showed that N-OH-AAB, a metabolite of DAB caused oxidative DNA damage, including 8-oxodG formation, in the presence of Cu(II). In addition, the DNA damage was dramatically enhanced by the endogenous reductant NADH. To clarify the nature of the reactive species we examined the effects of scavengers on the DNA damage induced by N-OH-AAB. Both catalase and bathocuproine inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). The effect of catalase on oxygen consumption confirmed that H_2O_2 was generated by the Cu(II)-mediated autoxidation of N-OH-AAB.

It is suggested that free •OH does not play an important role, because of the lack of inhibition of DNA damage by a typical •OH scavenger and the site specificity of DNA damage.[18, 21] 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.[22] Alternatively, the lack of inhibitory effect of •OH scavengers can be explained by the possibility that DNA damage is induced by •OH generated in very close proximity to the nucleic acid by the bound metal ion. Furthermore, because of site-specific binding of copper ions to DNA, it might be expected that site-specific DNA damage by hydroxyl radicals is generated by bound copper.

Based on these results, a possible mechanism is as follows (Fig. 6): DAB is metabolized to N-OH-AAB through several metabolic steps by demethylation and *N*-hydroxylation.[4] N-OH-AAB induces Cu(II)-mediated DNA damage through reactive oxygen species. Autoxidation of N-OH-AAB to the nitroso compound (NO-AB) via an intermediate occurs, coupled with generation of O_2^-. O_2^- is dismutated to H_2O_2 with the reduction of Cu(II) to Cu(I). H_2O_2 interacts with Cu(I) to form DNA-copper-hydroperoxo complexes, causing DNA damage.[23] When NADH was added, NO-AB might have been reduced to the intermediate or N-OH-AAB, and again autoxidation would occur, forming a redox cycle.

An endogenous reductant such as NADH would enhance the DNA damage through excessive generation of reactive oxygen species, by forming a redox cycle. The concentration of NAD(P)H in certain tissues was estimated to be as high as 100–200 μM.[24] Several studies indicate that NADH may react nonenzymatically with

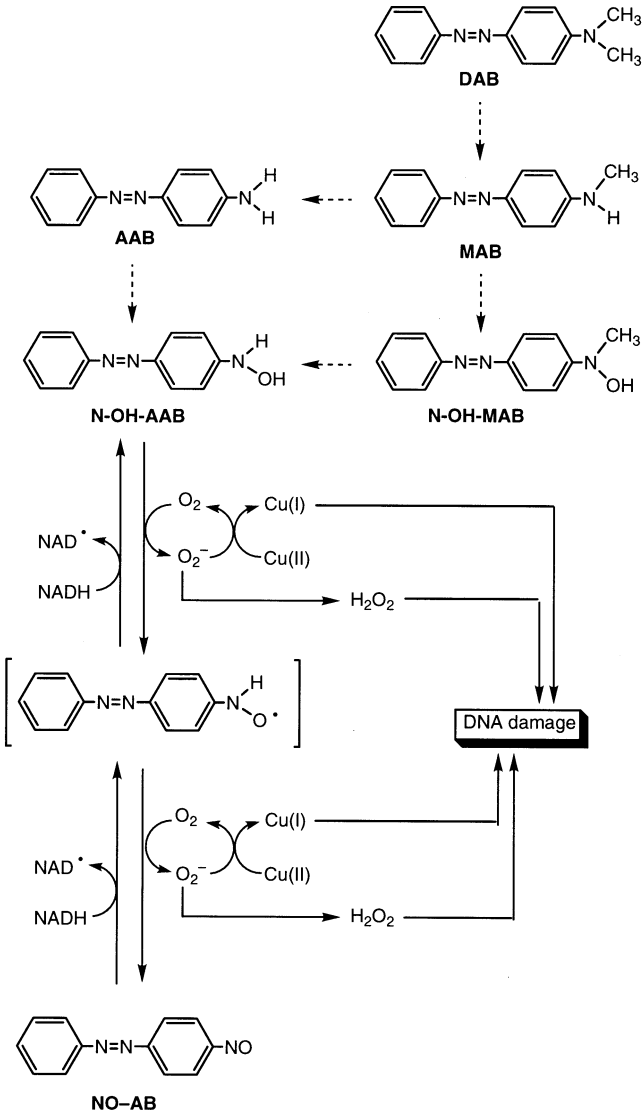

Fig. 6. A proposed mechanism for oxidative DNA damage induced by a DAB metabolite in the presence of Cu(II) and NADH.

some xenobiotics and mediate their reduction.[25–27] The biological importance of NADH and NADPH as nuclear reductants has been pointed out.[28]

N-OH-AAB plus Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues. Generally, site-specific and characteristic mutations have been found in human cancers as molecular mutational fingerprints associated with chemical carcinogens.[29] In this study, we did not detect N-OH-AAB-induced damage at well-known hotspots. However, we previously detected site-specific DNA damage, including hotspots, by the *N*-hydroxy metabolite of a heterocyclic amine, via a mechanism simi-

lar to that of N-OH-AAB.[27] Further researches may reveal the correlation between the site specificity and the carcinogenic process.

Carcinogenicity of amino azo dyes have been explained in terms of the formation of DNA adducts.[30] Amino azo dyes have an exocyclic amino group that is the key to their carcinogenicity, because this group undergoes biochemical *N*-oxidation and further conversion to reactive electrophiles.[30] DNA adducts formed by covalent binding through the activated nitrogen have been identified.[30] On the other hand, it has been pointed out that free radicals and subsequently formed reactive oxygen species are involved in aromatic amine carcinogenesis.[9] Hirano *et al.* reported that administration of 3′-MeDAB increased 8-hydroxyguanine in rodent liver DNA.[10]

The present study has revealed that N-OH-AAB, a metabolite of DAB, has oxidative DNA-damaging ability, and the existence of NADH enhances the damage through cycling redox reactions. It is noteworthy that a very low concentration, 0.1 μM N-OH-AAB induced 8-oxodG formation, suggesting that oxidative DNA damage may occur *in vivo*. Our previous study showed the oxidative DNA-damaging ability of a metabolite of an azo dye through azo-reduction.[31] We concluded that oxidative DNA damage may play an important role in the carcinogenic process of aminoazo dyes via their *N*-hydroxylated and azo-reduced metabolites. Nakayama *et al.* reported that active metabolites of naphthylamine and aminoazo dyes generated reactive oxygen species and that *N*-hydroxy-naphthylamine induced DNA lesions.[9, 32] It is reasonable to consider that *N*-hydroxy derivatives of carcinogenic amino-aromatic compounds such as amino azo dyes, naphthylamine and heterocyclic amines, play important roles in the carcinogenic process through H_2O_2 formation.[27, 32]

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