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Oxidative DNA damage by an *N*-hydroxy metabolite of the mutagenic compound formed from norharman and aniline

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Abstract

Norharman (9*H*-pyrido[3,4-*b*]indole), which is a heterocyclic amine included in cigarette smoke or cooked foodstuffs, is not mutagenic itself. However, norharman reacts with non-mutagenic aniline to form mutagenic aminophenylnorharman (APNH), of which DNA adducts formation and hepatocarcinogenic potential are pointed out. We investigated whether N-OH-APNH, an *N*-hydroxy metabolite of APNH, can cause oxidative DNA damage or not, using ^{32}P-labeled DNA fragments. N-OH-APNH caused Cu(II)-mediated DNA damage. When an endogenous reductant, β-nicotinamide adenine dinucleotide (NADH) was added, the DNA damage was greatly enhanced. Catalase and a Cu(I)-specific chelator inhibited DNA damage, suggesting the involvement of H_2O_2 and Cu(I). Typical $^{\bullet}$OH scavenger did not inhibit DNA damage. These results suggest that the main reactive species are probably copper-hydroperoxo complexes with DNA. We also measured 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation by N-OH-APNH in the presence of Cu(II), using an electrochemical detector coupled to a high-pressure liquid chromatograph. Addition of NADH greatly enhanced 8-oxodG formation. UV–VIS spectra and mass spectra suggested that N-OH-APNH was autoxidized to nitrosophenylnorharman (NO-PNH). We speculated that NO-PNH was reduced by NADH. Cu(II) facilitated the redox cycle. In the presence of NADH and Cu(II), very low concentrations of N-OH-APNH could induce DNA damage via redox reactions. We conclude that oxidative DNA damage, in addition to DNA adduct formation, may play an important role in the expression of genotoxicity of APNH.

Keywords: Norharman; *N*-hydroxy metabolite; DNA damage; Copper; Hydrogen peroxide; Redox cycle

Abbreviations: Norharman, 9*H*-pyrido[3,4-*b*]indole; APNH (aminophenylnorharman), 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole; N-OH-APNH (*N*-hydroxy-aminophenylnorharman), 9-(*N*-hydoroxy-4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole; NO-PNH (nitrosophenylnorharman), 9-(4′-nitrosophenyl)-9*H*-pyrido[3,4-*b*]indole; NO_2-PNH (nitrophenylnorharman), 9-(4′-nitrophenyl)-9*H*-pyrido[3,4-*b*]indole; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; ROS, reactive oxygen species; O_2^-, superoxide; NADH, β-nicotinamide adenine dinucleotide (reduced form); SOD, superoxide dismutase; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; DMSO, dimethylsulfoxide; HPLC–ECD, an electrochemical detector coupled to a high-pressure liquid chromatography; MS, mass spectrometry

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1. Introduction

Norharman (9*H*-pyrido[3,4-*b*]indole), a heterocyclic amine, exists in cigarette smoke and cooked meat and fish at much higher levels than those of other heterocyclic amines [1]. Norharman was detected in all urine samples from healthy volunteers eating ordinary diet [2]. Interestingly, norharman becomes mutagenic in the presence of non-mutagenic aniline with S9 mix, although norharman itself is not mutagenic either with or without S9 mix [3–5]. It is reported that norharman reacts with aniline to form aminophenylnorharman (APNH), and that APNH is metabolized to *N*-hydroxy-aminophenylnorharman (N-OH-APNH) in metabolic process [6]. APNH induces glutathione *S*-transferase placental form (GST-P) positive foci in the livers of F344 male rats in a dose-dependent manner, suggesting its carcinogenicity [7].

Regarding the mechanisms of DNA damage by carcinogenic heterocyclic amines, DNA adduct formation has been considered to be a major causal factor [8–10]. Both APNH and N-OH-APNH formed the same DNA adducts in *Salmonella typhimurium* YG1024, suggesting that APNH is *N*-hydroxylated to N-OH-APNH followed by formation of DNA adducts [6]. On the other hand, Hayatsu et al. showed that *N*-hydroxy derivative of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) produces intracellular reactive oxygen species (ROS) that can damage DNA in mouse cultured cells [11,12]. It is also reported that an *N*-hydroxy metabolite of heterocyclic amine, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), induced oxidative damage to isolated DNA fragments [13]. Maeda et al. reported O_2^- generation from heterocyclic amines with cytochrome P450 reductase and NADPH [14,15]. There remains a possibility that oxidative DNA damage also plays a role in genotoxicity induced by APNH.

In this study, we investigated whether N-OH-APNH can cause oxidative DNA damage or not in the presence of Cu(II) and β-nicotinamide adenine dinucleotide (NADH), using ^{32}P-5′-end-labeled DNA fragments obtained from the human *p53* tumor suppressor gene and the c-Ha-*ras*-1 protooncogene. We analyzed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) formation, a marker of oxidative DNA damage, in calf thymus DNA treated with N-OH-APNH. Furthermore, in order to clarify the mechanism of oxidative DNA damage, we traced the autoxidation of N-OH-APNH, and the redox reaction between NADH and oxidized N-OH-APNH, using UV–VIS spectroscopy and mass spectrometry (MS).

2. Materials and methods

2.1. Materials

Restriction enzymes (*Hin*dIII, *Sty*I, *Ava*I, *Eco*RI, and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA, USA). The [γ-^{32}P]ATP (222 TBq/mmol) was obtained from New England Nuclear. Alkaline phosphatase from calf intestine was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Piperidine, methional (3-(methylthio)propionaldehyde) were purchased from Wako Chemical Industries Ltd. (Osaka, Japan). Cu(II) chloride dihydrate was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased 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 purchased from Sigma Chemical Co. (St. Louis, MO, USA). Nuclease P_1 (400 units/mg) was purchased from Yamasa Shoyu Co. (Chiba, Japan).

2.2. Synthesis of 9-(N-hydoroxy-4′-aminophenyl)-9H-pyrido[3,4-b]indole (N-OH-APNH)

9-(4′-Nitrophenyl)-9*H*-pyrido[3,4-*b*]indole (Nard. Co. Hyogo, Japan) (275 mg) was suspended in tetrahydrofuran (20 ml) cooled to 0°C, and then 5% palladium/carbon (50 mg) and hydrazine monohydrate (0.2 ml) were added with stirring [6]. The progress of the reaction was monitored by thin-layer chromatograph (Merck precoated silica gel sheets 60F-254, chloroform:ethanol 10:1). After the reduction was completed (within 30 min), the reaction mixture was filtered and diluted with N_2-purged water. Solid material was collected by centrifugation and lyophilized to give pure 9-(*N*-hydoroxy-4′-aminophenyl)-9*H*-pyrido-[3,4-*b*]indole: a yellow powder (yield 80%). ^{1}H-NMR (Varian Gemini-400) ($CDCl_3$): δ 8.72 ppm (d, J = 0.9 Hz, 1H), 8.50 (d, J = 5.3, 1H), 8.20 (dd, J =

7.9, 1.0, 1H), 8.1 (dd, $J = 5.3$, 0.9, 1H), 7.56 (ddd, $J = 8.0$, 7.0, 1.1, 1H), 7.44 (d, $J = 9.0$, 2H), 7.42 (dd, $J = 7.9$, 1.0, 1H), 7.35 (ddd, $J = 8.0$, 7.0, 1.1, 1H), 7.25 (d, $J = 9.0$, 2H). Electron ionization MS (Shimadzu GC–MS QP-5000 with DI-50); m/z (%) = 275 (M^+, 24), 154 (100), 136 (62). FAB-HRMS (fast atom bombardment-high resolution MS, recorded with a JEOL JMS-SX 102A QQ apparatus with direct injection); calculated for $C_{17}H_{14}N_3O$ $[M + 1]^+$ 276.1137, found 276.1132. From the results of direct injection FAB-HRMS measurements in addition to the observation of the only single peak obtainable by two different HPLC systems, the compound was quite pure enough for following experiments.

2.3. 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 [16] and the c-Ha-*ras*-1 protooncogene [17]. A singly ^{32}P-5′-end-labeled double-stranded 118 bp *p53* fragment (*Hin*dIII* 13038–*Sty*I 13155) and 343 bp *p53* fragment (*Sty*I 13160–*Eco*RI* 13507) were prepared according to the method described previously [18]. A 261 bp c-Ha-*ras*-1 fragment (*Ava*I* 1645–*Xba*I 1905) and 341 bp fragment (*Xba*I 1906–*Ava*I* 2246) were prepared from plasmid pbcNI, which carries a 6.6 kb *Bam*HI chromosomal DNA restriction fragment. The asterisk (∗) indicates ^{32}P-labeling.

2.4. Detection of DNA damage

A standard reaction mixture (in a microtube; 1.5 ml) contained $CuCl_2$, NADH, a dimethylsulfoxide (DMSO) solution of N-OH-APNH, ^{32}P-labeled double-stranded DNA fragments, and 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, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min and treated as described previously [19,20]. The treated DNA fragments were electrophoresed on an 8% polyacrylamide / 8 M urea gel and an autoradiogram was obtained by exposing X-ray film to the gel. 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 [21] 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.

2.5. Analysis of 8-oxodG formation by N-OH-APNH

Calf thymus DNA fragment was incubated with N-OH-APNH, NADH, and $CuCl_2$. After ethanol precipitation, DNA was digested to its component nucleosides with nuclease P_1 and calf intestine phosphatase, and analyzed by an electrochemical detector coupled to a high-pressure liquid chromatography (HPLC–ECD), as described by Kasai et al. [22,23].

2.6. Measurement of UV–VIS spectra

UV–VIS spectra were measured with UV–VIS spectrometer (UV-2500PC Shimadzu, Kyoto, Japan). To observe the autoxidation of N-OH-APNH, the spectrum was measured every 5 min at 37°C. The sample was 10 μM N-OH-APNH in 10 mM sodium phosphate buffer (pH 7.8) containing 20% (v/v) DMSO and 2.5 μM DTPA. The concentration of nitrophenylnorharman (NO_2-PNH) was 10 μM. For analysis of NADH consumption, the absorption at 340 nm (reduced NADH) was traced every 3 min at 37°C. The reaction mixture contained various concentrations of N-OH-APNH, 100 μM NADH, and/or 20 μM $CuCl_2$ in 10 mM sodium phosphate buffer (pH 7.8) containing 2% (v/v) DMSO and 2.5 μM DTPA.

2.7. Measurement of HPLC–MS spectra of oxidized N-OH-APNH

To the solution of 100 μM N-OH-APNH in 1.5 ml of phosphate buffer (pH 7.4) was added 15 μl of 2 mM solution of $CuCl_2$. Reaction mixture was immediately analyzed with HPLC–MS. Electrospray ionization mass spectrometry was performed using a JEOL JMS-7000Q equipped with HP 1100 HPLC system. Elution conditions were follows. An analytical column of Zorbax Extend C18 (4.6 mm × 25 cm) was used with a linear gradient from 10 to 80% acetonitrile in sodium phosphate buffer (pH 2) over the course of 30 min at a rate of 1 ml/min.

3. Results

3.1. Damage to ^{32}P-labeled DNA

Fig. 1 shows an autoradiogram of a DNA fragment treated with N-OH-APNH in the presence and absence of NADH and/or Cu(II). Oligonucleotides that formed after DNA cleavage were detected on the autoradiogram. In the presence of Cu(II), N-OH-APNH caused DNA damage in a dose-dependent manner. When NADH was added, low concentrations of N-OH-APNH induced Cu(II)-mediated DNA damage efficiently. In the absence of N-OH-APNH, DNA damage was not observed with NADH and Cu(II) under the condition used. N-OH-APNH alone or N-OH-APNH + NADH did not cause DNA damage. Piperidine treatment showed approximately five-fold increase of oligonucleotide formation, while some oligonucleotides were observed also without piperidine treatment (data not shown). This result suggests that N-OH-APNH induces not only strand breakage but also base modification and/or liberation.

3.2. Effects of scavengers and bathocuproine on DNA damage

The effects of scavengers and bathocuproine on DNA damage by N-OH-APNH were investigated (Fig. 2). A typical $^{\bullet}OH$ scavenger mannitol did not inhibit DNA damage induced by N-OH-APNH in the presence of Cu(II), whereas methional inhibited the DNA damage. Bathocuproine, a Cu(I)-specific chelator, inhibited DNA damage, suggesting the involvement of Cu(I). DNA damage induced by N-OH-APNH was inhibited

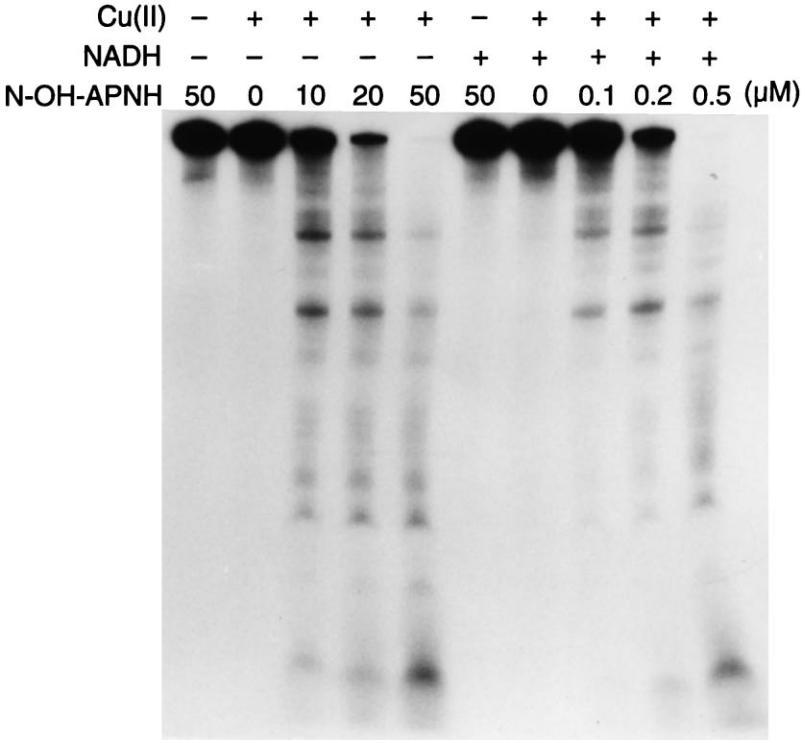

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragment incubated with N-OH-APNH, NADH, and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 261 bp DNA fragment, 10 μM/base calf thymus DNA, various concentrations of N-OH-APNH, 10% (v/v) DMSO, 100 μM NADH, and 20 μM $CuCl_2$ in phosphate buffer (pH 7.8). The reaction was incubated at 37°C for 1 h, followed by a piperidine treatment, as described in Section 2. The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and an autoradiogram was obtained by exposing an X-ray film to the gel.

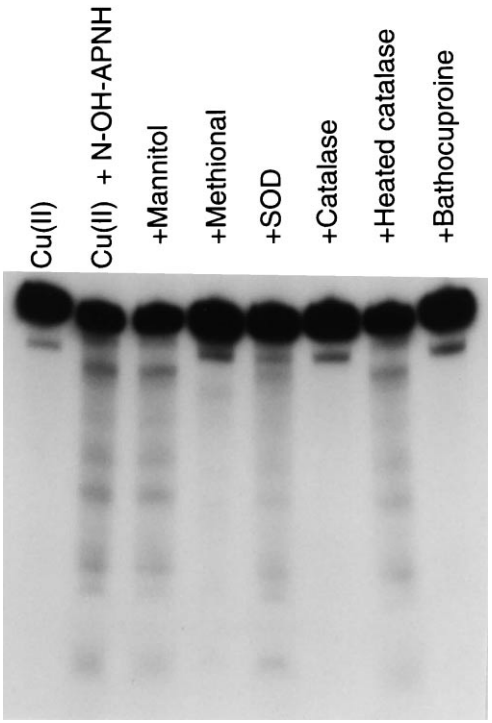

Fig. 2. Effects of scavengers and bathocuproine on DNA damage induced by N-OH-APNH + Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 118 bp DNA fragment, 10 μM/base calf thymus DNA, 10 μM N-OH-APNH, 10% (v/v) DMSO, and 20 μM $CuCl_2$ in phosphate buffer. The reaction was incubated at 37°C for 1 h, followed by a piperidine treatment. The DNA fragments were analyzed as described in 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 (heat-denatured for 10 min at 90°C before use); 30 units of SOD; 50 μM bathocuproine.

by catalase, while not inhibited by heated catalase. SOD showed little inhibitory effect on DNA damage. In the presence of NADH, same scavenger effects were observed (data not shown).

3.3. Site specificity of DNA damage by N-OH-APNH

An autoradiogram was obtained and scanned with a laser densitometer to measure the relative intensities of DNA cleavage products from the human c-Ha-*ras*-1 protooncogene (Fig. 3A) and the *p53* tumor suppressor gene (Fig. 3B). N-OH-APNH was observed to preferentially induce piperidine-labile sites at thymine and cytosine residues in the presence of Cu(II). In the presence of NADH, same DNA cleavage pattern was observed (data not shown).

3.4. Formation of 8-oxodG in calf thymus DNA

Using HPLC–ECD, we measured the 8-oxodG content of calf thymus DNA treated with N-OH-APNH in the presence of Cu(II) (Fig. 4A). The amount of 8-oxodG increased with the concentrations of N-OH-APNH. Formation of 8-oxodG increased after DNA denaturation. When NADH was added, 8-oxodG formation was observed at very low concentrations of N-OH-APNH (Fig. 4B).

3.5. Autoxidation of N-OH-APNH, and NADH consumption

Fig. 5A shows spectral changes of N-OH-APNH (solid lines) compared to a spectrum of NO_2-PNH (a dotted line). The absorption near 400 nm increased gradually with autoxidation of N-OH-APNH, whereas NO_2-PNH does not have maximum peak at 400 nm but has 356 nm. This result shows that the oxidation of N-OH-APNH did not proceed to NO_2-PNH. In the presence of Cu(II), UV–VIS spectrum of oxidized product was similar, but the oxidation of N-OH-APNH occurred within 1 min (data not shown).

Fig. 5B shows NADH consumption during redox reaction between NADH and oxidized products of N-OH-APNH. In the absence of Cu(II), NADH was slowly consumed. In the presence of Cu(II), NADH was consumed efficiently, suggesting that the redox reactions cycled very rapidly. Without N-OH-APNH, the spectrum of the reaction mixture of Cu(II) + NADH did not change under the condition used (data not shown).

The HPLC–MS analyses of the oxidized products showed four typical chromatogram peaks. The biggest peak had a molecular ion peak *m/z* 274 $[M + 1]^+$, indicating formation of nitroso form of APNH, nitrosophenylnorharman (NO-PNH) (data not shown).

4. Discussion

The present study showed that N-OH-APNH caused Cu(II)-mediated DNA damage, which was dramatically enhanced with endogenous reductant NADH. N-OH-APNH induced Cu(II)-mediated formation of

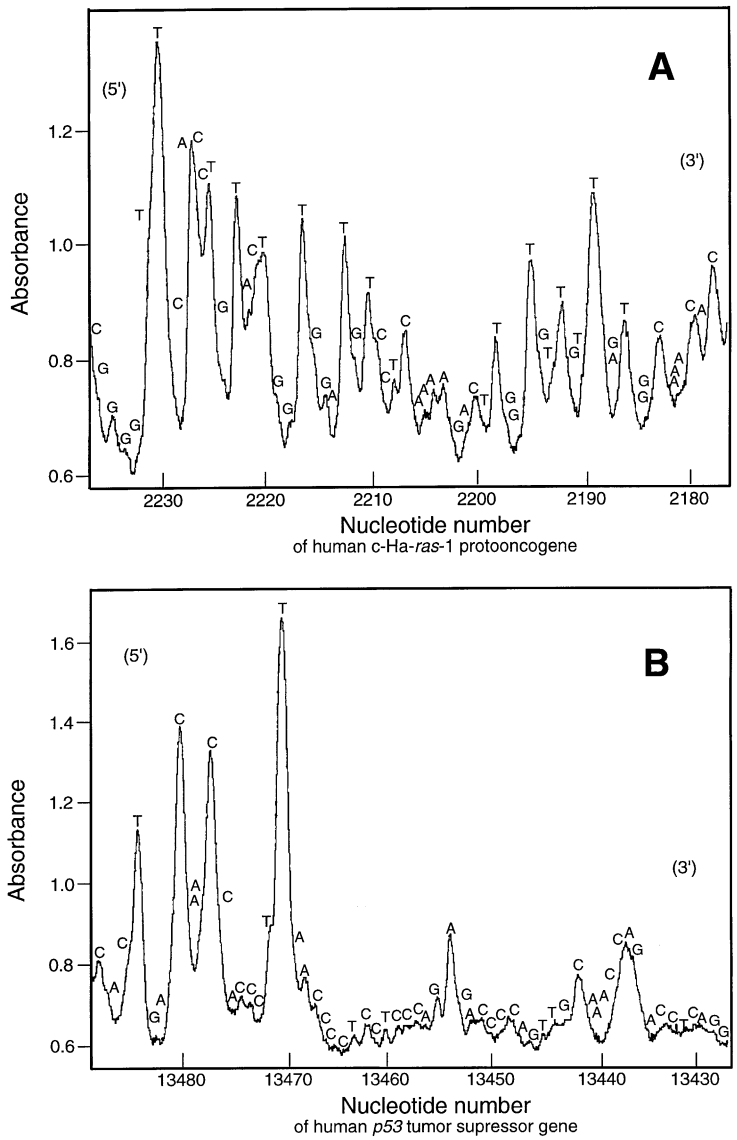

Fig. 3. Site specificity of DNA cleavage induced by N-OH-APNH + Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled DNA, 50 μM/base calf thymus DNA, 20 μM N-OH-APNH, 2% (v/v) DMSO, and 10 μM $CuCl_2$ in phosphate buffer. The reaction was incubated at 37°C for 2 h, followed by a piperidine treatment. The horizontal axis shows the nucleotide number: (A) 341 bp fragment (*Xba*I 1906–*Ava*I* 2246) of the c-Ha-*ras*-1 protooncogene; (B) 343 bp fragment (*Sty*I 13160–*Eco*RI* 13507) of the *p53* tumor suppressor gene.

piperidine-labile sites preferentially at thymine and cytosine residues. We detected formation of 8-oxodG, a piperidine-inert oxidative damage at guanine. NADH also enhanced the 8-oxodG formation induced by N-OH-APNH in the presence of Cu(II). To clarify the reactive species involved in oxidative DNA damage by N-OH-APNH, we examined the effects of scavengers on DNA damage. Both catalase and bathocuproine were found to reduce DNA damage, indicating the involvement of H_2O_2 and Cu(I). Failure to see an effect of typical $^{\bullet}$OH scavenger implies that free $^{\bullet}$OH does not play a major role in DNA damage. Alternatively, the

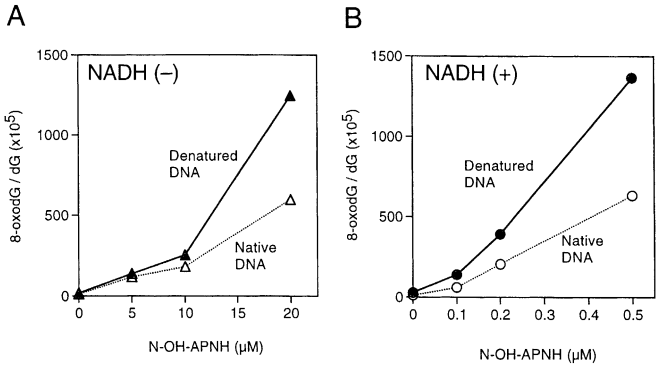

Fig. 4. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by N-OH-APNH. Calf thymus DNA (50 μM per base) was incubated with the indicated concentrations of N-OH-APNH and 20 μM $CuCl_2$ for 1 h at 37°C in the absence (A) and presence (B) of 100 μM NADH. After ethanol precipitation, DNA was enzymatically digested into nucleosides, and 8-oxodG formation was measured with an HPLC–ECD, as described in Section 2. Open symbols indicate that native DNA was used, and closed symbols indicate denatured DNA.

lack of effect of •OH scavengers may be due to DNA damage induced by •OH which is generated by metal ions bound in very close proximity to DNA. The inhibitory effect of methional on DNA damage can be explained by assuming that sulfur compounds are reactive with •OH and less reactive species [24].

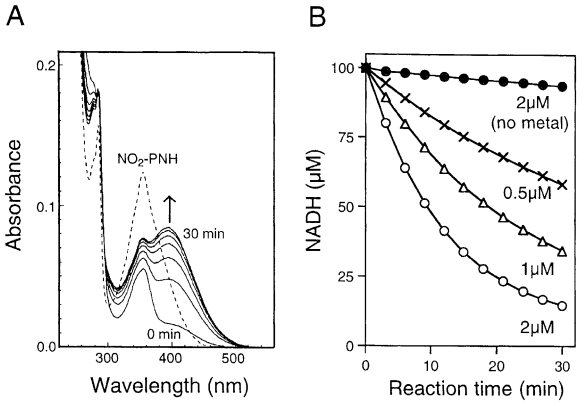

Fig. 5. Changes in spectrum with the time for autoxidation of N-OH-APNH and NADH consumption. (A) UV–VIS spectrum of 10 μM N-OH-APNH was traced every 5 min (solid lines). The dotted line shows the spectrum of 10 μM NO_2-PNH. (B) The concentrations of NADH were plotted every 3 min during redox reaction of N-OH-APNH. NADH (100 μM) was incubated with 2 μM N-OH-APNH (closed circles), 0.5 μM N-OH-APNH + 20 μM $CuCl_2$ (crosses), 1 μM N-OH-APNH + 20 μM $CuCl_2$ (triangles), and 2 μM N-OH-APNH + 20 μM $CuCl_2$ (open circles).

A possible mechanism as shown in Fig. 6 can be envisioned as accounting for most of the observations. Norharman reacts with aniline to form APNH, which is *N*-hydroxylated to N-OH-APNH [6]. N-OH-APNH induces Cu(II)-mediated oxidative DNA damage. Autoxidation of N-OH-APNH to nitroso compound (NO-PNH) via radical intermediate occurs coupled with generation of O_2^-. Formation of NO-PNH was suggested by UV–VIS spectroscopic studies and MS analyses. Relevantly, it is demonstrated that *N*-hydroxy compound of Trp-P-2 is autoxidized to form its nitroso derivative with generation of O_2^- [25]. O_2^- is dismutated to H_2O_2 and reduces Cu(II) to Cu(I). H_2O_2 interacts with Cu(I) to form DNA–copper–hydroperoxo complexes, causing DNA damage. Both Cu(I) and ROS generation during autoxidation of N-OH-APNH are required for DNA damage. DNA–copper–hydroperoxo complexes have been suggested in other studies [26–28].

The biological significance of copper has recently drawn much interest in connection with carcinogenicity and mutagenicity. Copper is an essential component of chromatine and is known to accumulate preferentially in the heterochromatic regions [29–31]. Copper sulfate showed clastogenic effects on the bone marrow chromosomes of mice in vivo [32]. Sone et al. suggested that excess intracellular copper in LEC rats induces DNA damage through ROS produced by Cu(II)/Cu(I) redox cycling and subsequently plays a crucial role in hepatocarcinogenesis [33]. Therefore, it is reasonable

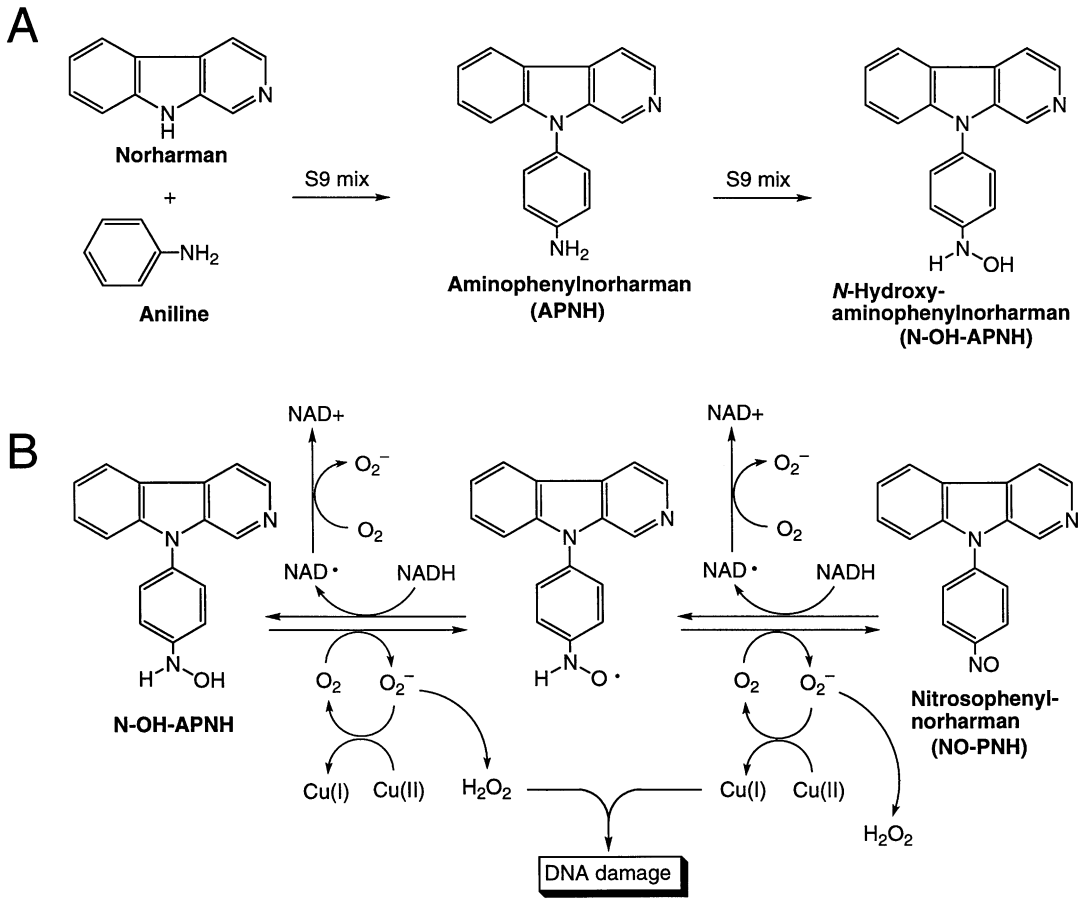

Fig. 6. (A) A process of the formation of N-OH-APNH [4]. (B) A proposed mechanism of metal-mediated DNA damage by N-OH-APNH in the presence of NADH.

to consider a possibility of copper involvement in DNA damage by N-OH-APNH.

When NADH is added, NO-PNH, the oxidized product of N-OH-APNH, may be reduced to the intermediate or N-OH-APNH, and again autoxidation occurred with excessive generation of ROS by forming redox cycle non-enzymatically. This is supported by reports; NADH reduced nitroso-aromatic compounds to reactive intermediates causing oxidative DNA damage [34,35]. Relevantly, in the case of a metabolite of carcinogenic heterocyclic amine, such as MeIQx, oxidative DNA damage was greatly enhanced by NADH via redox cycle [13]. The concentration of NAD(P)H in certain tissue has been estimated to be as high as 100–200 μM [36]. The biological importance of NADH and NADPH as nuclear reductants [37] has been demonstrated before.

It is considered that *N*-hydroxy derivatives of carcinogenic heterocyclic amines covalently form DNA adducts to induce mutagenicity and carcinogenicity [10]. The present study has revealed that N-OH-APNH has oxidative DNA-damaging ability, and NADH enhances the damage through cycling redox reactions. It is noteworthy that a very low concentration (0.1 μM) of N-OH-APNH induced 8-oxodG formation, suggesting that oxidative DNA damage can occur in vivo. Oxidative DNA damage may play an important role in carcinogenic process of APNH through ROS formation, in addition to DNA adduct formation.

Norharman can react with an aniline derivative, such as toluidine, to form coupling compound like APNH [3]. Although, aniline and toluidine are industrial materials [38,39], some aniline derivatives also exist in vivo, such as kynurenine, a metabolite of tryptophan [40,41]. Norharman may react with endogenous aniline derivative in vivo to form coupled genetoxic compounds which cause oxidative DNA damage as well as N-OH-APNH.

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