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Mechanism of Oxidative DNA Damage Induced by a Heterocyclic Amine, 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline

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Adduct formation has been considered to be a major causal factor of DNA damage by carcinogenic heterocyclic amines. By means of experiments with ^{32}P-labeled DNA fragments and an electrochemical detector coupled to a high-pressure liquid chromatograph, we investigated whether the *N*-hydroxy metabolite of 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) can cause oxidative DNA damage or not. This metabolite [MeIQx(NHOH)] was found to cause Cu(II)-mediated DNA damage, including 8-oxo-7,8-dihydro-2′-deoxyguanosine formation. When an endogenous reductant, β-nicotinamide adenine dinucleotide (NADH), was added, the DNA damage was greatly enhanced. Catalase and bathocuproine, a Cu(I)-specific chelator, inhibited the DNA damage, suggesting the involvement of H_2O_2 and Cu(I). MeIQx(NHOH) frequently induced DNA cleavage at thymine and cytosine residues in the presence of NADH and Cu(II). A UV-visible spectroscopic study showed that little decomposition of MeIQx(NHOH) occurred in the absence of Cu(II), whilst rapid spectral change was observed in the presence of Cu(II), suggesting that Cu(II) catalyzes the autoxidation. The addition of NADH reduced the oxidized product back to MeIQx(NHOH). These results suggest that a copper-peroxo intermediate, derived from the reaction of Cu(I) with H_2O_2, participates in Cu(II)-dependent DNA damage by MeIQx(NHOH), and NADH enhances the DNA damage via a redox cycle. We conclude that in addition to DNA adduct formation, oxidative DNA damage plays an important role in the carcinogenic process of MeIQx.

Key words: Heterocyclic amine — MeIQx — Oxidative DNA damage — Copper — NADH

Several heterocyclic amines isolated from cooked foods are among the most potent mutagens known.[1, 2] 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is one such heterocyclic amine isolated from cooked beef, chicken and mutton.[3, 4] Oral administration of MeIQx produces hepatocellular carcinomas and lung tumors in mice,[5–7] and hepatocellular carcinomas and squamous cell carcinomas of the Zymbal gland in rats.[5, 8, 9] An epidemiological study showed a significantly increased risk for cancers at all sites and for gastric cancer associated with the consumption of cooked fish.[4] Thus, most heterocyclic amines in food-pyrolysates were suggested to pose probable or possible carcinogenic risk to humans.[4]

Regarding the mechanisms of DNA damage by carcinogenic heterocyclic amines, DNA adduct formation has been considered to be a major causal factor. That is, MeIQx is oxidized to the *N*-hydroxy derivative [MeIQx(NHOH)] in the liver by cytochrome P450 IA2 isozyme, and the latter is esterified by *O*-acetyltransferase to the *N*-acetoxy derivative, which reacts with DNA to form adducts.[10, 11] DNA adducts generated by MeIQx were found *in vitro* and *in vivo* by means of the ^{32}P-post-labeling method.[12] On the other hand, it has been reported that several antioxidants[13–15] significantly inhibited MeIQx-induced hepatocarcinogenesis in rats. Kato *et al.*[16] reported that the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) level in rat liver increased dose-dependently with the concentration of MeIQx in the diet. These reports lead us to consider that reactive oxygen species may participate in heterocyclic amine-induced tumor development. Hayatsu and his colleagues[17, 18] showed that the *N*-hydroxy derivative of 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) produces intracellular reactive oxygen species that can damage DNA in mouse cells in culture. Therefore, DNA adduct formation is a prerequisite, but the DNA adducts themselves may not be sufficient for the carcinogenic action. There remains a possibility that oxidative DNA damage also plays a role in carcinogenesis induced by MeIQx.

In this study, we investigated whether MeIQx(NHOH) can cause oxidative DNA damage or not, 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-oxodG formation in calf thymus DNA by MeIQx(NHOH) in the presence of Cu(II) and β-nicotinamide adenine dinucleotide (NADH). Furthermore, in order to clarify the mechanism of oxidative DNA damage, spectral changes during the autoxidation of MeIQx(NHOH) were measured, using UV-visible spectroscopy.

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MATERIALS AND METHODS

Materials Restriction enzymes (*Sma*I, *Eco*RI, *Apa*I and *Sty*I) were purchased from Boehringer Mannheim (Mannheim, Germany). Restriction enzymes (*Hin*dIII, *Ava*I and *Xba*I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear (Boston, MA). MeIQx(NHOH) was supplied by NCI Chemical Carcinogen Reference Standard Repository in Midwest Research Institute (Menlo Park, CA). β-Nicotinamide adenine dinucleotide disodium salt (reduced form) (NADH) was purchased from Kohjin Co. (Tokyo). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were from Dojin Chemicals Co. (Kumamoto). 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). Human *p53* Amplimer Panel was from Clontech Lab. (Palo Alto, CA). Primers designed for use in the polymerase chain reaction (PCR) process for the amplification of the *p53* gene are contained in this product.

Preparation of ^{32}P-5′-end-labeled DNA fragments Exon-containing DNA fragments obtained from the human *p53* tumor suppressor gene[19] were prepared, as described previously.[20] The 5′-end-labeled 650-bp fragment (*Hin*dIII* 13972–*Eco*RI* 14621) was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase (*, ^{32}P-label). The 650-bp fragment was further digested with *Apa*I to obtain a singly labeled 443-bp fragment (*Apa*I 14179–*Eco*RI* 14621) and a 211-bp fragment (*Hin*dIII* 13972–*Apa*I 14182). A DNA fragment was also obtained from the human c-Ha-*ras*-1 protooncogene.[21] A DNA fragment was prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the c-Ha-*ras*-1 gene, and a singly labeled 98-bp fragment (*Ava*I* 2247–*Pst*I 2344) and a 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) were obtained according to the method described previously.[22] Nucleotide numbering starts with the *Bam*HI site.[21]

Detection of DNA damage by MeIQx(NHOH) in the presence of NADH and Cu(II) The standard reaction mixture (in a microtube; 1.5 ml; Eppendorf) contained MeIQx(NHOH), NADH and $CuCl_2$, ^{32}P-5′-end-labeled DNA fragments and sonicated calf thymus DNA (5 μM per base) in 200 μl of 10 m*M* sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. After incubation at 37°C for the indicated duration, the DNA fragments were heated at 90°C in 1 *M* piperidine for 20 min where indicated and treated as described previously.[23]

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[24] using a DNA-sequencing system (LKB 2010 Macrophor, Pharmacia Biotech, Uppsala, Sweden). The relative amounts of oligonucleotides from the treated DNA fragments were measured with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech).

Analysis of 8-oxodG formation in calf thymus DNA by MeIQx(NHOH) in the presence of NADH and Cu(II) Native or denatured DNA fragments (100 μM per base) from calf thymus were incubated with MeIQx(NHOH), NADH and $CuCl_2$ for the indicated duration at 37°C. Denatured DNA fragments were obtained by heating at 90°C for 5 min followed by chilling on ice before incubation. After ethanol precipitation, DNA was enzymatically digested to the nucleosides and analyzed by high-pressure liquid chromatography with an electrochemical detector (HPLC-ECD), as described previously.[25]

UV-visible spectral measurement during autoxidation of MeIQx(NHOH) UV-visible spectra of MeIQx(NHOH) were measured with a UV-visible spectrometer (UV-2500PC, Shimadzu, Kyoto). The reaction mixture contained MeIQx(NHOH) in 10 m*M* phosphate buffer (pH 7.8). Where indicated, 5 μM $CuCl_2$ was added to the reaction mixtures. The spectra of the mixtures were measured repeatedly at 37°C for the indicated duration. To analyze the redox reaction with NADH, the absorption maximum of NADH at 340 nm was measured with a UV-visible spectrometer. The decrease in absorbance at 340 nm (ε=6.22×10^3 $M^{-1}\cdot cm^{-1}$) is due to NADH oxidization to NAD^+.

RESULTS

Damage to ^{32}P-labeled DNA fragments by MeIQx-(NHOH) in the presence of NADH and Cu(II) Fig. 1 shows an autoradiogram of DNA fragments treated with MeIQx(NHOH) in the presence and absence of NADH and Cu(II). Oligonucleotides were detected on the autoradiogram as a result of DNA cleavage. In the absence of MeIQx(NHOH), DNA damage was not observed with NADH and Cu(II) under the conditions used. MeIQx(NHOH) alone and MeIQx(NHOH) plus NADH did not cause DNA damage. MeIQx(NHOH) induced DNA damage in the presence of Cu(II). The intensity of DNA damage increased with increasing concentration of MeIQx(NHOH) (Fig. 1) and incubation time (data not shown). When NADH was added, low concentrations of MeIQx(NHOH) efficiently induced Cu(II)-mediated DNA damage. The increase of oligonucleotides by piperidine treatment suggested that not only strand breakage, but also base modification and/or liberation were induced.

Effects of scavengers and bathocuproine on DNA damage induced by MeIQx(NHOH) in the presence of Cu(II) Fig. 2 shows the effects of scavengers and batho-

cuproine, a Cu(I)-specific chelator, on DNA damage induced by MeIQx(NHOH) in the presence of Cu(II). Inhibition of DNA damage by catalase and bathocuproine suggests the involvement of hydrogen peroxide (H_2O_2) and Cu(I). Methional inhibited the DNA damage, although other typical hydroxyl radical (•OH) scavengers, ethanol, mannitol and sodium formate, did not. SOD showed no inhibitory effect on DNA damage. Similar effects of scavengers and bathocuproine were obtained in the case of MeIQx(NHOH) in the presence of Cu(II) and NADH (data not shown).

Site specificity of DNA cleavage by MeIQx(NHOH) in the presence of NADH and Cu(II) An autoradiogram was obtained and scanned with a laser densitometer to measure relative intensity of DNA cleavage in the human *p53* tumor suppressor gene as shown in Fig. 3. MeIQx(NHOH) plus Cu(II) induced piperidine-labile sites preferentially at thymine and cytosine residues (Fig. 3A). When NADH was added (Fig. 3B), a similar cleavage pattern was observed. It is noteworthy that the cytosine residue of the ACG sequence is complementary to codon 273, which is a known hotspot[26] of the *p53* gene. DNA cleavage was also observed frequently at thymine and cytosine in the c-Ha-*ras*-1 protooncogene (Fig. 4A). When denatured DNA (Fig. 4B) was used, damage occurred more frequently at guanine sites.

Formation of 8-oxodG in calf thymus DNA by MeIQx(NHOH) in the presence of NADH and Cu(II) Using HPLC-ECD, we measured the 8-oxodG content in calf thymus DNA treated with MeIQx(NHOH) in the presence of NADH and Cu(II) (Fig. 5). The amount of 8-oxodG increased with the concentration of MeIQx-(NHOH) in the presence of Cu(II). The formation of 8-oxodG increased after DNA denaturation (data not shown). When NADH was added, a dramatic increase of 8-oxodG formation was observed.

UV-visible spectroscopic study on the autoxidation of MeIQx(NHOH) Fig. 6 shows the UV-visible spectral changes of MeIQx(NHOH) in the presence and absence of Cu(II). The spectra of MeIQx(NHOH) changed only a little during 60 min, suggesting very slow autoxidation, in the absence of Cu(II) (Fig. 6A). When Cu(II) was added (Fig. 6B), MeIQx(NHOH) showed a rapid decrease in the absorbance maximum at 260 nm and an increase in the absorbance maximum at 420 nm within 10 min.

The spectral changes of NADH oxidation were measured to clarify the effect of NADH on the reaction mix-

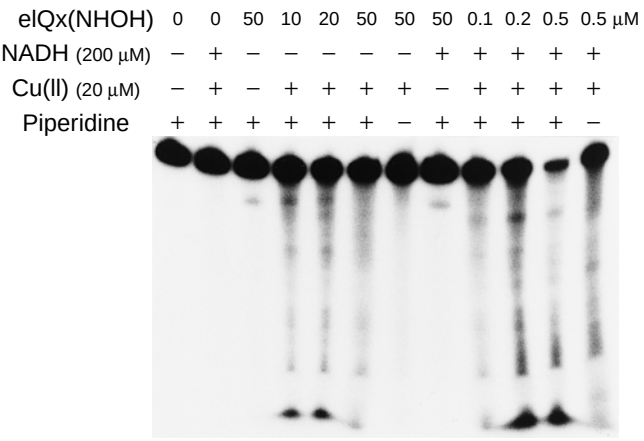

Fig. 1. Autoradiogram of ^{32}P-labeled DNA fragments incubated with MeIQx(NHOH) in the presence of NADH and Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 98-bp DNA fragments, 5 μM per base of sonicated calf thymus DNA, indicated concentrations of MeIQx(NHOH), 200 μM NADH, and 20 μM $CuCl_2$ 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 1 h, followed by piperidine treatment where indicated, as described in "Materials and Methods." The DNA fragments were electrophoresed on an 8% polyacrylamide/8 M urea gel and the autoradiogram was obtained by exposing an X-ray film to the gel.

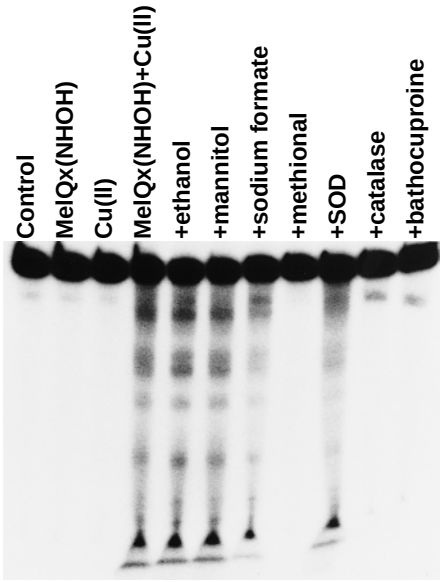

Fig. 2. Effects of scavengers and bathocuproine on DNA damage induced by MeIQx(NHOH) in the presence of Cu(II). The reaction mixture contained ^{32}P-5′-end-labeled 211-bp DNA fragments, 5 μM per base of sonicated calf thymus DNA, 50 μM MeIQx(NHOH) and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. The mixture was incubated at 37°C for 1 h, followed by piperidine treatment. The DNA fragments were analyzed as described in the legend to Fig. 1. The concentrations of scavengers and bathocuproine were as follows: 5% (v/v) ethanol; 0.1 M mannitol; 0.1 M sodium formate; 0.1 M methional; 30 units of SOD; 30 units of catalase; 50 μM bathocuproine.

ture of MeIQx(NHOH) and Cu(II). A redox reaction between NADH and oxidized products of MeIQx(NHOH) was observed. Fig. 6C shows that the absorbance maximum at 340 nm of NADH was decreased. It is estimated that 67.5 μM NADH was oxidized to NAD^+ in 60 min, when 200 μM of NADH was used.

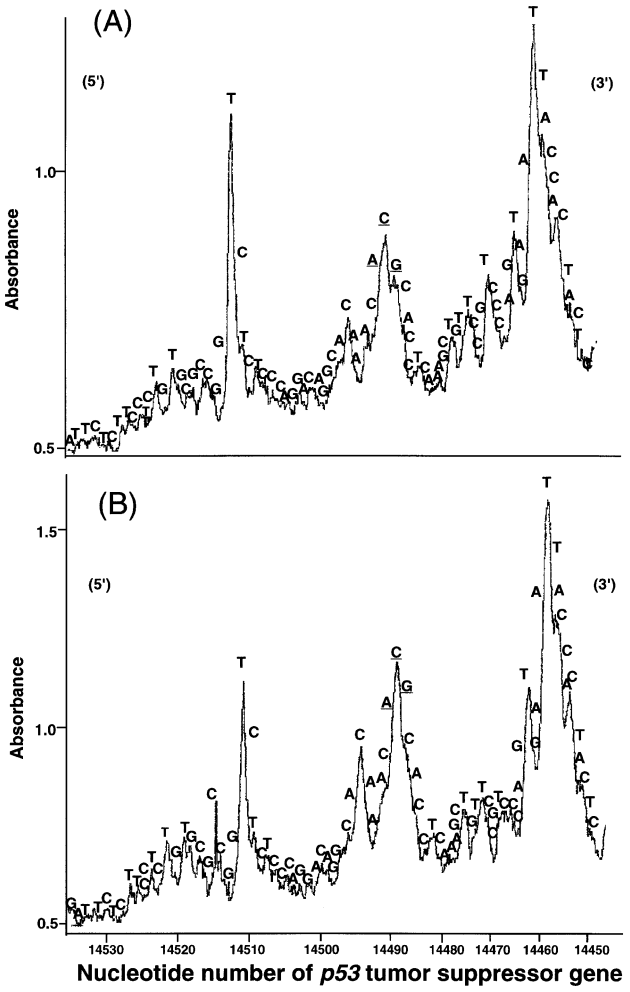

Fig. 3. Site specificity of Cu(II)-mediated DNA cleavage induced by MeIQx(NHOH) in the presence and absence of NADH. The reaction mixture contained ^{32}P-5′-end-labeled 443-bp fragment (*Apa*I 14179–*Eco*RI* 14621) of the *p53* tumor suppressor gene, 5 μM per base of sonicated calf thymus DNA and 50 μM MeIQx(NHOH) plus 20 μM $CuCl_2$ (A), or 0.5 μM MeIQx(NHOH) plus 200 μM NADH plus 20 μM Cu(II) (B), in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Reaction mixtures were incubated at 37°C for 1 h. After the piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 1. The relative amounts of oligonucleotide were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL, Pharmacia Biotech). The horizontal axis shows the nucleotide number of the human *p53* tumor suppressor gene[19] and underscoring shows the complementary sequence to codon 273 (nucleotide number 11486–11488).

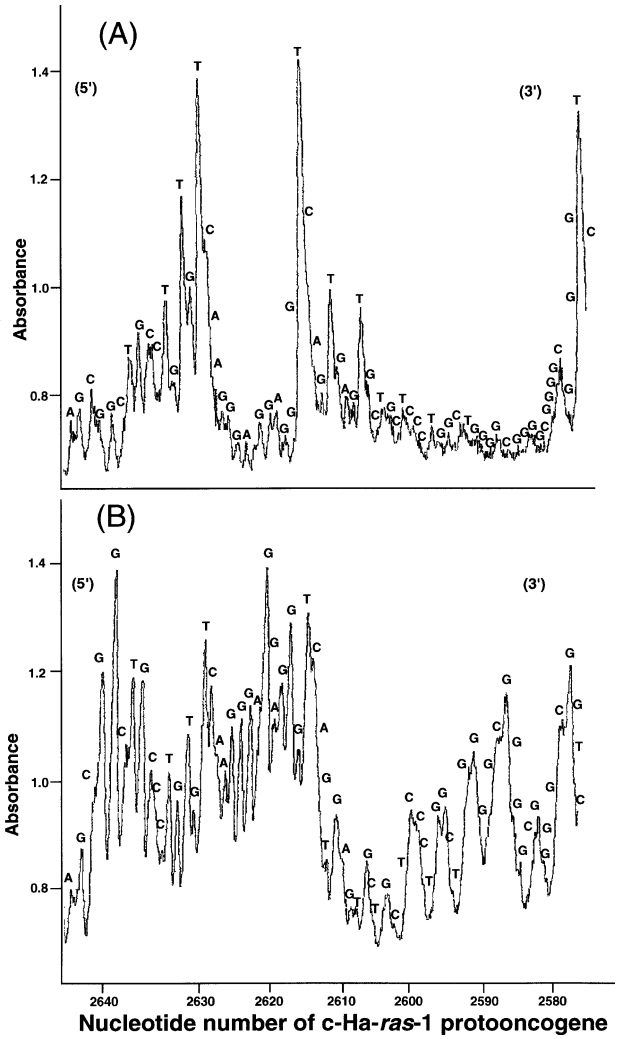

Fig. 4. Effect of denaturation of DNA on site specificity of DNA cleavage induced by MeIQx(NHOH) in the presence of Cu(II) and NADH. The reaction mixture contained ^{32}P-5′-end-labeled 337-bp fragment (*Pst*I 2345–*Ava*I* 2681) of the human c-Ha-*ras*-1 protooncogene, 5 μM per base of sonicated calf thymus DNA, 0.1 μM MeIQx(NHOH), 20 μM $CuCl_2$ and 200 μM NADH in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 μM DTPA. Native (A) and denatured (B) DNA was used. DNA fragments were heated at 90°C for 5 min for denaturation before the incubation. Reaction mixtures were incubated at 37°C for 1 h. After the piperidine treatment, the DNA fragments were analyzed as described in the legend to Fig. 3. The horizontal axis shows the nucleotide number of the human c-Ha-*ras*-1 protooncogene starting with the *Bam*HI site.[21]

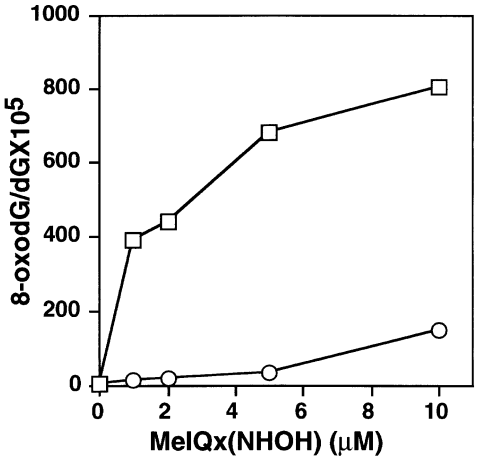

Fig. 5. Cu(II)-mediated formation of 8-oxodG in calf thymus DNA by MeIQx(NHOH) in the presence and absence of NADH. Calf thymus DNA fragments (100 μM per base) were incubated with the indicated concentration of MeIQx(NHOH) and 20 μM $CuCl_2$ in the presence and absence of 200 μM NADH for 1 h at 37°C. After ethanol precipitation, DNA was digested to the nucleosides with nuclease P_1 and calf intestine phosphatase and analyzed by HPLC-ECD. The symbols are as follows: circles, MeIQx+Cu(II); squares, MeIQx+Cu(II)+NADH.

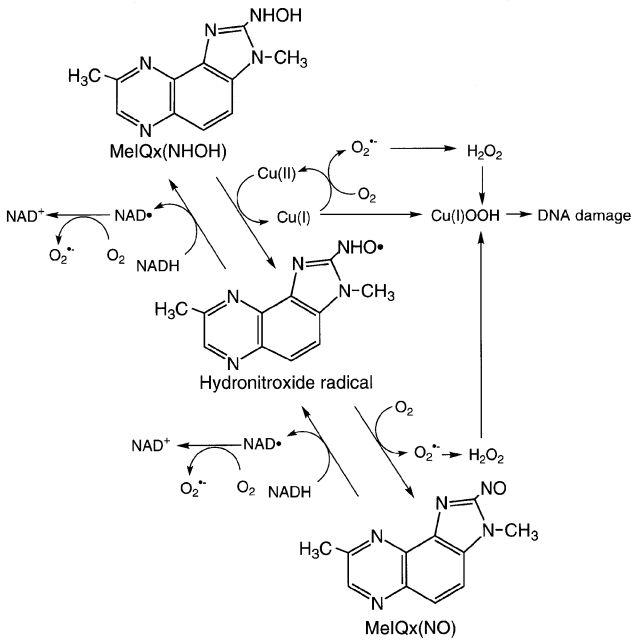

Fig. 7. Proposed mechanism for oxidative DNA damage induced by MeIQx(NHOH) in the presence of Cu(II) plus NADH.

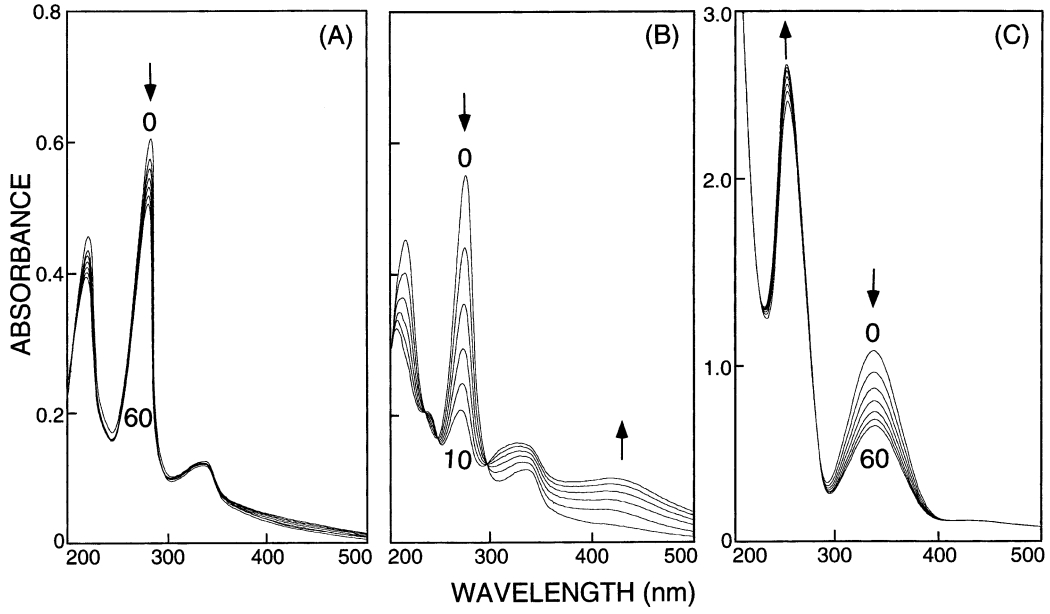

Fig. 6. Effects of Cu(II) and NADH on spectral changes of MeIQx(NHOH). Spectra were measured with a UV-visible spectrometer at 37°C, 20 μM MeIQx(NHOH), every 10 min for 60 min (A), 20 μM MeIQx(NHOH) and 5 μM $CuCl_2$, every 2 min for 10 min (B), 20 μM MeIQx(NHOH), 5 μM $CuCl_2$ and 200 μM NADH, every 10 min for 60 min (C) in 10 mM phosphate buffer (pH 7.8).

DISCUSSION

The present study has demonstrated that the *N*-hydroxy derivative of a heterocyclic amine has the ability to cause oxidative DNA damage, including 8-oxodG formation, in the presence of Cu(II). In addition, the DNA damage was dramatically enhanced by an endogenous reductant, NADH. The UV-visible spectroscopic study showed that Cu(II) mediated autoxidation of MeIQx(NHOH) to the oxidized form, probably the nitroso derivative of MeIQx [MeIQx(NO)]. When NADH was added, MeIQx(NO) might be reduced to the hydronitroxide radical of MeIQx [MeIQx(NHO•)] and/or MeIQx(NHOH), and autoxidation would occur again, suggesting that a redox cycle is formed in the presence of NADH.

The hypothetical mechanism of oxidative DNA damage by MeIQx(NHOH) shown in Fig. 7 can account for most of the observations. Metal-mediated autoxidation of the *N*-hydroxy derivative to the hydronitroxide radical occurs. The hydronitroxide radical is further autoxidized to a nitroso derivative with generation of $O_2^{\cdot-}$ from O_2. The generation of H_2O_2 by $O_2^{\cdot-}$ dismutation proceeds with concomitant reduction of Cu(II) to Cu(I). The inhibitory effects of catalase and bathocuproine suggest that H_2O_2 and Cu(I) are required for the DNA damage. Typical •OH scavengers did not protect DNA from *N*-hydroxy derivatives in the presence of Cu(II). One possible explanation for this is that •OH is not involved in DNA damage. These results suggest that DNA-associated Cu(I) generates other oxidants, including a copper-peroxo intermediate, such as Cu(I)-OOH, which is formed by reaction of H_2O_2 and Cu(I). This may be supported by the inhibitory effect of methional on the DNA damage. It can be speculated that the reactive species is a kind of crypto-hydroxy radical, copper-peroxo intermediate, because it has been reported that the radical is reactive with sulfur derivatives such as methionine[27] and methional,[28] but not with traditional •OH scavengers.

It was reported that very powerful one-electron oxidants would be able to oxidize NADH via a mechanistic pathway involving one-electron transfer.[29] Several studies indicate that NADH may react nonenzymatically with some xenobiotics and mediate their reduction.[30, 31] We compared the intensity of DNA damage induced by *N*-hydroxy derivatives with or without NADH in the presence of Cu(II). When NADH was added, the Cu(II)-mediated DNA damage was dramatically enhanced by very low concentrations of *N*-hydroxy derivatives. The formation of 8-oxodG was also enhanced in the presence of NADH. It is known that aromatic nitroso compounds can be easily reduced to *N*-hydroxy derivatives by NADH.[32] Endogenous reductants, such as NADH, reduce nitroso or nitro derivatives to *N*-hydroxy derivatives with the generation of $O_2^{\cdot-}$ from O_2, forming a redox cycle. The cycling of redox reactions would enhance the DNA damage with excessive generation of reactive oxygen species. Maeda and his colleagues[33, 34] detected $O_2^{\cdot-}$ generation from various heterocyclic amines including MeIQx, with cytochrome P-450 reductase/NADPH.

The possibility that metal ion plays an important role in non-enzymatic activation of NADH should be considered. The concentration of NAD(P)H in certain tissues was estimated to be as high as 100–200 μM.[35] The biological importance of NADH and NADPH as nuclear reductants has been pointed out.[36] Copper has been found in the nucleus and in close association with chromosomes and DNA bases, where it has physiological functions to maintain DNA structure.[37] Copper ions exhibit a very high affinity for DNA, and DNA-bound Cu(II) can undergo Cu(II)/Cu(I) redox cycling in a reducing environment and also reduce O_2 to $O_2^{\cdot-}$, generating H_2O_2. The DNA-Cu(I) complex reacts with H_2O_2[22] inducing DNA damage through a Fenton-type reaction.[38, 39] Thus, copper in chromatin and the nuclear reductant NADH may serve not only physiological functions, but also pathogenic functions under certain conditions.

Kato *et al.*[16] reported that MeIQx treatment induced a dose-dependent increase of 8-oxodG, and also an increase of glutathione *S* transferase placental form-positive foci, an indicator of carcinogenic potential, in rat liver. Hirose *et al.*[13–15] showed that several antioxidants inhibited MeIQx-promoted hepatocarcinogenesis in a rat medium-term liver bioassay model. The mechanism of oxidative DNA damage by MeIQx may be explained as follows: the *N*-hydroxy derivative of the heterocyclic amine MeIQx induces Cu(II)-mediated DNA damage through reactive oxygen species, and in the presence of NADH, the damage occurs in the presence of a very small amount of *N*-hydroxy derivative through a cycling redox reaction. Genetic alteration in the *p53* gene was observed in rats treated with MeIQx.[40] It is noteworthy that MeIQx-(NHOH) attacked the cytosine residue of the ACG sequence complementary to codon 273, a known hotspot[26] of the *p53* gene. In conclusion, oxidative DNA damage appears to play an important role in carcinogenicity of MeIQx, in addition to the previously reported MeIQx-DNA adduct formation.

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