Copper-mediated DNA damage by metabolites of p-dichlorobenzene

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p-Dichlorobenzene (p-DCB) has been reported to be carcinogenic for rodents, although it does not seem to be mutagenic in bacterial test systems. In this study, the mechanism of DNA damage by metabolites of p-DCB in the presence of metals was investigated by a DNA sequencing technique using 32P-labeled DNA fragments and by an electrochemical detector coupled to an HPLC. 2,5-Dichlorohydroquinone (DCHQ), one of the major metabolites, caused DNA damage in the presence of Cu(II). 2,5-Dichloro-p-benzoquinone (DCBQ) slightly induced DNA damage in the presence of Cu(II), but addition of NADH induced DNA damage very efficiently. DCHQ plus Cu(II) induced piperidine-labile sites at thymine residues at high frequency. A similar DNA cleavage pattern was observed with DCBQ plus Cu(II) in the presence of NADH. Both DCHQ and DCBQ plus NADH increased 8-oxo-7,8-dihydro-2'-deoxyguanosine in calf thymus DNA in the presence of Cu(II). Typical hydroxyl radical scavengers showed no inhibitory effects on this Cu(II)-mediated DNA damage. Bathocuproine and catalase inhibited the DNA damage, indicating the participation of Cu(I) and hydrogen peroxide (H2O2) in the DNA damage. UV-visible and ESR spectroscopy has demonstrated that DCHQ is rapidly autoxidized to DCBQ via a semiquinone radical, even in the absence of metal ions, indicating that the semiquinone radical itself is not the main active species inducing DNA damage. These results suggest that a semiquinone radical produced by autoxidation of DCHQ and/or reduction of DCBQ by NADH reacts with O2 to form superoxide and subsequently H2O2. Consequently, it is considered that the active species derived from the reaction of H2O2 with Cu(I) participates in the DNA damage.

Introduction

p-Dichlorobenzene (p-DCB) has been widely used as a repellent, an aerosol deodorant and a mildew control agent. Hill et al. (1) reported that, among 1000 adults in the USA, 98% had detectable levels of 2,5-dichlorophenol in their urine. 2,5-Dichlorophenol is mutagenic in bacterial test systems (2) and is carcinogenic in rodents (3). In addition, an increase in the level of 8-oxoG was observed in benzene-exposed mice and in benzene metabolite- and hydroquinone-treated cells (4).

Studies on the metabolism of p-DCB in vivo and in vitro have revealed that 2,5-dichlorohydroquinone (DCHQ) is one of the major metabolites of p-DCB in rodents (11-13). Oxidation of DCHQ to 2,5-dichloro-p-benzoquinone (DCBQ) can occur enzymatically (14). Several studies indicate that NADH may react non-enzymatically with some quinones and mediate their reduction (15). Therefore, it is of interest to clarify whether NADH plays an important role in non-enzymatic activation of p-DCB metabolites and causes metal-dependent DNA damage. In this study, we examined DNA damage by DCHQ and DCBQ plus NADH in the presence of Cu(II) using 32P-5'-end-labeled DNA fragments obtained from a proto-oncogene (c-Ha-ras-1). We also analyzed 8-oxoG formation in calf thymus DNA using an electrochemical detector coupled to an HPLC (HPLC-ECD) and investigated the reaction mechanism by UV-visible and electron spin resonance (ESR) spectroscopy.

Materials and methods

Materials

Restriction enzymes (BamHI, Avai, XbaI and PstI) and T4 polynucleotide kinase were purchased from New England Biolabs. Calf intestine phosphatase was from Boehringer Mannheim GmbH. [32P]ATP (222 TBq/mmol) was from New England Nuclear. DCHQ was from Eastman Kodak Co. DCBQ was from Tokyo Kasei Co. (Tokyo, Japan). Dihyldenetriamine-N,N',N",N"'-pentaaetic acid (DTPA) and bathocuproine disulfonic acid were from Dojin Chemicals Co. (Kumamoto, Japan). Acrylamide, dimethylsulfoxide (DMSO), bisacrylamide and piperidine were from Wako Chemicals Co. (Osaka, Japan). CuCl2, ethanol, t-mannitol and sodium formate were from Nacalai Tesque Inc. (Kyoto, Japan). Calf thymus DNA, superoxide dismutase (SOD) (3000 U/mg from bovine erythrocyte and catalase (45 000 U/mg from bovine liver) were from Sigma Chemical Co. Nuclease P1 was from Yamasa Shoyu Co. (Chiba, Japan).

Detection of damage to isolated DNA induced by DCHQ and DCBQ plus NADH in the presence of metal ions

DNA fragments were prepared from plasmid pBlCI, which carries a 6.6 kb BamHI chromosomal DNA segment containing the human c-Ha-ras-1 proto-
oncogene (16). Singly labeled 261 bp (Avul* 1645–XbaI 1905), 341 bp (XbaI 1906–Avul* 2246), 98 bp (Avul* 2247–PstI 2344) and 337 bp (PstI 2345–Avul* 2681) fragments were obtained according to the method described previously (16). The asterisk indicates 32P-labeling and nucleotide numbering starts with the BamHI site (17).

The standard reaction mixture in a microtube (1.5 ml Eppendorf tube) contained 10 μM DCBQ, 250 μM NADH, 20 μM CuCl2, [32P]DNA fragment and 20 μM base sonicated calf thymus DNA in 200 μl of 1 M sodium phosphate buffer, pH 7.8, containing 5 μM DTPA. After incubation at 37°C for 60 min, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min where indicated and treated as previously described (16).

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 (18) using a DNA sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 Ultrascan XL) was used for measurement of the relative amounts of oligonucleotides from treated DNA fragments.

Analysis of 8-oxodG in calf thymus DNA formed by DCHQ and DCBQ plus NADH in the presence of Cu(II)

Calf thymus DNA (100 μM/mg) was incubated with 40 μM DCHQ or 40 μM DCBQ plus 200 μM NADH in the presence or absence of 20 μM Cu(II) in 4 mM sodium phosphate buffer, pH 7.8, at 37°C for the indicated durations. After the addition of 1 mM DTPA, the DNA was precipitated by ethanol. Then the DNA was digested to nucleosides with nuclease P1 and alkaline phosphatase and analyzed by HPLC-ECD as previously described (19).

UV-visible spectra measurements

UV-visible spectra were measured at 25°C with a UV-vis-NIR recording spectrophotometer (Shimadzu UV-365).

ESR spectra measurements

ESR spectra were measured at 25°C using a JEOL-3XG (JEOL, Tokyo, Japan) spectrometer with 100 kHz field modulation according to the method previously described (20). Spectra were recorded with a microwave power of 16 mW, a modulation amplitude of 0.1 G and a receiver gain of 1×100. The magnetic fields were calculated from the splitting of Mn(II) in MgO (ΔH1/2 = 86.9 G).

Results

Damage of 32P-labeled DNA fragments induced by DCHQ in the presence of Cu(II)

Figure 1 shows an autoradiogram of DNA fragments treated with DCHQ in the presence of Cu(II). DNA cleavage increased with increasing concentration of DCHQ and with time (data not shown). The upper band and lower band in the control show single-stranded and double-stranded forms of intact DNA fragment respectively. The cleavage without piperidine treatment suggests breakage of the deoxyribose phosphate backbone by DCHQ plus Cu(II) (Figure 1A). The amount of oligonucleotides increased with piperidine treatment (Figure 1B). Since altered bases are readily removed from their sugar by piperidine treatment, it is considered that base alteration and/or liberation were induced by DCHQ in the presence of Cu(II). We estimated the relative ratio of direct DNA strand break formation versus the yield of base alterations and liberations by measuring the density of the bands with and without piperidine treatment. The yields of base alterations and liberations were ~70% of total DNA damage.

Neither DCHQ nor Cu(II) caused DNA damage by themselves.

DNA damage by DCBQ plus Cu(II) in the presence of NADH

Although DCBQ plus Cu(II) induced DNA damage slightly (Figure 2, lane 3), NADH enhanced DCBQ plus Cu(II)-induced DNA damage even in the absence of NADH-FMN oxidoreductase, which catalyzes the one electron reduction of quinones (Figure 2, lane 5). It is speculated that the 3-hydroxy derivative of DCHQ (3-OH-DCHQ) participates in DCBQ plus Cu(II)-induced DNA damage in the absence of NADH. The magnetic fields were calculated from the splitting of Mn(II) in MgO (ΔH1/2 = 86.9 G).

Fig. 1. Autoradiogram of 32P-labeled DNA fragments incubated with DCHQ in the presence of Cu(II). The reaction mixture contained the 32P-5'-end-labeled 261 bp fragment (Avul* 1645–XbaI 1905), 20 μM/base sonicated calf thymus DNA, the indicated concentrations of DCHQ, 10 μM CuCl2 and 5 μM DTPA in 200 μl 10 mM phosphate buffer, pH 7.9. After incubation at 37°C for 60 min, followed by piperidine treatment (B) or without piperidine treatment (A), the treated DNA fragments were electrophoresed on an 8% polyacrylamide–8 M urea gel (12×16 cm) and an autoradiogram obtained by exposing X-ray film to the gel.

Fig. 2. DNA damage by DCBQ plus Cu(II) in the presence of NADH. The reaction mixture contained the 32P-5'-end-labeled 261 bp fragment (Avul* 1645–XbaI 1905), 20 μM/base sonicated calf thymus DNA, 10 μM DCBQ, 220 μM NADH and 20 μM CuCl2 in 200 μl 10 mM phosphate buffer, pH 7.9, containing 5 μM DTPA. After incubation at 37°C for 60 min and piperidine treatment, the DNA fragments were analyzed by the method described in the legend to Figure 1.
since it is known that 3-OH-DCHQ is formed on the addition of H₂O to DCBQ (21). Cu(II)-dependent DNA damage was not induced by 220 μM NADH (Figure 2, lane 4). Relevantly, we have reported that Cu(II)-dependent DNA damage is caused by >1 mM NADH (22).

Effects of scavengers and bathocuproine on DNA damage

Figure 3 shows the effects of OH scavengers, SOD and catalase on DCBQ plus Cu(II)-induced DNA damage in the presence of NADH. The OH scavengers (ethanol, mannitol, sodium formate and DMSO) did not inhibit DNA damage (lanes 2–5). Catalase inhibited DNA damage completely (lane 8), suggesting the involvement of H₂O₂. SOD did not significantly inhibit DNA damage by DCBQ plus Cu(II) in the presence of NADH (lane 6). The addition of bathocuproine or DTPA inhibited DNA damage by DCBQ plus Cu(II) in the presence of NADH, suggesting the involvement of Cu(I) (lanes 10 and 11). Similar results were observed for DCHQ-induced DNA damage in the presence of Cu(II) (data not shown).

Site specificity of Cu(II)-mediated DNA damage induced by DCHQ or DCBQ plus NADH

For the measurement of relative intensity of Cu(II)-mediated DNA cleavage, ³²P-5'-end-labeled DNA fragments incubated with DCHQ or DCBQ plus NADH in the presence of Cu(II) followed by piperidine treatment were electrophoresed; the autoradiogram obtained is shown in Figure 4. Autoradiograms obtained were scanned with a laser densitometer (Figures 5 and 6). DCHQ plus Cu(II) induced piperidine-labile sites frequently at thymine residues, especially located 5' and/or 3' of guanine (Figure 5A). Relevantly, 5-formyluracil, a well-established radical oxidation product of thymine, has been shown to be one form of piperidine-labile damage in photooxidized DNA (23). A similar DNA cleavage pattern was observed with DCBQ plus Cu(II) in the presence of NADH (Figures 5B and 6A). This site specificity was similar to that of DNA cleavage induced by H₂O₂ in the presence of Cu(I) (data not shown).

When denatured DNA was used, the site specificity of DNA cleavage was changed (Figure 6). Cleavage at guanine residues was increased and cleavage at 5'-GG-3' in codon 12 of the c-Ha-ras-1 proto-oncogene was noteworthy (Figure 6B).

Formation of 8-oxodG in calf thymus DNA by DCHQ and DCBQ plus NADH in the presence of Cu(II)

Formation of 8-oxodG, a relevant indicator of oxidative base damage, is by four pathways (24–30) and causes DNA misreplication that might lead to mutation or cancer (31). Figure 7 shows the time course of 8-oxodG formation in calf thymus DNA treated with DCHQ and DCBQ plus NADH in the presence of Cu(II). The formation of 8-oxodG increased with time. DCBQ plus NADH produced twice the 8-oxodG of DCHQ. With DNA denaturation, the formation of 8-oxodG by DCHQ and DCBQ plus NADH increased ~2.2- and 2.1-fold respectively (data not shown). This is in agreement with the above-mentioned observation that when denatured [³²P]DNA fragments were used, cleavage at guanine residues was increased. Relevantly, it has been shown that piperidine treatment of 8-oxodG-containing DNA results in breakage of the deoxyribose phosphate backbone (32). Neither DCHQ nor DCBQ plus NADH alone caused 8-oxodG formation in the absence of Cu(II).

UV-visible spectroscopic studies on the autodioxidation of DCHQ

When a stock solution of DCHQ was added to the buffer solution, the solution turned yellow rapidly, with an increase in the absorption maximum at 445 nm even in the absence of Cu(II) (Figure 8A). The spectra with absorption maxima at 445 nm can be attributed to DCBQ and semiquinone radicals. After 2 min, the absorbance decreased, with a change to violet. DCBQ turned violet more efficiently than DCHQ. These results demonstrate that DCHQ is rapidly autoxidized to DCBQ, which is gradually converted into other compounds. On the other hand, addition of NADH to DCBQ in buffer solution rapidly decreased the absorption maximum at 340 nm attributed to NADH and the absorption maximum at 445 nm attributed to DCBQ, indicating reduction of DCBQ by NADH (data not shown).

Production of semiquinone radicals during autooxidation of DCHQ and DCBQ

Figure 8B shows the time course of the relative intensity of the ESR signal of the semiquinone radical produced during autooxidation of DCHQ at room temperature (25°C). The three line signals with a hyperfine splitting constant of 2.01 G were assigned to the semiquinone radical of DCHQ on the basis of the hyperfine coupling constant, which is similar to that determined for the semiquinone radical derived from reduction of dichloroquinone (33). The ESR spectrum was observed in the absence of trapping agents, since the semiquinone radical of DCHQ is relatively stable. The signal intensity increased until it reached a maximum after 2 min. The signal intensity decreased thereafter (Figure 8B) and, concomitantly, two new line signals with a hyperfine splitting constant of 2.48 G appeared. These signals may be assigned to the semiquinone radical of 3-OH-DCHQ on the basis of the hyperfine coupling constant (33). Both UV-visible and ESR spectroscopy suggest that DCHQ is rapidly autoxidized to DCBQ via the semiquinone radical even in the absence of metal ions, and the addition...
A: DCHQ

![Autoradiogram showing site specificity of DNA cleavage induced by DCHQ and DCBQ plus NADH in the presence of Cu(II).](image1)

B: DCBQ + NADH

![Site specificity of DNA cleavage induced by DCHQ and DCBQ plus NADH in the presence of Cu(II).](image2)

**Discussion**

The present results have shown that DCHQ causes DNA damage in the presence of Cu(II). DCBQ alone slightly induced DNA damage in the presence of Cu(II). NADH is required to promote oxidative effects of DCBQ on DNA. In the presence of NADH plus Cu(II), DCBQ induced DNA damage more efficiently than DCHQ. The effect of piperidine treatment suggests that DCHQ or DCBQ plus NADH induced not only breakage of the deoxyribose phosphate backbone but also base alteration and/or liberation. The formation of 8-oxodG in calf thymus DNA by DCHQ and DCBQ plus NADH was observed in the presence of Cu(II). On the other hand, DNA damage was not induced during autoxidation of DCHQ in the absence of metal ions. UV-visible and ESR spectroscopy have demonstrated that DCHQ is rapidly autoxidized to DCBQ via semiquinone even in the absence of metal ions. Comparison of these results suggests that covalent binding of DCHQ or the semiquinone radical to DNA is a minor pathway, if any, in induction of DNA cleavage, although den Besten et al. (12) have reported that DCB is metabolized to DCHQ, which is covalently bound to DNA.

It is known that enzyme-catalyzed cyclic reduction—oxidation of several quinones occurs in mammalian nuclei (34). However, NADH-dependent DNA damage induced by DCBQ plus Cu(II) was observed even in the absence of NADH-FMN oxidoreductase. The mechanism as shown in Figure 9 can be envisioned as accounting for most of the observations. UV-visible and ESR spectroscopy suggest that DCBQ is reduced by NADH to a semiquinone which reacts with O₂ to produce O₂⁻ and, subsequently, H₂O₂. Furthermore, the NAD⁺ formed also reacts with O₂ to form NADH and O₂ at an almost diffusion controlled rate (35). Finally, H₂O₂ should participate in DNA damage.

In order to examine what kind of active species cause DNA damage, experiments using various scavengers were performed. Bathocuproine and catalase completely inhibited DNA damage. These results suggest that active species derived from H₂O₂ and Cu(I) play an important role in the DNA damage. Typical
DNA damage by metabolites of p-dichlorobenzene

Fig. 6. Comparison of site specificity of DCBQ-induced cleavage of native and denatured forms of DNA. The 32P-5′-end-labeled 261 bp fragment (Avel* 1645–XbaI 1905) in 200 μl 10 mM sodium phosphate buffer, pH 7.9, containing 5 μM DTPA and 20 μM/base sonicated calf thymus DNA was incubated with 8 μM DCBQ plus 20 μM CuCl2 in the presence of 220 μM NADH at 37°C for 60 min (A). For the experiment with denatured DNA (B), the 5′-end-labeled DNA fragment was treated at 90°C for 10 min and quickly chilled before addition of DCBQ, CuCl2, and NADH. After piperidine treatment, DNA fragments were analyzed as described in the legend to Figure 5. Underlining indicates codon 12 of the human c-Ha-ras-1 proto-oncogene.

'OH scavengers showed little or no inhibitory effect on DNA damage, suggesting that 'OH might not have played an important role. In the presence of Cu(II), DCHQ and DCBQ plus NADH induced piperidine-labile sites frequently at thymine residues, especially located 5′ and/or 3′ of guanine. Usually there is no specificity in 'OH-mediated formation of DNA damage (36–38). It is likely that in the present case binding of Cu(II) to DNA is the key factor for the observed sequence specificity in piperidine-induced DNA strand breaks. We speculate that Cu(II) bound to DNA in a site-specific manner is reduced to Cu(I), which reacts with H2O2 to form a DNA-metal-oxygen complex. This complex may release 'OH to attack an adjacent DNA constituent before being scavenged by 'OH scavengers. Thus, the site-specific DNA damage can be explained by both active oxygen species formed and the specific manner of binding of Cu(II).

Fig. 6. Comparison of site specificity of DCBQ-induced cleavage of native and denatured forms of DNA. The 32P-5′-end-labeled 261 bp fragment (Avel* 1645–XbaI 1905) in 200 μl 10 mM sodium phosphate buffer, pH 7.9, containing 5 μM DTPA and 20 μM/base sonicated calf thymus DNA was incubated with 8 μM DCBQ plus 20 μM CuCl2 in the presence of 220 μM NADH at 37°C for 60 min (A). For the experiment with denatured DNA (B), the 5′-end-labeled DNA fragment was treated at 90°C for 10 min and quickly chilled before addition of DCBQ, CuCl2, and NADH. After piperidine treatment, DNA fragments were analyzed as described in the legend to Figure 5. Underlining indicates codon 12 of the human c-Ha-ras-1 proto-oncogene.

Certain carcinogens are known to induce both H2O2 formation and oxidative DNA damage in the presence of endogenous metal ions (39–42). The metabolites of p-DCB caused oxidative damage to isolated DNA through H2O2 formation, although the quinone-type metabolite required NADH. NADH might function as a nuclear reductant (22,43). Several papers have pointed to the possibility that chemicals are non-enzymatically reduced by NAD(P)H in vivo (15,44,45). The concentration of NAD(P)H in certain tissues was estimated to be as high as 100–200 μM (46). Thus, we have shown that NADH can be a source of endogenous one-electron reductant for DCBQ, resulting in oxidative DNA damage.

The present work suggests that Cu(II) is an important factor in oxidative DNA damage induced by metabolites of p-DCB. It has been shown that Cu(II) binds specifically to guanine
residues in DNA (47-49). The binding of copper to DNA and/ or protein in chromatin is proposed to serve physiological functions (50), whereas copper bound to DNA and/or protein may provide an adventitious site for deleterious redox reactions (51). Recently, Chiu et al. (52) have reported that copper ions bind to non-histone proteins, leading to ascorbate-mediated DNA damage, which is much stronger than that in the case of iron. A copper redox cycle mechanism has been proposed to explain the oxidative degradation of DNA upon incubation with hydroquinone and Cu(II) (53). Therefore, copper-mediated DNA damage by metabolites of p-DCB through \( \text{H}_2\text{O}_2 \) seems to be relevant for expression of the carcinogenicity of p-DCB.

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**Reference**

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