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Oxidative DNA Damage and Apoptosis Induced by Benzene Metabolites[1]

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ABSTRACT

Benzene is a widely recognized human carcinogen. The mechanism of DNA damage induced by major benzene metabolites 1,4-benzoquinone (1,4-BQ) and hydroquinone (1,4-HQ) was investigated in relation to apoptosis and carcinogenesis. Pulsed-field gel electrophoresis showed that cellular DNA strand breakage was induced by benzene metabolites. Internucleosomal DNA fragmentation and morphological changes of apoptotic cells were observed at higher concentrations of benzene metabolites. Flow cytometry showed an increase of peroxides in cultured cells treated with benzene metabolites. 1,4-BQ induced these changes at a much lower concentration than 1,4-HQ. Damage to DNA fragments obtained from the c-Ha-*ras*-1 proto-oncogene was investigated by a DNA sequencing technique. 1,4-BQ + NADH and 1,4-HQ induced piperidine-labile sites frequently at thymine residues in the presence of Cu(II). Catalase and bathocuproine inhibited DNA damage, suggesting that H_2O_2 reacts with Cu(I) to produce active species causing DNA damage. Electron spin resonance studies showed that semiquinone radical was produced by NADH-mediated reduction of 1,4-BQ and autoxidation of 1,4-HQ, suggesting that benzene metabolites produce O_2^- and H_2O_2 via the formation of semiquinone radical. These results suggest that these benzene metabolites cause DNA damage through H_2O_2 generation in cells, preceding internucleosomal DNA fragmentation leading to apoptosis. The fates of the cells to apoptosis or mutation might be dependent on the intensity of DNA damage and the ability to repair DNA.

INTRODUCTION

Benzene, widely used in chemical industry, has been shown to cause hematological disorders and carcinogenic effects in humans and animals. Exposure to benzene causes leukemia, lymphoma, and carcinomas of mammary gland and liver in humans and animals (1–5). Sister chromatid exchanges (1, 2, 6, 7) and chromosomal loss and breakage (8) were demonstrated in mice. However, benzene has not been shown to be mutagenic in a bacterial test system (9). Benzene is metabolized by cytochrome P450 to various phenolic metabolites, which accumulate in bone marrow. Benzene metabolism is considered important to express its toxicity (10), and there are many reports of benzene toxicity mediated by its metabolites. We have already reported that benzene metabolites 1,2,4-benzenetriol and 1,4-HQ[3] caused oxidative DNA damage (11), and 1,2,4-benzenetriol produced 8-OH-dG, an oxidative product of guanine residue, in the presence of metal ions (12). Benzene metabolites produced 8-OH-dG in cultured cells *in vitro* and the bone marrow of mice *in vivo* (13). It is noteworthy that a significant correlation was observed between urinary 8-OH-dG and benzene exposure in humans (14). Thus, previous studies have suggested the participation of active oxygen species in benzene metabolite-induced DNA damage. However, the mechanism of cellular DNA damage through the generation of active oxygen species remains to be clarified.

Apoptosis contributes to the pathogenesis of a number of diseases, including cancer (15). Cell death in response to DNA damage, in most instances, has been shown to result from apoptosis (15, 16). Apoptosis is induced by many cytotoxic chemicals and ionizing radiation and characterized by morphological and biochemical changes such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, and DNA fragmentation at internucleosomal sites.

To study the mechanism of benzene carcinogenicity, we investigated DNA damage induced by benzene metabolites 1,4-BQ and 1,4-HQ in relation to apoptosis and carcinogenesis. DNA strand breaks in human cultured cells were investigated with pulsed-field gel electrophoresis. Internucleosomal DNA fragmentation and morphological changes of apoptotic cells were examined. The cells treated with benzene metabolites were analyzed to detect intracellular generation of peroxides by flow cytometry. To investigate the mechanism of cellular DNA damage, we examined the DNA damage induced by benzene metabolites in the presence of NADH and metal ions using ^{32}P-5′-end-labeled DNA fragments obtained from the human c-Ha-*ras*-1 proto-oncogene. NADH was used because the biological importance of NADH has been described (17), and some chemicals are nonenzymatically reduced by NADH (18, 19). Free radicals derived from benzene metabolites were identified by ESR spectrometry.

MATERIALS AND METHODS

Materials. Restriction enzymes (*Ava*I and *Pst*I) and T_4 polynucleotide kinase were purchased from New England Biolabs. [γ-^{32}P]ATP (222 TBq/mmol) was from DuPont New England Nuclear. 1,4-BQ was from Nacalai Tesque, Inc. (Kyoto, Japan). 1,4-HQ was from Aldrich Chemical Co. (Milwaukee, WI). Proteinase K was from Boehringer Mannheim GmbH. DCFH-DA was from Molecular Probes, Inc. Glucose oxidase was from Toyobo Co. (Osaka, Japan). DTPA and bathocuproinedisulfonic acid were from Dojin Chemical Co. (Kumamoto, Japan). SOD (3,000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from bovine liver) were from Sigma Chemical Co. Mn(III)-PP_i was prepared according to Archibald and Fridovich (20).

Detection of Cellular DNA Damage by Pulsed-Field Gel Electrophoresis. HL60 cells were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 6% FCS (Whittaker Bioproducts) at 37°C. For the determination of DNA strand breaks, cells (1×10^6 cells/ml) were incubated with either 1,4-BQ or 1,4-HQ in 2.5 ml of RPMI 1640 containing 6% FCS for 4 h at 37°C. After the incubation, the medium was removed, and the cells were washed twice with PBS and resuspended in 65 μl PBS. The cell suspension was solidified with agarose, followed by treatment with proteinase K according to the method described previously (21). Electrophoresis was performed in 0.5 × Tris-borate EDTA buffer [45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.0)] by a CHEF-Mapper pulsed-field electrophoresis system (Bio-Rad) at 200 V at 14°C. Switch time was 60 s for 15 h, followed by a 90-s switch time for 9 h. DNA in the gel was visualized in ethidium bromide.

Detection of DNA Ladder Formation Induced by Benzene Metabolites. HL60 cells (1×10^6 cells/ml) were incubated with either 1,4-BQ or 1,4-HQ in 2.5 ml of RPMI 1640 supplemented with 6% FCS for 4 h at 37°C. After the incubation, the medium was removed, and the cells were washed twice with PBS. The cells were suspended in 1 ml of cytoplasm extraction buffer [10 mM Tris (pH 7.5), 150 mM NaCl, and 5 mM $MgCl_2$ in 0.5% Triton X-100] and centrifuged. The pellet was treated with lysis buffer [10 mM Tris (pH 7.5), 400

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[3] The abbreviations used are: 1,4-HQ, hydroquinone (also known as 1,4-benzenediol); 1,4-BQ, 1,4-benzoquinone; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine (also known as 8-oxo-7,8-dihydro-2′-deoxyguanosine); DCFH-DA, 2′,7′-dichlorofluorescin diacetate; ESR, electron spin resonance; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; SOD, superoxide dismutase.

mM NaCl, and 1 mM EDTA in 1% Triton X-100] for 10 min and centrifuged at 4°C. The supernatant was treated with 0.2 mg/ml RNase overnight at room temperature and subsequently treated with 0.1 mg/ml proteinase K for 2 h at 37°C. The DNA was extracted with phenol-chloroform and subsequently extracted with water-saturated ether. Then, the DNA was precipitated with ethanol for 30 min at −90°C. The pellet was dissolved in 40 μl of Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. DNA was electrophoresed on a 1.4% agarose gel containing 0.375 μg/ml ethidium bromide in 0.5 × Tris-borate EDTA buffer.

Flow Cytometric Detection of Peroxides in Cultured Cells Treated with Benzene Metabolites. HL60 cells (1×10^6 cells/ml) were incubated with either 1,4-BQ or 1,4-HQ in RPMI 1640 containing 6% FCS for 3.5 h at 37°C. DCFH-DA, a sensitive fluorimetric probe of peroxides (22, 23), was dissolved in ethanol, 5 μM DCFH-DA were added to the medium, and the cells were incubated for 30 min at 37°C. After the incubation, the medium was removed, and the cells were washed with PBS once and suspended in PBS. The cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA).

Detection of DNA Damage Using c-Ha-*ras*-1 Fragments. DNA fragments were prepared from plasmid pbcNI, which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing human c-Ha-*ras*-1 proto-oncogene (24, 25). The singly labeled 98-bp fragment (*Aval** 2247–*Pst*I 2344) and 337-bp fragment (*Pst*I 2345–*Aval** 2681) were obtained according to the method described previously (24, 25). The asterisk indicates ^{32}P-labeling, and nucleotide numbering starts with the *Bam*HI site (26).

The standard reaction mixture in a microtube (1.5-ml Eppendorf) contained benzene metabolite, a ^{32}P-labeled DNA fragment, and sonicated calf thymus DNA (10 μM/base) in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. After incubation at 37°C, the DNA fragments were heated at 90°C in 1 M piperidine for 20 min where indicated and treated as described previously (24, 25).

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

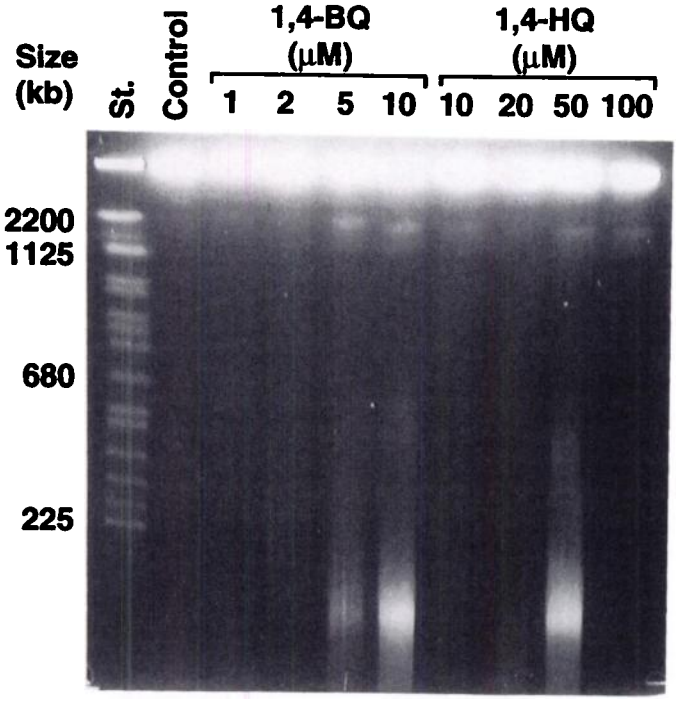


Fig. 1. Detection of benzene metabolite-induced cellular DNA damage by pulsed-field gel electrophoresis. HL60 cells were treated with either 1,4-BQ or 1,4-HQ for 4 h at 37°C. The cells were prepared in agarose plugs, lysed, and subjected to pulsed-field gel electrophoresis through 1% agarose gel, as described in "Materials and Methods." The gel was stained in ethidium bromide. *Lane St.*, size marker DNA (*Saccharomyces cerevisiae*).

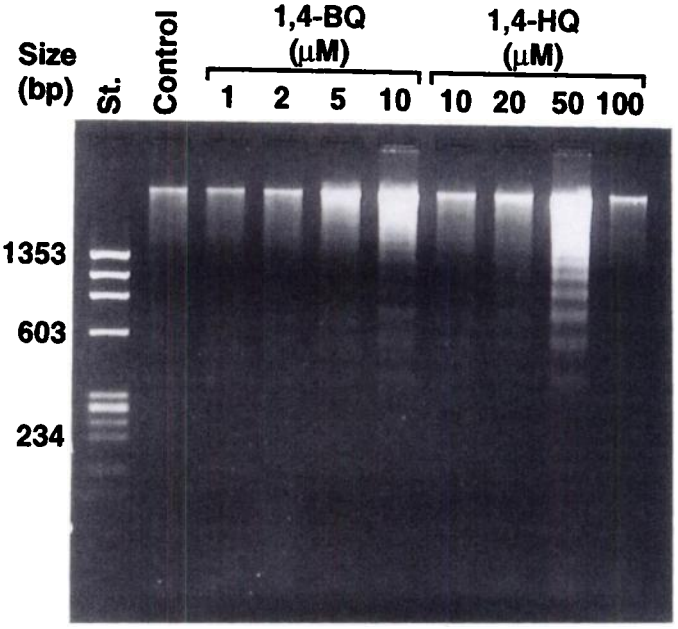


Fig. 2. Internucleosomal DNA fragmentation in cultured cells treated with benzene metabolites. HL60 cells were treated with either 1,4-BQ or 1,4-HQ for 4 h at 37°C. The cells were lysed, and DNA was extracted as described in "Materials and Methods." The DNA was electrophoresed in a 1.4% agarose gel containing 0.375 μg/ml ethidium bromide. *Lane St.*, size marker DNA (ϕX 174/*Hae*III digest).

Detection of Free Radicals Derived from Benzene Metabolites with ESR Spectrometry. ESR spectra were recorded to detect free radicals derived from benzene metabolites. The reaction mixture containing 500 μM 1,4-BQ or 1,4-HQ in 10 mM sodium phosphate buffer (pH 7.8) was incubated for 1 min at 25°C. In certain experiments, NADH (1 mM) or $CuCl_2$ (20 μM) was added to the mixture. The spectra were measured at 25°C using a JES-FE-3XG spectrometer (JEOL, Tokyo, Japan) with 100-kHz field modulation. The spectra were recorded with a microwave power of 16 mW and a modulation amplitude of 1.0 G. No spin-trapping agent was used.

RESULTS

Detection of DNA Damage in Cultured Cells Treated with Benzene Metabolites. Fig. 1 shows DNA strand breakage in cultured cells treated with benzene metabolites, detected by pulsed-field gel electrophoresis. Weak DNA strand breakage to produce 1–2-Mb fragments was induced by 1–2 μM 1,4-BQ and 10–20 μM 1,4-HQ. The apparent production of 1–2-Mb and 50-kb DNA fragments was observed at 5 μM 1,4-BQ and 50 μM 1,4-HQ. The formation of 50-kb fragments was increased at 10 μM 1,4-BQ. A 50-kb DNA fragment disappeared at 100 μM 1,4-HQ, probably due to necrosis. These results reveal that 1,4-BQ caused cellular DNA damage more efficiently than 1,4-HQ.

DNA Ladder Formation and Apoptotic Changes in Cultured Cells Treated with Benzene Metabolites. Fig. 2 shows DNA ladder formation in cells treated with benzene metabolites. The DNA ladder was slightly induced by 5 μM 1,4-BQ and 20 μM 1,4-HQ, and apparent fragmentation was induced by 10 μM 1,4-BQ and 50 μM 1,4-HQ. DNA fragments disappeared at 100 μM 1,4-HQ, probably due to necrotic changes.

Apoptotic cells, identified by chromatin condensation and nuclear fragmentation, were observed frequently in cells treated with 5 μM 1,4-BQ and 50 μM 1,4-HQ (data not shown).

Production of Intracellular Peroxides from Benzene Metabolites. Fig. 3 shows flow cytometric distribution of cells treated with benzene metabolites or glucose oxidase, an enzyme to catalyze H_2O_2

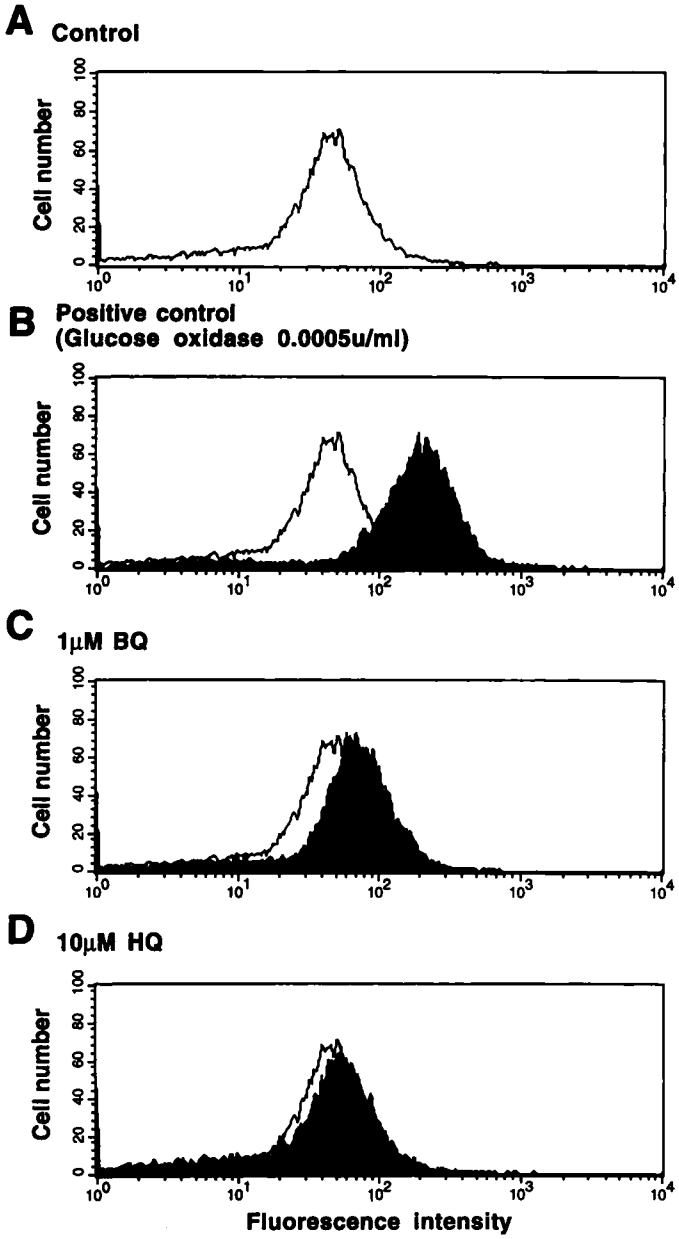


Fig. 3. Flow cytometric fluorescence distributions of cultured cells treated with benzene metabolites. HL60 cells were treated with either 1,4-BQ or 1,4-HQ for 3.5 h at 37°C, and 5 μM DCFH-DA were added to the medium, followed by incubation for 30 min at 37°C. The cells were analyzed with a flow cytometer (FACScan). *A*, control. *B*, cells treated with 0.0005 units/ml glucose oxidase. *C*, cells treated with 1 μM 1,4-BQ. *D*, cells treated with 10 μM 1,4-HQ. *Abscissa*, relative fluorescence intensity; *ordinate*, cell number. Distributions of the fluorescence intensity of the control are also shown in *B*, *C*, and *D*.

generation from glucose, and subsequently treated with DCFH-DA, a probe to detect intracellular production of peroxides (22, 23). The cells treated with glucose oxidase were used as positive control and showed a significant increase in fluorescence intensity. Treatment with 1 μM 1,4-BQ and 10 μM 1,4-HQ showed increases in fluorescence intensity (Fig. 3), suggesting that intracellular production of H_2O_2 and other peroxides was increased by benzene metabolites.

Damage to ^{32}P-labeled DNA Fragments Induced by Benzene Metabolites in the Presence of NADH and Cu(II). Fig. 4 shows the autoradiogram of DNA damage induced by 1,4-BQ. 1,4-BQ caused DNA damage in the presence of both NADH and Cu(II). The intensity of DNA damage increased with time (Fig. 4) and the concentration of benzene metabolites (Fig. 5*A*). In the absence of NADH and/or Cu(II), 1,4-BQ did not cause DNA damage (Fig. 4), suggesting the requirement of NADH and Cu(II) for 1,4-BQ-induced DNA damage. DNA damage was enhanced by piperidine treatment (Fig. 5*A*, *Lanes 3* and *6*), suggesting that 1,4-BQ caused not only DNA strand breakage but also base modification and/or liberation. 1,4-HQ + Cu(II) induced DNA damage (Fig. 5*B*), but 1,4-HQ alone did not (data not shown). 1,4-BQ and 1,4-HQ caused little or no DNA damage in the presence of other metal ions [Mn(II), Mn(III), Fe(II), and Fe(III)].

Effects of Scavengers and Bathocuproine on DNA Damage by Benzene Metabolites. The effects of scavengers and bathocuproine, a Cu(I)-specific chelator, on DNA damage by 1,4-BQ and 1,4-HQ were investigated (28). ·OH scavengers ethanol, mannitol, and sodium formate only weakly inhibited DNA damage induced by 1,4-BQ in the presence of NADH and Cu(II) (Fig. 6, *Lanes 2–4*). Catalase (*Lane 5*)

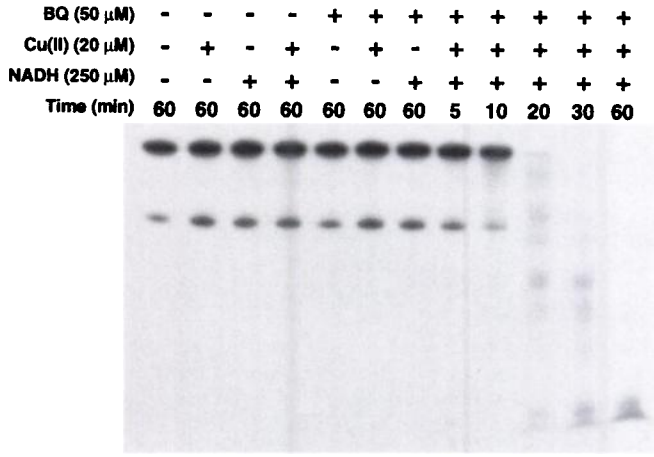


Fig. 4. Autoradiogram of ^{32}P-labeled DNA fragments incubated with 1,4-BQ in the presence of NADH and Cu(II). The reaction mixture contained the ^{32}P-5′-end-labeled 337-bp DNA fragment, 10 μM/base of sonicated calf thymus DNA, 50 μM 1,4-BQ, 250 μM NADH, and 20 μM $CuCl_2$ in 200 μl of 10 mM phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for the indicated durations at 37°C. DNA fragments were treated with 1 M piperidine for 20 min at 90°C and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel.

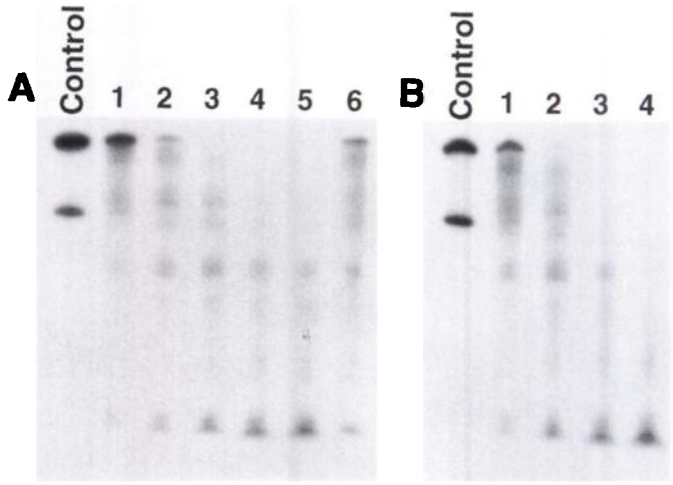


Fig. 5. Effects of concentration on DNA damage by 1,4-BQ and 1,4-HQ. The reaction mixture contained the ^{32}P-5′-end-labeled 337-bp DNA fragment, 10 μM/base of sonicated calf thymus DNA, 1,4-BQ + 250 μM NADH (*A*) or 1,4-HQ (*B*), and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 20 min at 37°C. DNA fragments were treated with 1 M piperidine for 20 min at 90°C (except *Lane 6*) and analyzed by the method described in the legend to Fig. 4. The concentrations of benzene metabolites are as follows; *Lane 1*, 10 μM; *Lane 2*, 20 μM; *Lane 3*, 50 μM; *Lane 4*, 100 μM; *Lane 5*, 200 μM; *Lane 6*, 50 μM, without piperidine treatment. Control did not contain benzene metabolite, NADH, and $CuCl_2$.

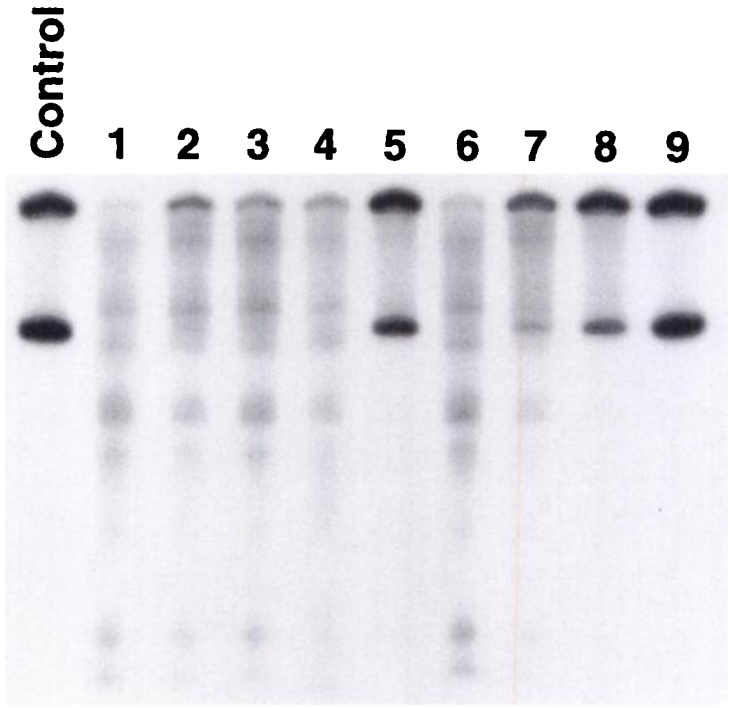


Fig. 6. Effects of scavengers and bathocuproine on DNA damage by 1,4-BQ. The reaction mixture contained the ^{32}P-5′-end-labeled 337-bp DNA fragment, 10 μM/base of sonicated calf thymus DNA, 20 μM 1,4-BQ, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 20 min at 37°C. DNA fragments were treated with 1 M piperidine for 20 min at 90°C and analyzed by the method described in the legend to Fig. 4. Scavenger or bathocuproine was added as follows: *Lane 1*, no scavenger; *Lane 2*, 1 M ethanol; *Lane 3*, 0.1 M mannitol; *Lane 4*, 0.1 M sodium formate; *Lane 5*, 150 units/ml catalase; *Lane 6*, 150 units/ml SOD; *Lane 7*, 10 μM bathocuproine; *Lane 8*, 20 μM bathocuproine; *Lane 9*, 50 μM bathocuproine. Control did not contain 1,4-BQ, NADH, and $CuCl_2$.

and bathocuproine (*Lanes 7–9*) inhibited DNA damage, suggesting the involvement of H_2O_2 and Cu(I). Heat-inactivated catalase showed no inhibitory effect on DNA damage (data not shown). Although 150 units/ml of SOD showed little or no effect on DNA damage (*Lane 6*), a lower concentration (50 units/ml) of SOD enhanced DNA damage (data not shown). Similar effects of scavengers and bathocuproine were observed with 1,4-HQ + Cu(II), although the stronger enhancing effect of SOD on 1,4-HQ-induced DNA damage was observed (data not shown).

Site Specificity of DNA Damage by Benzene Metabolites. Fig. 7 shows the patterns of DNA strand breaks induced by benzene metabolites in the presence of Cu(II). The relative intensity of DNA cleavage obtained by scanning autoradiogram with laser densitometer is shown in Fig. 8. 1,4-BQ induced piperidine-labile sites frequently at thymine residues adjacent to guanine residues, especially in the 5′-GTC-3′ sequence in the presence of NADH and Cu(II) (Fig. 8), although there remains a possibility that certain base damage might be over- or underrepresented, depending on its sensitivity to piperidine. 1,4-HQ showed similar patterns of DNA cleavage sites (Fig. 7).

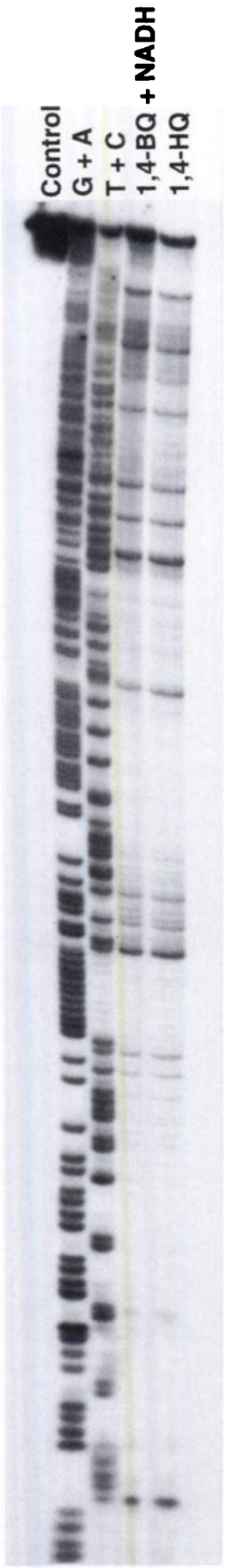
Production of Free Radicals from Benzene Metabolites. Fig. 9 shows ESR spectra of radicals generated by benzene metabolites. 1,4-BQ + NADH shows the formation of a 1:4:6:4:1 quintet spectrum with a_H = 2.3 G, reasonably assigned to the semiquinone radical (28). 1,4-BQ alone and 1,4-BQ + Cu(II) did not produce the signal. 1,4-HQ alone produced the signal of the semiquinone radical, and addition of Cu(II) increased the signal. These results suggest that the semiquinone radical is generated by NADH-mediated 1,4-BQ reduction and 1,4-HQ autoxidation.

DISCUSSION

In the present study, DNA damage induced by benzene metabolites was examined in relation to apoptosis and carcinogenesis. Pulsed-field gel electrophoresis showed that benzene metabolites induced cellular DNA strand breakage. Production of 1–2-Mb DNA fragments was observed at 1–2 μM 1,4-BQ and 10–20 μM 1,4-HQ. Moreover, 5–10 μM 1,4-BQ and 50 μM 1,4-HQ produced 50-kb DNA fragments. DNA ladder and apoptotic changes were observed at 5 μM 1,4-BQ and 50 μM 1,4-HQ. Flow cytometry showed an increase in the generation of peroxides in cells treated with 1 μM 1,4-BQ and 10 μM 1,4-HQ. These results suggest that benzene metabolites generate intracellular peroxides, mainly H_2O_2, causing the DNA strand break to produce 1–2-Mb DNA fragments and subsequently 50-kb fragments, preceding endonuclease-catalyzed internucleosomal DNA fragmentation and resulting in apoptosis. Furthermore, it is suggested that lower concentrations of benzene metabolites induce carcinogenesis rather than apoptosis through DNA damage. The fates of the cells exposed to benzene metabolites— mutation or apoptosis— depend on their concentrations and the intensity of DNA damage (Fig. 10).

To clarify the mechanism of cellular DNA damage induced by benzene metabolites, we investigated the damage to DNA fragments obtained from the c-Ha-*ras*-1 proto-oncogene. 1,4-BQ required both NADH and Cu(II) for DNA damage, whereas 1,4-HQ required only Cu(II). The inhibitory effects of catalase and bathocuproine on DNA damage indicate the involvement of H_2O_2 and Cu(I). Low concentrations of SOD enhanced DNA damage, especially by 1,4-HQ + Cu(II). This effect can be explained by SOD-accelerated oxidation of 1,4-HQ (29). Because typical ·OH scavengers showed only weak inhibitory effects on DNA damage, active species other than ·OH might have played an important role. Cu(II)-mediated DNA damage by 1,4-BQ + NADH was frequently induced at thymine residues adjacent to guanine residues, especially in the 5′-GTC-3′ sequence. The pattern of DNA cleavage induced by 1,4-HQ resembled that induced by 1,4-BQ. Relevantly, several papers showed that the reaction of H_2O_2 with Cu(II) causes DNA damage with a site-specificity for thymine residues (28, 30–32). This supports the involvement of active oxygen species generated from H_2O_2 + Cu(I) other than ·OH, which causes DNA cleavage at any nucleotide with little site-specificity (33, 34).

A possible mechanism of DNA damage induced by benzene metabolites in the presence of NADH and Cu(II) is proposed as shown in Fig. 10. NADH-mediated reduction of 1,4-BQ and autoxidation of 1,4-HQ produce the semiquinone radical. The formation of the semiquinone radical was confirmed by a quintet ESR spectrum with



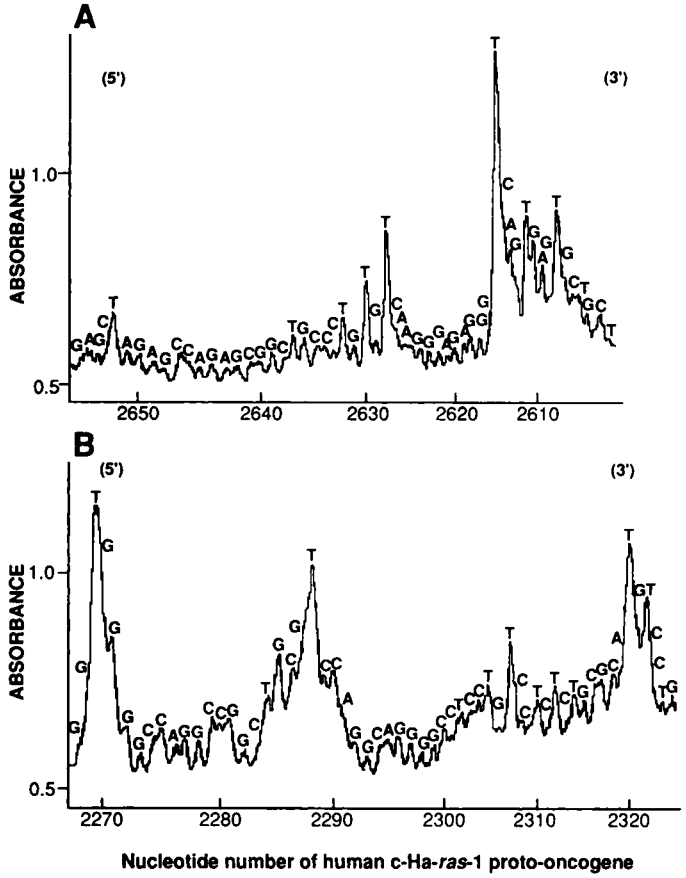


Fig. 8. Site-specificity of DNA cleavage induced by 1,4-BQ in the presence of NADH and Cu(II). The reaction mixture contained the ^{32}P-5'-end labeled 337-bp fragment (*PstI* 2345–*AvaI** 2681; *A*) or the 98-bp fragment (*AvaI** 2247–*PstI* 2344; *B*), 10 μM/base of sonicated calf thymus DNA, 20 μM 1,4-BQ, 250 μM NADH, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 20 min at 37°C. DNA fragments were treated with 1 M piperidine for 20 min at 90°C and then electrophoresed on an 8% polyacrylamide/8 M urea gel. The autoradiogram was obtained by exposing X-ray film to the gel. The relative amounts of DNA fragments were measured by scanning the autoradiogram with a laser densitometer (LKB 2222 UltroScan XL). *Abscissa*, nucleotide number of human c-Ha-*ras*-1 proto-oncogene starting with the *Bam*HI site (26).

a_H = 2.3 G (28). However, the semiquinone radical is not a main active species causing DNA damage because Cu(I) was required for DNA damage, whereas the semiquinone radical was formed by 1,4-BQ + NADH in the absence of copper. It is speculated that the NAD radical was formed by the reaction of NADH with 1,4-BQ. Recently, it was reported that the NAD radical was detected using ESR and mass spectrometry (18). The reactions of semiquinone and NAD radicals with O_2 yield O_2^-, which is dismutated to generate H_2O_2. Cu(II) is reduced to Cu(I) by the reaction with O_2^-, and H_2O_2 reacts with Cu(I) to form the active species causing DNA damage. Thus, the NADH-dependent redox cycle of 1,4-BQ generates active oxygen species and mediates DNA damage.

The biological importance of NADH as a nuclear reductant has

Fig. 7. Autoradiogram showing site-specificity of DNA cleavage by benzene metabolites + Cu(II). The reaction mixture contained the ^{32}P-5'-end-labeled 337-bp fragment (*PstI* 2345–*AvaI** 2681), 10 μM/base of sonicated calf thymus DNA, either 20 μM 1,4-BQ + 250 μM NADH or 20 μM 1,4-HQ, and 20 μM $CuCl_2$ in 200 μl of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA. The mixture was incubated for 20 min at 37°C. DNA fragments were treated with 1 M piperidine for 20 min at 90°C and analyzed by the method described in the legend to Fig. 4. *Lanes G + A* and *T + C*, patterns obtained for the same fragment cleaved by the methods of Maxam and Gilbert (27). Control did not contain benzene metabolite, NADH, and $CuCl_2$.

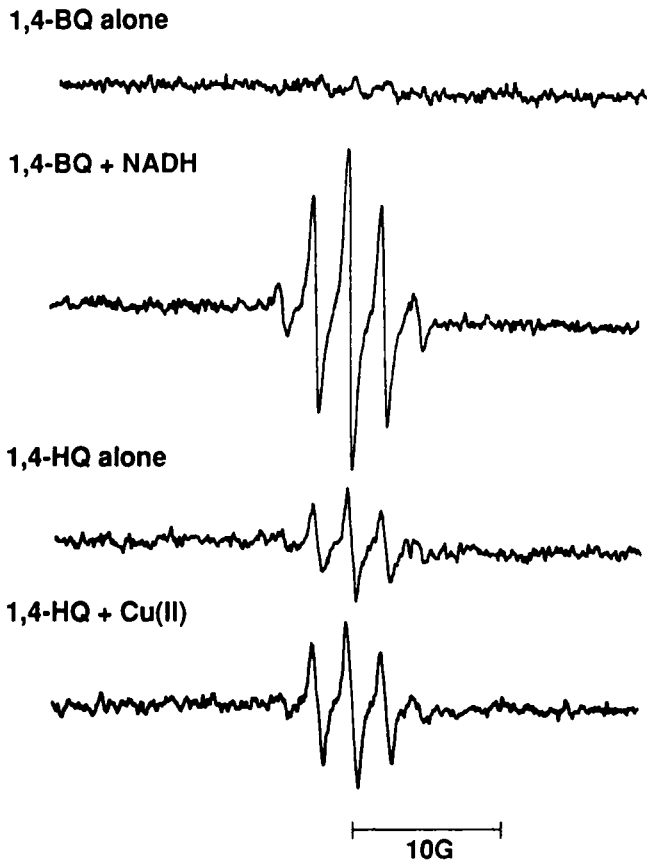


Fig. 9. ESR spectra of radicals generated from benzene metabolites. The reaction mixture, which contained 500 μM benzene metabolite and 5 μM DTPA in 10 mM phosphate buffer (pH 7.8), was kept for 1 min at 25°C, and the spectrum was measured. Where indicated, NADH (1 mM) or $CuCl_2$ (20 μM) was added to the mixture. No spin-trapping agent was added.

been described (17). The possibility that some chemicals are nonenzymatically reduced by NADH *in vivo* has been shown (18, 19). NADH can be a source of endogenous reductant, resulting in oxidative DNA damage. The present study showed that 1,4-BQ induced a cellular DNA strand break and apoptotic changes at a much lower concentration than 1,4-HQ, indicating that 1,4-BQ has greater potential to cause cellular DNA damage and apoptosis. The NADH-dependent redox cycle is considered to be important to explain the higher potentiality of 1,4-BQ because 100–200 μM of NAD(P)H are contained in certain tissues (35) and NAD(P)H may play very important roles as a reductant.

Copper occurs in the mammalian cell nucleus and may contribute to high-order chromatin structures (36). Copper ions bind to nonhistone proteins and caused much stronger ascorbate-mediated DNA damage than iron (37). Copper-mediated production of active oxygen species and DNA damage are reported (12, 38, 39). Therefore, the copper-dependent DNA damage by benzene metabolites is of interest in connection with these observations.

Many studies have shown cytotoxicity and genotoxicity of benzene (1–7). The generation of active oxygen species from benzene metabolites has been discussed in relation to benzene carcinogenicity. Our previous reports showed that benzene metabolites 1,2,4-benzenetriol and 1,4-HQ caused DNA damage mediated by active oxygen species (11), and 1,2,4-benzenetriol produced 8-OH-dG in the presence of metal ions (12). The hypothesis we proposed that benzene metabolites produce active oxygen species to cause DNA damage has been supported by the following reports: (*a*) DNA damage mediated by active oxygen species was caused by 1,4-HQ in the presence of Cu(II) (28); (*b*) benzene metabolites produced 8-OH-dG *in vitro* and *in vivo* (13); (*c*) a significant correlation between benzene exposure and urinary 8-OH-dG was observed in humans (14); and (*d*) benzene administration produced active oxygen species in the bone marrow of rats (40). Therefore, the importance of active

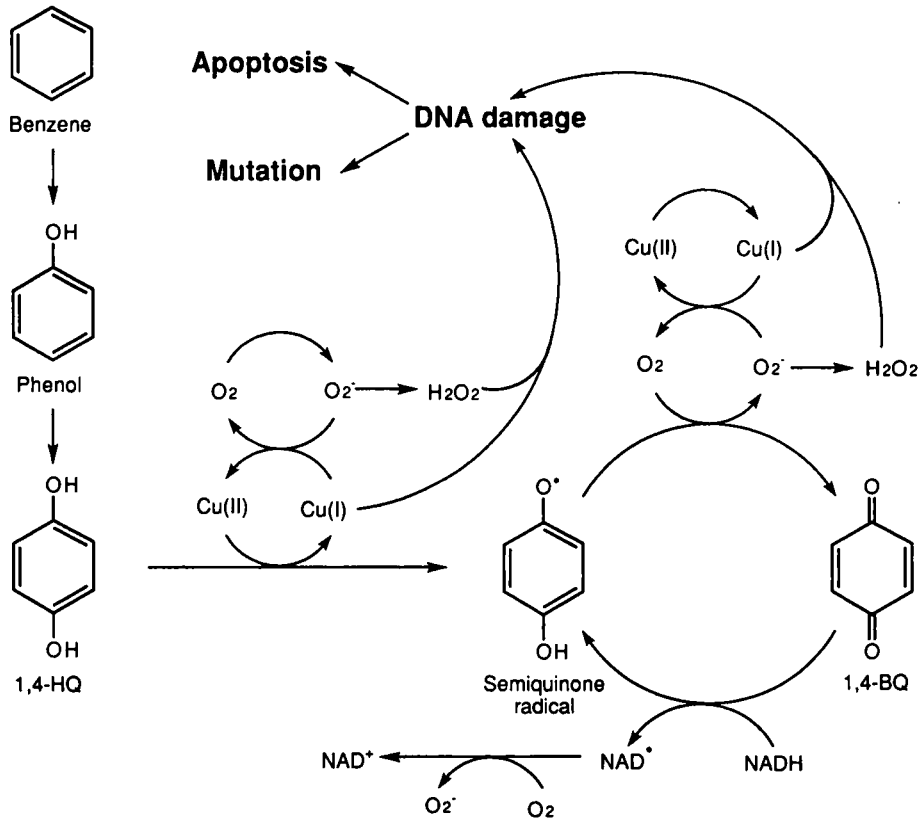


Fig. 10. Proposed mechanism of DNA damage, apoptosis, and carcinogenesis induced by benzene metabolites in the presence of NADH and Cu(II).

oxygen species for DNA damage and carcinogenesis induced by benzene metabolites has been confirmed.

The relationship among DNA damage, apoptosis, and carcinogenesis attracts our interests. DNA damage induces the production of p53 protein, the activation of protease, and the subsequent activation of endonucleases to catalyze DNA fragmentation at internucleosomal sites, leading to apoptosis (15). The present study suggested that benzene metabolites generated active oxygen species to induce a cellular DNA strand break preceding apoptosis. This proposed two fates of the cells with DNA damage; one is apoptosis, and the other is mutation leading to carcinogenesis. The cells that incur strong DNA damage and undergo apoptosis are no longer candidates for producing cancer cells. When weak DNA damage was induced, the cellular response allows repair of the damage. However, if the damage failed to be repaired, mutagenic lesions could be propagated and might lead to carcinogenesis. The fates of the cells might be dependent on the intensity of the DNA damage and the ability to repair DNA.

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