— Rapid Communication —

Two-Electron Quinone Reductase (Aldo-keto Reductase 1C Isozyme) Augments the Oxidative DNA Damage Induced by Quinones in Diesel Exhaust Particles by Accelerating Redox Cycling

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DNA cleavage by quinones contained in diesel exhaust particles (DEP) was examined in a cell-free system using supercoiled FX174 DNA as the target DNA. In the presence of Cu(II) and NADPH, 9,10-phenanthrenequinone (PQ) caused the transformation of the supercoiled FX174 DNA into open circular and then linear forms in a concentration-dependent manner. This DNA damage by PQ was decreased by catalase, a superoxide anion scavenger and a Cu(I)-specific chelator, but not by superoxide dismutase and a hydroxyl radical scavenger, suggesting that the ultimate reactive product responsible for the DNA scission may be Cu(I)-OOH generated from hydrogen peroxide and Cu(I) rather than hydroxyl radicals. In addition, 1,2-naphthoquinone (1,2-NQ) damaged DNA more severely than PQ, while 1,4-NQ and 9,10-anthraquinone (AQ) did not induce significant DNA damage. When a purified aldo-keto reductase (AKR) 1C isozyme, which catalyzes the two-electron reduction of PQ, was included in the reaction mixture, the PQ-induced DNA damage became more extensive. Addition of the AKR1C isozyme also increased the 1,2-NQ-induced DNA damage and conferred the ability to cause DNA damage on 1,4-NQ, but had no effect on AQ. The severity of the DNA damage induced by DEP quinones was solely related to both NADPH consumption and reactive oxygen species (ROS) generation. These findings indicate that the generation of ROS via redox cycling of DEP quinones is a causative event in DNA scission and that the AKR1C isozyme accelerates the redox cycling of DEP quinones that are utilized as substrates, thereby resulting in the promotion of oxidative stress and DNA damage.

Key words —— quinone in diesel exhaust particle, two-electron reduction, redox cycling, DNA scission

INTRODUCTION

Epidemiological studies have shown that increased exposure to ambient air particulate matter (PM) is associated with respiratory, cardiovascular and malignant lung diseases.[1–3] Diesel exhaust particles (DEP), a major component of urban PM, contribute to the adverse effects of inhaled PM. Many studies have suggested that oxidative stress is a key biological event in the toxicity of DEP. For instance, intratracheal administration of DEP into mice was found to cause marked mortality owing to lung edema formation and this condition was attenuated by pretreatment with superoxide dismutase (SOD).[4] Furthermore, exposure of macrophages to organic chemicals extracted from DEP, which contain high levels of polycyclic aromatic hydrocarbons (PAH) and their oxygenated derivatives, was shown to induce reactive oxygen species (ROS) generation, resulting in apoptotic and/or necrotic cell death, and these responses were suppressed by pretreatment with *N*-acetylcysteine, a typical antioxidant.[5–8] We previously found that 9,10-phenanthrenequinone (PQ), a

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PAH-quinone found in DEP,[9] produces superoxide anions,[10] and is toxic toward human pulmonary epithelial A549 cells.[11]

Oxidative DNA damage induced by catechols and quinones has been studied extensively to understand their toxicity.[12–20] Catechol and 1,4-hydroquinone (metabolites of benzene),[12–14] catechol estrogen[15,16] and dopa[17] were reported to induce DNA damage in the presence of transition metals, especially Cu, and this DNA damage was enhanced by NADH. Quinones such as 1,2-naphthoquinone (1,2-NQ) and benzo[*a*]pyrene-7,8-dione were also found to be able to cause oxidative DNA damage in the presence of both NADPH and Cu(II).[18–20] Taken together, it has been proposed that both ROS and Cu(I) generated via Cu(II)/Cu(I)-coupled redox cycling of catechol (hydroquinone)/semiquinone radical/quinone are critical for DNA damage, and lead to mutagenesis and carcinogenesis.

We recently demonstrated that an aldo-keto reductase (AKR) 1C isozyme purified from rabbit liver[21] mediates two-electron reduction of PQ to produce 9,10-dihydroxyphenanthrene (PQH_2) by detecting its stable diacetoxy derivative and promotes ROS production through the redox cycling based on disproportionation reaction between PQ and PQH_2 to yield PQ semiquinone radical, reaction of this radical species with molecular oxygen to produce superoxide anion and PQ, and interaction of PQH_2 with superoxide anion because of its high p*K*a value to form hydrogen peroxide and PQ semiquinone radical.[22] The present study is an extension to our previous report[22] and was designed to clarify whether the two-electron reduction of PQ by the AKR1C isozyme is a deleterious pathway.

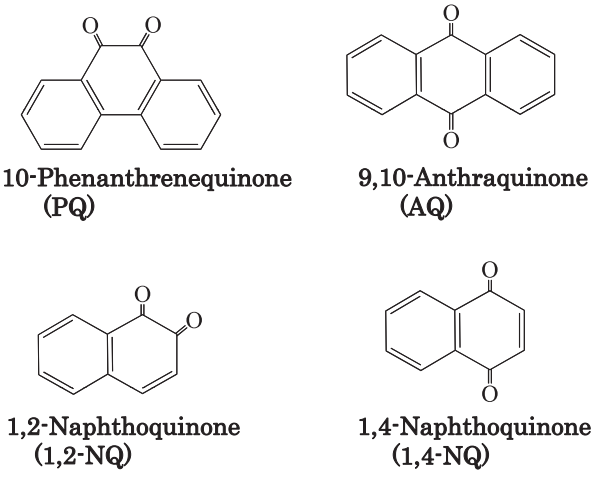

Fig. 1. Structures of the Quinones Used in this Study

In this study, we examined oxidative DNA damage by four quinones found in DEP,[9,23,24] namely PQ, 9,10-anthraquinone (AQ), 1,2-NQ and 1,4-NQ (Fig. 1), in a cell-free system using supercoiled (SC) FX174 DNA as an index of deleterious effects. The results indicate that the AKR1C isozyme augments the PQ-induced DNA damage by enhancing the redox cycling of PQ in the cell-free system using SC FX174 DNA as the target DNA. We also compare the abilities of the four DEP quinones to cause oxidative DNA damage in the absence and presence of the AKR1C isozyme.

MATERIALS AND METHODS

Materials —— The following chemicals were obtained commercially: PQ, AQ and 1,4-NQ from Nakalai Tesque Inc. (Kyoto, Japan); 1,2-NQ from Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.); NADPH from Oriental Yeast Co. Ltd. (Osaka, Japan); mannitol, Tiron, bathocuproine, trichloroacetic acid, ferrous ammonium sulfate, isothiocyanate and cupric chloride ($CuCl_2$) from Wako Pure Chemical Industries Ltd. (Osaka, Japan); SOD (from bovine erythrocytes), catalase (from bovine liver) and cytochrome *c* (from horse heart) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The SC FX174 DNA was purchased from New England Biolabs (Beverly, MA, U.S.A.). A linearized form of the SC DNA was obtained by digestion with *Pst*I (Promega Co., Madison, WI, U.S.A.). As the AKR1C isozyme, NR1 was purified from rabbit liver as described previously.[21] All other chemicals were of the highest grade available.

DNA Nicking Assay —— DNA strand breaks induced by quinones were measured by the conversion of the SC FX174 DNA into open circular (OC) and linear (LIN) forms. Reaction mixtures (15 μl) containing 200 ng of SC FX174 DNA, 0–30 μM quinone in methanol (final concentration 1.7%), 1 mM NADPH, 10 μM $CuCl_2$ and 10 mM sodium phosphate buffer (pH 7.4) in the absence or presence of the AKR1C isozyme were incubated for 15 or 60 min at 37°C. At the end of the incubation period, 3 μl of loading buffer (30% glycerol, 0.25% bromophenol blue, 0.15% xylene cyanol) was added and the samples were then immediately electrophoresed in a 0.55% agarose gel with Tris-borate-EDTA (TBE) buffer (0.04 M Tris-borate, 1 mM EDTA). After the electrophoresis, the gels were stained with ethidium bromide, exposed

to ultraviolet light at 302 nm and photographed. In this system, a single nick in the double stranded SC FX174 DNA caused transformation to the OC form, nicks in both strands caused transformation to the LIN form and multiple nicks to both strands led to fragmentation to low molecular weight species.

NADPH Consumption —— The incubation mixture (1.5 ml) consisting of 67 mM sodium phosphate buffer (pH 7.4), 0.167 mM NADPH and 10 μM quinone in the presence or absence of the AKR1C isozyme was incubated at 37°C. Incubations without a quinone were also performed. The reaction was initiated by the addition of NADPH. The rate of NADPH consumption was monitored by measuring the decrease in absorbance at 340 nm and calculated using an extinction coefficient of 6220 M^{-1} cm^{-1}.

Measurement of Superoxide Anion Formation —— Superoxide anion generation was determined by measuring SOD-inhibitable reduction of cytochrome *c*. The incubation mixture (1.5 ml) consisting of 67 mM sodium phosphate (pH 7.4), 1 mM NADPH, 10 μM quinone and 0.1 mM cytochrome *c* in the presence or absence of SOD (1000 U) was incubated at 37°C. Incubations with the AKR1C isozyme were also performed. The reaction was initiated by the addition of NADPH. The rate of cytochrome *c* reduction was monitored by measuring the increase in absorbance at 550 nm and calculated using an extinction coefficient of 19600 M^{-1} cm^{-1}.

Measurement of Hydrogen Peroxide Formation —— Hydrogen peroxide generation was determined by measuring ferrithiocyanate complex formation.[25] The incubation mixture (1.5 ml) consisting of 10 mM sodium phosphate (pH 7.4), 1 mM NADPH and 10 μM quinone in the presence or absence of the AKR1C isozyme was incubated at 37°C. At the end of the incubation period, trichloroacetic acid (final concentration: 5%), ferrous ammonium sulfate (final concentration: 1.6 mM) and potassium thiocyanate (final concentration: 0.2 M) were added and the absorbance at 480 nm based on ferrithiocyanate complex formation was measured. Hydrogen peroxide produced was calculated from a standard curve that was linear over the range of 0–150 nmol.

RESULTS

DNA Damage Induced by PQ

We examined whether PQ is capable of inducing DNA damage using NADPH (1 mM) as an electron donor. As shown in Fig. 2, concentration-dependent destruction of DNA was observed in the presence of 10 μM Cu(II). In the absence of PQ or Cu(II), no significant DNA damage occurred. In the presence of 1 μM PQ, the SC DNA was significantly converted into OC DNA. At higher concentrations, the formation of OC DNA increased together with the appearance of LIN DNA as the concentration of PQ increased. In the presence of 30 μM PQ, the SC DNA was completely destroyed. We also examined the NADPH consumption and ROS (superoxide anion and hydrogen peroxide) generation. As expected, increasing concentrations of PQ increased the NADPH consumption and ROS generation (see Fig. 5 shown later), indicating that PQ causes NADPH-dependent redox cycling and thereby continuously generates ROS until the NADPH is completely consumed. Irrespective of the presence of PQ, Cu(I) was detected with NADPH alone.

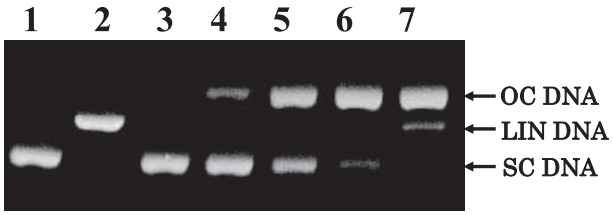

Fig. 2. Concentration-dependent DNA Damage by PQ in the Presence of NADPH and Cu(II)

SC DNA (200 ng) was incubated for 15 min with various concentrations of PQ (1–30 μM) in the presence of NADPH (1 mM) and Cu(II) (10 μM). Lane 1, SC DNA; lane 2, *Pst*-treated SC DNA; lane 3, without PQ; lanes 4–7, with PQ at 1, 5, 10 and 30 μM, respectively.

It was noted that the DNA damage became more severe with increasing concentrations of NADPH or Cu(II) (data not shown). When Fe(III) was substituted for Cu(II), such DNA cleavage was not observed. In accord with this finding, it has been reported that 1,4-hydroquinone and 1,4-benzoquinone cause little or no DNA damage in the presence of Fe(III).[12]

Effects of SOD, Catalase, a ROS Scavenger and a Cu Chelator on the DNA Damage Induced by PQ

To clarify the reactive species responsible for the DNA damage induced by PQ, we examined the effects of SOD, catalase, a ROS scavenger and a Cu chelator on the DNA damage. Catalase was able to prevent the DNA damage induced by PQ (Fig. 3, lane 5). Bathocuproine, a Cu-specific chelator, at 100 μM and Tirone, a superoxide anion scavenger,

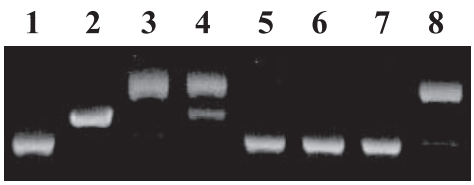

Fig. 3. Effects of Radical Scavengers and a Cu(II) Chelator on the DNA Damage Induced by PQ

The reaction mixture containing SC DNA (200 ng), quinone (10 μM), NADPH (1 mM) and Cu(II) (10 μM) was incubated with a scavenger or Cu(I) chelator for 15 min. Lane 1, SC DNA; lane 2, *Pst*-treated SC DNA; lane 3, no addition; lane 4, with SOD (150 U/ml); lane 5, with catalase (150 U/ml); lane 6, with bathocuproine (100 μM); lane 7, with Tirone (10 mM); lane 8, with mannitol (5 mM).

at 10 mM were also able to block the DNA damage (Fig. 3, lanes 6 and 7). In contrast, mannitol, a hydroxyl radical scavenger, at 5 mM marginally reduced the damage (Fig. 3, lane 8) whereas SOD at 150 U/ml increased the DNA damage (Fig. 3, lane 4). It was noted that SOD at the higher concentration of 1000 U/ml showed little or no inhibitory effect on the DNA damage.

Effects of the AKR1C Isozyme on the DNA Damage Induced by DEP Quinones

First, we compared the potencies of the four DEP quinones at 10 μM to cause DNA damage in the presence of NADPH (1 mM) and Cu(II) (10 μM). 1,2-NQ completely destroyed the SC DNA and its DNA-cleaving ability was stronger than that of PQ (Fig. 4A, lane 7). In contrast, AQ and 1,4-NQ did not induce significant DNA damage (Fig. 4A, lanes 5 and 6).

Next, we examined the DNA cleavage by the DEP quinones in the presence of the AKR1C isozyme. PQ caused more pronounced DNA damage in the presence of the AKR1C isozyme than in its absence (Fig. 4, lane 4). Although 1,4-NQ was not effective in the absence of the AKR1C isozyme, it gained the ability to cause DNA damage in the presence of the AKR1C isozyme (Fig. 4, lane 6). However, AQ caused no DNA damage, even in the presence of the AKR1C isozyme (Fig. 4, lane 5). Based on these findings, it was considered that, in the absence of the AKR1C isozyme, NADPH can act as a reductant for limited quinones, namely 1,2-NQ and PQ, and that the AKR1C isozyme may catalyze the NADPH-dependent reduction of 1,4-NQ as well as PQ. To address these hypotheses, NADPH consumption was measured in the absence or presence of the AKR1C isozyme. The results are summarized in Table 1. PQ and 1,2-NQ consumed NADPH in the absence of the AKR1C isozyme,

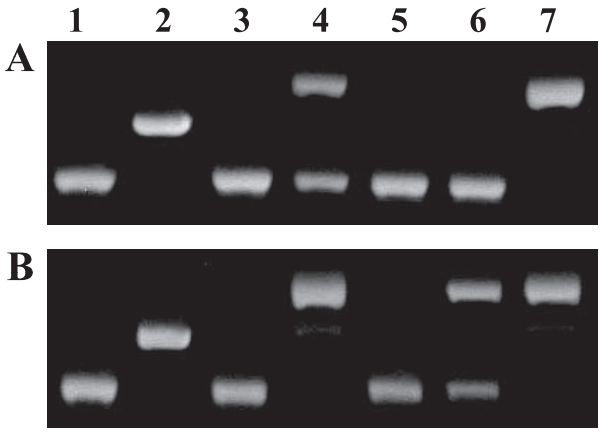

Fig. 4. Effects of the AKR1C Isozyme on the DNA Damage Induced by Quinones

The reaction mixture containing SC DNA (200 ng), quinone (10 μM), NADPH (1 mM) and Cu(II) (10 μM) was incubated for 15 min in the absence (A) or presence (B) of the AKR1C isozyme (1 munit). Lane 1, SC DNA; lane 2, *Pst*-treated SC DNA; lane 3, without quinone; lane 4, with PQ; lane 5, with AQ; lane 6, with 1,4-NQ; lane 7, with 1,2-NQ.

Table 1. NADPH Consumption by Quinones in the Presence and Absence of the AKR1C Isozyme

Quinone	NADPH consumed (nmol/min)	
	Without the AKR1C isozyme	With the AKR1C isozyme
None	0.159 ± 0.059	0.172 ± 0.057
PQ	0.641 ± 0.101	1.430 ± 0.027
AQ	0.164 ± 0.043	0.178 ± 0.028
1,4-NQ	0.165 ± 0.047	0.551 ± 0.042
1,2-NQ	5.489 ± 0.378	5.883 ± 0.128

The rate of NADPH consumption was determined in reaction mixtures (1.5 ml) containing 10 μM quinone or methanol (final concentration: 3.3%), 0.167 mM NADPH and 67 mM sodium phosphate buffer (pH 7.4) in the absence or presence of the AKR1C isozyme (0.8 mU). Each value is the mean ± S.D. of three determinations.

and the consumption rate with 1,2-NQ was 12-fold faster than that with PQ. With 1,4-NQ and AQ, the consumption rates were very slow and similar to those in the absence of each quinone, indicating that NADPH did not serve as a reductant for these quinones. When the AKR1C isozyme was included in the reaction mixture, PQ exhibited marked acceleration of NADPH consumption, while the consumption rate with 1,2-NQ was increased but not as markedly different from that in the absence of the AKR1C isozyme. As expected from the finding that 1,4-NQ only caused DNA damage in the presence of the AKR1C isozyme, this quinone consumed more NADPH in the presence of the AKR1C isozyme than in its absence, while the amounts of NADPH consumed with AQ were almost the same in the presence and absence of the AKR1C isozyme. Consistent with these findings, superoxide anion generation and hydrogen peroxide formation were de-

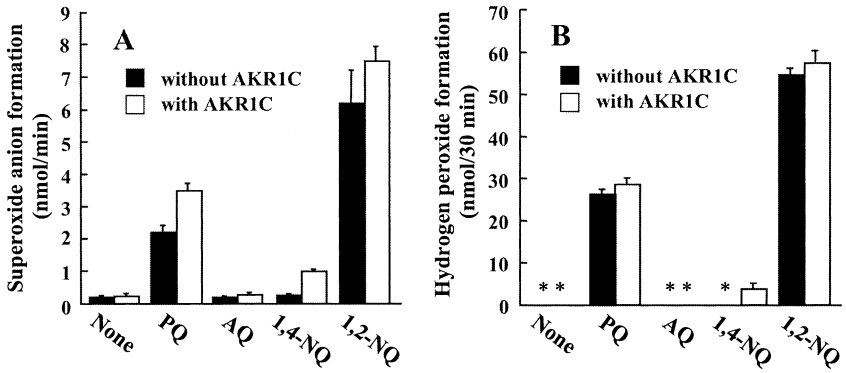

Fig. 5. Superoxide Anion and Hydrogen Peroxide Production by Quinones in the Presence and Absence of the AKR1C Isozyme

The formation of superoxide anion (A) and hydrogen peroxide (B) were measured in reaction mixtures (1.5 ml) containing 10 μM quinone or methanol (final concentration: 3.3%), 0.167 mM NADPH and 67 mM sodium phosphate buffer (pH 7.4) in the absence or presence of the AKR1C isozyme (0.8 mU). A, the rate of superoxide anion generation was determined by measuring SOD-inhibitable reduction of cytochrome *c* as described in Materials and Methods. B, the amount of hydrogen peroxide formed during 30 min was determined by measuring ferrithiocyanate complex formed as described in Materials and Methods. Results are expressed as the mean ± S.D. of three determinations. *Hydrogen peroxide was not detected in the reaction mixtures without quinone and with AQ in both the absence and presence of AKR1C isozyme, and with 1,4-PQ in the absence of AKR1C isozyme.

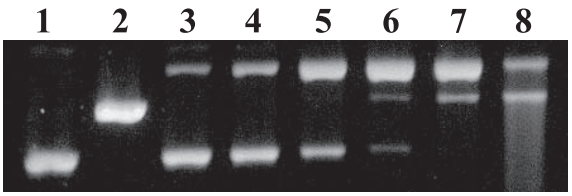

Fig. 6. Dose-dependent PQ-induced DNA Damage by the AKR1C Isozyme

The reaction mixture containing SC DNA (200 ng), quinone (10 μM), NADPH (1 mM) and Cu(II) (10 μM) was incubated with the AKR1C isozyme (0–6 mU). Lane 1, SC DNA; lane 2, *Pst*1-treated SC DNA; lane 3, without the AKR1C isozyme; lanes 4–8, with the AKR1C isozyme at 0.75, 1.5, 3, 4.5 and 6 mU, respectively.

tected in certain reaction mixtures (*i.e.*, PQ and 1,2-NQ in both the absence and presence of the AKR1C isozyme, and 1,4-NQ in the presence of the AKR1C isozyme, Fig. 5) and it was found that there is a good correlation between their production and DNA damage.

To further confirm the involvement of the AKR1C isozyme in stimulating the DNA damage by PQ, various amounts of the AKR1C isozyme were added to the reaction mixture and the DNA damage was monitored. A progressive increase in the DNA damage was observed as the amount of the AKR1C isozyme increased (Fig. 6), accompanied by an increase in NADPH consumption (data not shown).

DISCUSSION

In the present study, we compared the potencies of four quinones contained in DEP to induce DNA damage, and also investigated whether the AKR1C isozyme can participate in quinone-induced DNA damage. The results revealed that *o*-quinones such as PQ and 1,2-NQ induced oxidative DNA damage in the presence of both NADPH and Cu(II), whereas *p*-quinones (1,4-NQ and AQ) were inactive. Since shunting electrons from NADPH by quinones, resulting in ROS generation cause substantial oxidative DNA damage, a reasonable explanation for this observation is that the degrees of oxidative DNA damage caused by PQ, 1,2-NQ, 1,4-NQ and AQ are correlated with their one-electron reduction potential values (1,2-NQ, −89 mV; PQ, −124 mV; 1,4-NQ, −140 mV; 9,10-AQ, −348 mV) as determined by pulse radiolysis studies.[26,27] Addition of the AKR1C isozyme markedly increased the PQ-induced DNA damage but had marginal effects for 1,2-NQ. Interestingly, the AKR1C isozyme conferred the ability to cause DNA damage on 1,4-NQ, while AQ remained inactive.

Recently, we demonstrated that the AKR1C isozyme used in the present study catalyzes NADPH-dependent two-electron reduction of PQ to PQH_2 by detecting its stable diacetoxy derivative by HPLC and also detected the generation of superoxide anions and semiquinone radicals in the reaction mixture by ESR.[22] The present study showed that DEP quinones capable of cleaving DNA consume NADPH and produce ROS, indicating that these quinones cause redox cycling triggered by the formation of their reduction products, as proposed for PQ in our previous report.[22] In connec-

tion with this, a good correlation between NADPH consumption and DNA scission was observed in the present study, strongly suggesting that the generation of ROS is a key event in the DNA damage. The results obtained in the experiments using the AKR1C isozyme also support this suggestion, since the AKR1C isozyme stimulated the PQ-induced oxidative DNA damage in a dose-dependent manner and the intensity of the DNA damage induced by the enzyme was correlated with increased consumption of NADPH and generation of ROS. In addition, we found that, only in the presence of the AKR1C isozyme, 1,4-NQ consumed NADPH and cleaved DNA. Accordingly, the AKR1C isozyme is proposed to participate in accelerating or causing the NADPH-dependent redox cycling of PQ or 1,4-NQ, thereby leading to the production of reactive species responsible for DNA damage.

It has been reported that both generation of ROS and production of Cu(I) are critical for DNA cleavage by quinones and catechols.[12–20] Consistent with this notion, we also detected ROS and Cu(I) in the reaction mixture that caused DNA damage. To clarify the active species responsible for the DNA damage, the effects of ROS scavengers and a Cu(I)-specific chelator were examined. The results showed that catalase, Tiron (a superoxide anion scavenger) and bathocuproine [a Cu(I)-specific chelator] reduced the DNA damage, indicating the involvement of superoxide anions, hydrogen peroxide and Cu(I). On the other hand, mannitol (a hydroxyl radical scavenger) did not exhibit any inhibitory effects and SOD increased the DNA damage. The latter may be explained by dismutation of superoxide anions to hydrogen peroxide. Although an ESR study revealed that hydroxyl radicals were formed during the reduction of PQ in the presence of Cu(II) (data not shown), the above findings indicate that mannitol did not affect PQ-induced DNA scission, thereby suggesting that hydroxyl radicals are not critical factor for the oxidative DNA damage by PQ. This suggestion is further supported by the finding that incubation mixture in the presence of Fe(III), in which hydroxyl radicals should be generated by Fenton and Harbar-Weiss reactions, caused little or no DNA damage. Taken together, these results also suggest that Cu(I)-OOH complexes derived from the reaction of hydrogen peroxide with Cu(I) may play a role in causing DNA damage, as proposed by Kawanishi and co-workers.[13] The Cu(I) formed from Cu(II) bound to DNA interacts with hydrogen peroxide, resulting in the formation of DNA-Cu(I)-OOH complexes. Hydroxyl radicals released from these complexes immediately attack adjacent DNA before they can be scavenged by hydroxyl radicals scavengers.[28]

In general, AKR1C isozymes are constitutively and ubiquitously expressed in various tissues and exhibit broad substrate specificities for the oxidation of hydroxysteroids, alicyclic alcohols and PAH dihydrodiols as well as the reduction of aromatic aldehydes, ketones and quinones using NADP(H) as a cofactor.[29, 30] The AKR1C isozyme used in the present study had the ability to reduce 1,2-NQ and 1,4-NQ as well as PQ at different rates. In accord with these findings, 1,4-NQ gained the ability to cause DNA damage in the presence of the AKR1C isozyme. The marginal effect observed with 1,2-NQ may be explained by high non-enzymatic reduction at a rate that not much different from that obtained with the AKR1C isozyme. These findings indicate that the AKR1C isozyme can participate in oxidative DNA damage by DEP quinones that can be reduced by the enzyme.

It is known that NADPH-cytochrome P450 reductase catalyzes the one-electron reduction of quinones to yield semiquinone radicals, which can reduce oxygen to form superoxide anions and consequently the dismutation product hydrogen peroxide, resulting in the induction of oxidative stress.[31, 32] In contrast, NADPH-quinone oxidoreductase, which catalyzes the two-electron reduction of quinones to form hydroquinones, has been thought to protect against quinone toxicity not only by preventing ROS generation by competing with NADPH-cytochrome P450 reductase-dependent redox cycling, but also by preventing arylation by eradicating the electrophilic property of quinones, although it actually depends on the stability of the resulting hydroquinones.[33, 34] In accord with our previous report,[22] the present study clearly indicates that the AKR1C isozyme may participate in the induction of oxidative stress by DEP components. In addition, it is known that AKR1C isozymes participate in the formation of toxic PAH *o*-quinones from innocuous PAHs as dihydrodiol dehydrogenases.[35, 36] Two other enzymes, cytochrome P450 and epoxide hydrolase, are involved in this process. Accordingly, AKR1C isozymes may play an important role in DEP toxicity via their ability to produce toxic PAH *o*-quinones and to induce oxidative stress through redox cycling.

In conclusion, the potency for causing DNA

damage varies among DEP quinones and may simply depend on their ability to accept electrons from reducing agents. Although studies with more quinones are needed, it seems likely that NADPH can act as a reducing agent with specificity for *o*-quinones in the absence of an enzyme. However, the AKR1C isozyme not only stimulates but also confers the ability to cause DNA damage, depending on its reducing capability for DEP quinones. Considering the existence of several quinone-reducing enzymes in tissues, their participation should be taken into account for evaluating DEP toxicity *in vivo*. The present study may provide useful information for clarifying the toxicity of DEP.

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