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Oxidation of Proximal Protein Sulfhydryls by Phenanthraquinone, a Component of Diesel Exhaust Particles

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Diesel exhaust particles (DEP) contain quinones that are capable of catalyzing the generation of reactive oxygen species in biological systems, resulting in induction of oxidative stress. In the present study, we explored sulfhydryl oxidation by phenanthraquinone, a component of DEP, using thiol compounds and protein preparations. Phenanthraquinone reacted readily with dithiol compounds such as dithiothreitol (DTT), 2,3-dimercapto-1-propanol (BAL), and 2,3-dimercapto-1-propanesulfonic acid (DMPS), resulting in modification of the thiol groups, whereas minimal reactivities of this quinone with monothiol compounds such as GSH, 2-mercaptoethanol, and *N*-acetyl-L-cysteine were seen. The modification of DTT dithiol caused by phenanthraquinone proceeded under anaerobic conditions but was accelerated by molecular oxygen. Phenanthraquinone was also capable of modifying thiol groups in pulmonary microsomes from rats and total membrane preparation isolated from bovine aortic endothelial cells (BAEC), but not bovine serum albumin (BSA), which has a Cys34 as a reactive monothiol group. A comparison of the thiol alkylating agent *N*-ethylmaleimide (NEM) with that of phenanthraquinone indicates that the two mechanisms of thiol modification are distinct. Studies revealed that thiyl radical intermediates and reactive oxygen species were generated during interaction of phenanthraquinone with DTT. From these findings, it is suggested that phenanthraquinone-mediated destruction of protein sulfhydryls appears to involve the oxidation of presumably proximal thiols and the reduction of molecular oxygen.

Introduction

Exposure of experimental animals and humans to diesel exhaust particles (DEP)[1] is associated with lung cancer, allergic inflammation, asthma, and cardiopulmonary dieseases (*1*−*3*). It was reported previously that intratracheal administration of DEP into mice caused a marked mortality due to lung edema formation. This condition was attenuated by pretreatment with superoxide dismutase (SOD) conjugated with poly(ethylene glycol) (*4*). We found that quinones contained in DEP were reduced by one electron by NADPH−cytochrome P450 reductase, leading to overproduction of superoxide and hydroxyl radical (*5*). Recent studies have indicated that exposure of macrophages to organic chemicals extracted from DEP resulted in induction of apoptosis (*6*) and an increase in the gene expression of the oxidative stress-inducible protein heme oxygenase-1 (*7*). Taken together, it is likely that quinones in DEP can play a critical role in catalyzing the generation of reactive oxygen species, resulting in cellular oxidative stress. Although Schuetzle and co-workers (*8, 9*) reported previously that a variety of quinones have been identified as DEP components, few studies on the involvement of the quinones in oxidative stress-dependent DEP toxicity have been reported.

Quinones are found in the diet and in contaminants in urban air and are utilized as dye, antibiotics, and anticancer drugs (*8, 10*). Due to their ubiquitous presence, the toxicology of quinones is an area of extreme interest. The toxicity of quinones has been proposed to result from their potential to (1) serve as alkylating agents and (2) interact with, for example, flavoproteins to generate reactive oxygen species (*10*−*12*). Thus, the toxicity of many quinoid compounds is due to either or both oxidative stress (i.e., oxidation of sulfhydryls) as well as covalent modification of biological macromolecules. Oxidation of cysteine residues in proteins is associated with not only decreased sulfhydryl status but also

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[1] Abbreviations: DEP, diesel exhaust particles; BAEC, bovine aorta endothelial cells; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BAL, 2,3-dimercapto-1-propanol; DMPS, 2,3-dimercapto-1-propanesulfonic acid; BSA, bovine serum albumin; SOD, superoxide dismutase; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; Hepes, 4-(2-hydroxyethyl)-1-piperazineesulfonic acid.

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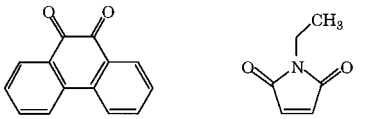

Figure 1. Structures of phenanthraquinone and NEM.

changes in oxidative stress-mediated signal transduction (*13, 14*). If the sulfhydryls are modified, physiological functions would be altered, thereby inducing oxidative stress.

In this study, we chose phenanthraquinone as a model compound because this chemical is known as a relatively abundant quinone in DEP (*8*). Several previous reports indicate that electrophiles such as *N*-ethylmaleimide (NEM) (Figure 1) can promote alkylation of protein thiols. Unlike NEM, phenanthraquinone will not alkylate thiols but has the potential to act as a redox cycling quinone, generating thiol oxidants such as hydrogen peroxide. In the present study, using NEM and phenanthraquinone (Figure 1), we (1) investigate the possibility of oxidation of protein sulfhydryls by phenanthraquinone and (2) look for the generation of reactive oxygen species and oxidized thiol intermediates formed during interaction of these compounds with sulfhydryls.

Materials and Methods

Materials. The chemicals and proteins were obtained as follows: Phenanthraquinone, DTT, NEM, and bovine serum albumin (BSA) from Nacalai Tesque, Inc. (Kyoto, Japan); 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO) from Labotech Co. (Tokyo, Japan); catalase from Sigma Chemical Co. (St. Louis, MO); 2,3-dimercapto-1-propanol (BAL) and 2,3-dimercapto-1-propanesulfonic acid (DMPS) from Aldrich Chemical. Co. Inc. (Milwaukee, WI); Econo-Pac 10DG column from Bio-Rad Laboratories (Richmond, CA); cytochrome *c* from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cu,Zn-superoxide dismutase (Cu,Zn-SOD) was purified from human liver by the method of Kumagai et al. (*15*). All other chemicals used were obtained from commercial sources and were of the highest grade available.

Preparation of Enzyme. Bovine aortic endothelial cells (BAEC) were obtained from Dainippon Pharmaceutical Industrial Co. (Tokyo, Japan). BAEC were maintained in Dulbecco's modified Eagle's medium: Nutrient mixture F-12 supplemented with 15% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), fibroblast growth factor-acidic (5 ng/mL), and heparin (10 units/mL). Cells were incubated in a humidified atmosphere of 95% air/5% CO_2. The medium was changed every 2−3 days, and cells were routinely passaged by trypsin/EDTA with a split ratio of 1:4. BAEC between passages 3 and 6 were scraped from the culture plate and homogenized in 50 mM Tris-HCl (pH 7.4)/0.1 mM EDTA/0.1 mM EGTA/1 mM phenylmethylsulfonyl fluoride/leupeptin (1 μg/mL). The homogenate was centrifuged at 100000*g* for 60 min to isolate the total membrane fraction. The resulting pellets were suspended in the homogenate buffer containing 2.5 mM $CaCl_2$. Suspensions obtained were frozen under liquid nitrogen and kept at −70 °C before use. For pulmonary preparations, lungs of Wistar rats (4 weeks) were homogenized in 3 volumes of 10 mM Tris-HCl (pH 7.0)/0.1 mM EDTA. The homogenates were centrifuged at 9000*g* for 20 min. The supernatants were recentrifuged at 105000*g* for 60 min. Cytosol fraction (3 mL) was applied to a Econo-Pac 10DG column to remove low molecular weight thiol compound such as GSH. Pellets obtained were washed with 100 mM potassium pyrophosphate buffer (pH 7.4) to remove hemoglobin and recentrifuged at 105000*g* for 60 min. The pellets were suspended in 10 mM Tris-HCl (pH 7.0)/0.1 mM EDTA to make a final concentration of 5.4−6.5 mg/mL. The microsomal preparation was stored at −70 °C before use; under these conditions, a decline of protein sulfhydryl content with a rate of 0.3 nmol of SH group (mg of protein)$^{-1}$ day^{-1} was observed. Protein concentration was measured by the method of Bradford (*16*) with bovine serum albumin as the standard.

Measurement of Thiol Content. Phenanthraquinone and other quinones used were dissolved in Me_2SO. All spectrophotometric measurements were performed using a Shimadzu UV-1600 double-beam spectrophotometer (Kyoto, Japan). Thiol contents of DTT preparations and in BAEC were determined as described previously (*17*). The incubation mixture (1 mL) containing different concentrations of phenanthraquinone (final concentration of Me_2SO, 2%) was incubated with a variety of sulfhydryls at 37 °C for 10−60 min. For anaerobic conditions, argon gas was bubbled through the solution during each incubation. In the case of nonprotein sulfhydryl compounds (i.e., DTT), an aliquot (0.5 mL) of the incubation mixture was mixed with 10% trichloroacetic acid (0.5 mL), and then a portion (0.5 mL) of the mixture was mixed with 1 mL of 0.4 M Tris-HCl (pH 8.9)/20 mM EDTA and 25 μL of 10 mM DTNB. For the protein sulfhydryl compound (with the BAEC preparation), an aliquot (0.1 mL) of the incubation mixture was mixed with 0.3 mL of 0.2 M Tris-HCl (pH 8.2)/20 mM EDTA, followed by 20 μL of 10 mM DTNB, 0.15 mL of 5% SDS. Each resulting mixture was measured at 412 nm against a blank to determine the content of the thiol groups (with Me_2SO). For nonprotein sulfhydryls, each thiol compound was used as the standard, and quantitation of protein sulfhydryl content was determined using an extinction coefficient of 13.6 mM^{-1} cm^{-1}.

Detection of Thiyl Radical. Electron spin resonance (ESR) studies were performed at 25 °C by using an JES-FA200 spectrometer (JEOL Co. Ltd., Tokyo, Japan) as described previously (*5*). Thiyl radicals generated during the chemical reaction of phenanthraquinone with DTT were identified as its DMPO adduct by the method of Yim et al. (*18*). The reaction mixture (0.2 mL) containing 100 μM phenanthraquinone (in Me_2SO, final concentration of 2%), 10 mM DTT, 100 mM DMPO, and 100 mM potassium phosphate buffer (pH 7.4) was incubated at 25 °C for 30 s. The spectrometer settings are indicated in the figure legends.

Generation of Reactive Oxygen Species. Superoxide production was determined by Cu,Zn-SOD-inhibitable reduction of cytochrome *c* as described previously (*5*) except that cytochrome *c* was used instead of its acetylated form and reduction of cytochrome *c* was measured at 550 nm using an extinction coefficient of 21.1 mM^{-1} cm^{-1}. The incubation mixture (1.5 mL) consisted of 0.1 μM phenanthraquinone, 0.1 mM DTT, 0.05 mM cytochrome *c*, and 50 mM Tris-HCl (pH 7.5) in the absence and presence of Cu,Zn-SOD (2400 units). The reaction was initiated by addition of the quinone to a sample cuvette. Hydrogen peroxide was determined according to the method of Hildebrandt and Roots (*19*). The incubation mixture (1.5 mL) contained 0.1 μM phenanthraquinone, 100 μM DTT, and 0.1 M potassium phosphate buffer (pH 7.5). Reaction was performed at 37 °C for different time periods and terminated by addition of 1 mL of 2.5% perchloric acid. After the reaction mixture was centrifuged at 14000*g* for 5 min, the supernatant (0.2 mL) was mixed with 1 mL of water, 0.24 mL of 10 mM ferroammonium sulfate, and 0.12 mL of 2.5 M potassium thiocyanate. Then, each resulting mixture was measured at 480 nm. For the calibration curve, hydrogen peroxide was used as the standard.

HPLC. Separation of phenanthraquinone and NEM was accomplished using a Shimadzu HPLC system (Kyoto, Japan). After reaction, an aliquot (20−100 μL) was applied to a YMC packed column AM-type (250 × 4.6 mm i.d., 5 μm particle size, Yamamura Labs, Kyoto, Japan) at a flow rate of 1 mL/min. Water/acetonitrile (1:1, v/v) was used as the mobile phase, and detection was performed at 255 nm. Under these conditions, the

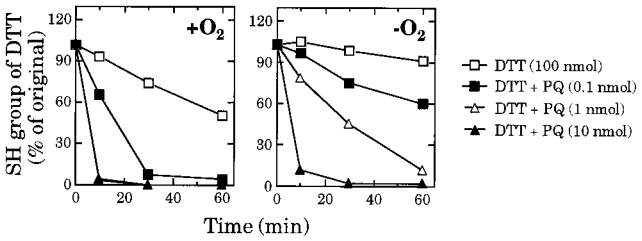

Figure 2. Consumption of thiol content in DTT during incubation with phenanthraquinone under aerobic and anaerobic conditions. Incubation mixture (1 mL) consisted of phenanthraquinone (0.1, 1, and 10 nmol), DTT (100 nmol) in 0.1 M potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for different time periods. For the anaerobic condition, argon gas was bubbled through the solution during each incubation. Then the consumption of the thiol group was determined under conditions described under Materials and Methods. Each value is the mean of three determinations. Note: the SD value of the mean for each data point was less than 5%.

retention times of phenanthraquinone and NEM were 9.7 and 4.6 min, respectively. For the reaction of phenanthraquinone with the BAEC preparation, the reaction mixture was filtered with a membrane filter (4 mm i.d., 0.5 μm pore size) and the filtrate immediately analyzed by the HPLC system described above.

Results

Interaction of Phenanthaquinone and NEM with Sulfhydryls. The interaction of both phenanthraquinone and NEM with the thiol groups of DTT and the thiol groups contained in the BAEC preparation was examined. When NEM (25 and 50 μM) was incubated with DTT (100 μM) in 0.1 M potassium phosphate buffer (pH 7.5) at 37 °C for 10 min under anaerobic conditions, the SH groups of DTT consumed were 7.2 ± 2.0 and 20.5 ± 2.3 μM, respectively (n = 3). The level of thiol modification by NEM reached a plateau after 10 min. The rate of thiol consumption by phenanthraquinone was also pH-dependent. Not unexpectedly, the rate of DTT oxidation increased with increasing pH [i.e., 6.9 ± 3.4, 48.3 ± 1.0, and 103.8 ± 0.3 μM modified thiol was formed after 10 min in the presence of phenanthraquinone (1 μM) at pH 5.5, 6.5, and 7.5, respectively, n = 3]. As shown in Figure 2, the SH group of DTT was sensitive to dissolved oxygen under the conditions examined. However, the destruction of DTT was found to be catalytic with respect to phenanthraquinone since complete thiol loss in a 100 μM DTT solution was seen with the addition of <100 μM phenanthraquinone under anaerobic conditions (Figure 2). For example, phenanthraquinone at only 10 μM resulted in the complete loss of free thiol groups in a 100 μM DTT solution at pH 7.5 after 30 min, whereas molecular oxygen stimulated phenanthraquinone-mediated consumption of thiols in DTT markedly. The ability of phenanthraquinone to oxidize thiols was not mediated by a metal-dependent process since buffer treated with the metal chelator dithizone did not affect the reaction (data not shown). However, treatment of thiols with 1,4-benzoquinone, 2-methyl-1,4-benzoquinone, 2-chlorobenzoquinone, 2,3,5,6-tetramethyl-1,4-benzoquinone, pyrroloquinoline quinone, 2-anilino-1,4-naphthoquinone, lapachol, 2-chloroanthraquinone, 5,12-naphthacenequinone, 9,10-anthraquinone, or mytomycin *c* at a concentration of 1 μM gave negligible amounts of thiol oxidation products. Interestingly, SH groups of dithiol compounds such as DTT, BAL, and DMPS were selectively oxidized by phenanthraquinone, whereas those of monothiol compounds such as GSH, 2-mercaptoethanol, and *N*-acetyl-L-cysteine were not (Table 1).

Compared to DTT oxidation, the thiols contained in the membrane fraction of BAEC were less susceptible to phenanthraquinone-mediated modification. However, an excess amount of the protein sulfhydryls in BAEC preparation was consumed after incubation with phenanthraquinone, but not NEM, suggesting a redox cycling of phenanthraquinone in the presence of protein thiol (Table 2). As shown in Table 3, the unusual stoichiometric relationship between protein sulfhydryls consumed and phenanthraquinone added (0.2 nmol) was also seen with rat lung microsomes. In contrast, phenanthraquinone (1 and 10 nmol) was unaffected by a reactive monothiol group of BSA (100 nmol) although the thiol content

Table 1. Consumption of SH Groups in Monothiol and Dithiol Compounds by Phenanthraquinone[a]

	consumption of SH group (nmol)	
compound	1 nmol	10 nmol
monothiol		
GSH	2.7 ± 0.5	1.8 ± 2.3
2-mercaptoethanol	0	0.3 ± 1.0
N-acetyl-L-cysteine	0	0.3 ± 2.0
dithiol		
DTT	103.0 ± 0.3	103.0 ± 0.0
BAL	43.9 ± 1.0	98.0 ± 0.6
DMPS	17.1 ± 2.1	89.1 ± 1.0

[a] The incubation mixture (1 mL) consisted of phenanthraquinone (1 and 10 nmol) and various thiol compounds (100 nmol) in 100 mM potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for 10 min under aerobic conditions. Each value is the mean ± SD of three determinations.

Table 2. Consumption of Thiol Contents in BAEC during Incubation with Phenanthraquinone and NEM[a]

	SH groups consumed (nmol)			
	phenanthraquinone added (nmol)		NEM added (nmol)	
time (min)	1	10	25	50
10	3.7 ± 4.4	12.9 ± 8.1	15.4 ± 6.1	25.7 ± 3.9
30	19.8 ± 4.8	23.9 ± 4.2	22.4 ± 3.6	28.6 ± 1.3
60	26.8 ± 5.1	34.2 ± 5.0	19.8 ± 1.3	32.0 ± 5.2

[a] The incubation mixture (1 mL) consisted of BAEC preparation (with phenanthraquinone, 67.28 nmol of SH; with NEM, 48.16 nmol of SH) and different amounts of phenanthraquinone or NEM in 0.1 M potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for different time periods under aerobic conditions. Then the consumption of the thiol group was determined under conditions described under Materials and Methods. Each data is the mean ± SD of three determinations.

Table 3. Consumption of SH Groups in Lung Microsomes of Rats during Interaction with Phenanthraquinone[a]

phenanthraquninone added (nmol)	SH groups consumed (nmol)
0.02	0 ± 0.1
0.2	2.4 ± 0.4
2	2.7 ± 0.5
20	5.3 ± 1.1

[a] The incubation mixture (1.5 mL) consisted of lung microsomes from rats (27.75 nmol of SH group) and different amounts of phenanthraquinone in 100 mM potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for 60 min under aerobic conditions. Each value is the mean ± SD of three determinations.

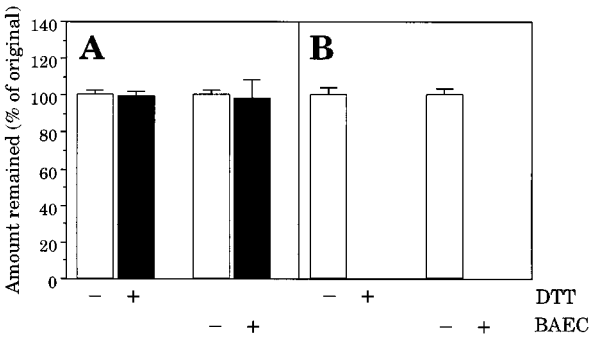

Figure 3. Measurement of phenanthraquinone or NEM remaining during incubation with DTT: (A) phenanthraquinone; (B) NEM. Open and closed bars indicate amounts of phenanthraquinone and NEM in the absence and presence of DTT (BAEC), respectively. The incubation mixture (1 mL) consisted of phenanthraquinone (10 nmol) or NEM (50 nmol), DTT (100 nmol), or BAEC preparation (40.37 nmol of SH) in 0.1 M potassium phosphate buffer (pH 7.5). Reactions were carried out at 37 °C for 30 min under aerobic conditions. Then, the remaining phenanthraquinone or NEM was determined under conditions described under Materials and Methods. The amount of each compound remaining is expressed as its peak height. Each value is the mean ± SD of three determinations.

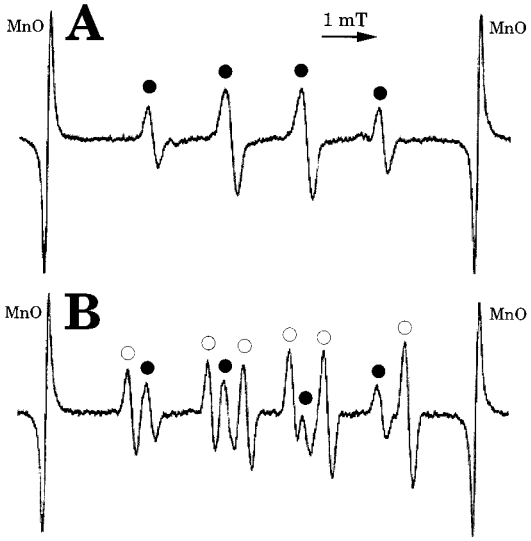

Figure 4. ESR spectra of radical species generated. Closed circles, thiyl-DMPO adduct; open circles, methyl-DMPO adduct. (A) Horseradish peroxidase (10 μg) was incubated with 10 mM DTT, 0.05 mM hydrogen peroxide, and 20 mM Hepes buffer (pH 7.2). (B) 0.1 mM phenanthraquinone was incubated with 10 mM DTT, 100 mM DMPO, and 0.1 M potassium phosphate buffer (pH 7.5). Phenanthraquinone was dissolved in Me_2SO (final concentration of 2%). Each reaction was performed at 25 °C for 0.5 min under aerobic conditions. The instrument settings were as follows: modulation width, 100 kHz−0.2 mT; sweep time, 1 min; time constant, 0.03 s; microwave power, 7.99 mW; microwave frequency, 9.4124 GHz; magnetic field, 335.3 ± 5 mT.

of commercial BSA used in the study was 0.52 mol of SH group/mol.

The fact that phenanthraquinone can destroy thiols in a catalytic fashion could indicate that there is no direct chemical reaction between phenanthraquinone and thiols. That is, phenanthraquinone serves as a catalyst for the generation of the ultimate thiol-degrading species. Consistent with this idea, it was found that phenanthraquinone was not consumed during the thiol-destroying reaction (Figure 3A). On the other hand, NEM is known to react directly with thiol functions, leading to covalent and irreversible modification. Indeed, when NEM was reacted with DTT or the thiols contained in the BAEC preparation, it was consumed (Figure 3B).

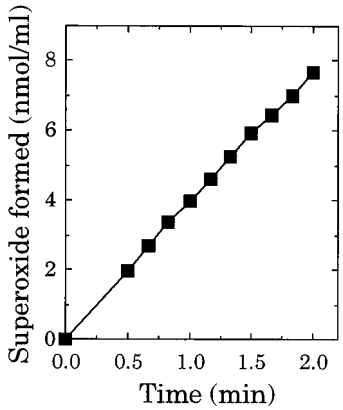

Figure 5. Superoxide generation during reaction of phenanthraquinone with DTT. The incubation mixure (1.5 mL) consisting of phenanthraquinone (0.15 nmol) was incubated with DTT (150 nmol), cytochrome *c* (50 nmol) in 50 mM Tris-HCl (pH 7.5) in the presence and absence of Cu,Zn-SOD (2400 units) under aerobic conditions. Reactions were performed at 25 °C under aerobic conditions. Each value is the average of duplicate determinations.

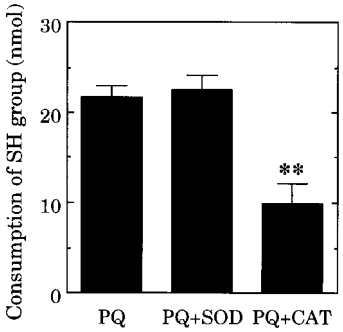

Figure 6. Effects of reactive oxygen scavengers on phenanthraquinone-mediated consumption of protein sulfhydryls. PQ, phenanthraquinone; SOD, Cu,Zn-SOD; CAT, catalase. The incubation mixture (1.5 mL) consisted of phenanthraquinone (150 nmol) and lung microsomes from rats (104 nmol of SH group) in 0.1 M potassium phosphate buffer (pH 7.5) in the absence and presence of Cu,Zn-SOD or catalase (1000 units). Reactions were carried out at 37 °C for 60 min under aerobic conditions. Each value is the mean ± SD of three determinations.

Thiyl Radical Generation. Thiyl radicals can be trapped with a radical trapping agent such as DMPO (*24*, *25*). Figure 4A shows a typical ESR spectrum of the thiyl radical formed in a system of DTT, horseradish peroxidase, hydrogen peroxide, and DMPO. This DMPO adduct has a distinctive ESR spectrum consisting of four relatively broad lines with coupling constants of $\alpha^N = 1.51$ mT and $\alpha^H_\beta = 1.60$ mT, as reported by Mason and Rao (*20*). When phenanthraquinone (100 μM) was mixed with DTT (10 mM) at pH 7.4, signals which were identical to those generated by the thiyl radical generating system were observed (Figure 4B). Moreover, a radical species corresponding to a methyl-DMPO adduct was also detected ($\alpha^N = 1.63$ mT and $\alpha^H = 2.32$ mT) (*21*). The detection of the methyl-DMPO adduct is indicative of hydroxyl radical production since it has been demonstrated that the reaction of hydroxyl radical with Me_2SO (used to solubilize phenanthraquinone) in the presence of DMPO results in the formation of this adduct

(*22*) (Figure 4B). These signals were not detected when phenanthraquinone or DTT alone was incubated (data not shown).

Production of Reactive Oxygen Species. The destruction of thiols in the presence of catalytic amounts of phenanthraquinone and the generation of thiyl and reduced oxygen intermediates (as evidenced by the generation of the DMPO adducts) are consistent with a process whereby phenanthraquinone serves as a catalyst for the oxidation of thiols by the electron acceptor, molecular oxygen. Such a process would generate, as an intermediate, the reduced oxygen species superoxide. As expected, a reaction of phenanthraquinone (0.1 nmol) with DTT (100 nmol) caused a time-dependent generation of superoxide as determined by Cu,Zn-SOD-inhibitable reduction of cytochrome *c* (Figure 5). Under the conditions, production of hydrogen peroxide with a rate of 44 nmol per 5 min was also observed. To confirm whether these reactive oxygen species could contribute to phenanthraquinone-mediated oxidation of protein sulfhydryls, we examined effects of scavenging agents for superoxide and hydrogen peroxide on consumption of microsomal protein sulfhydryls from rat lung caused by phenanthraquinone (Figure 6). Addition of an excess amount of catalase (1000 units) suppressed the phenanthraquinone-mediated consumption of microsomal protein sulfhydryls. However, Cu,Zn-SOD was without effect on the oxidation.

Discussion

The present results indicated that protein sulfhydryls undergo facile modification by phenanthraquinone, a component of DEP. We find that like NEM, phenanthraquinone is capable of destroying the thiols of both DTT and those contained in a BAEC preparation (Figure 2 and Table 2). However, the mechanisms of thiol destruction appear to be different for the two chemical agents. Phenanthraquinone-mediated modification of thiols is catalytic since thiol destruction occurs without loss of phenanthraquinone whereas thiol loss via the NEM-mediated process results in simultaneous loss of NEM (Figure 3). It is well recognized that quinones have two chemical properties consisting of (1) electrophilic attack to nucleophiles, resulting in thiol adduct formation, and/or (2) redox cycling, wherein there is rapid and sequential reduction to the quinone, leading to production of reactive oxygen species (*11*). Therefore, we conclude that the reactivity of phenanthraquinone toward DTT is classified into the latter case.

The catalytic destruction of thiols by phenanthraquinone occurred even in anaerobic media; however, such a phenomenon was further accelerated in the presence of molecular oxygen. That is, phenanthraquinone acts as a catalyst for the thiol-mediated reduction of O_2, which leads to the generation of reactive oxygen species and thiol oxidation. This idea is supported by our observation that both reduced oxygen species and the oxidized thiol species, thiyl radical, are observed by ESR (Figure 4). Furthermore, the reaction of phenanthraquinone with thiols results in the generation of a species, such as superoxide, which can reduce cytochrome *c* in an SOD-inhibitable fashion (Figure 5). Taken together, these data are consistent with the following chemical reactions resulting in the overall oxidation of thiols by O_2:

$$\text{Q (phenanthraquinone)} + \text{R}-\text{SH} \rightarrow \text{Q}^{\bullet -} + \text{H}^+ + \text{R}-\text{S}^{\bullet} \quad (1)$$

$$\text{R}-\text{S}^{\bullet} + \text{R}-\text{SH} \rightarrow \text{R}-\text{S}-\text{S}-\text{R}^{\bullet -} \quad (2)$$

$$\text{R}-\text{S}-\text{S}-\text{R}^{\bullet -} + \text{Q} \rightarrow \text{Q}^{\bullet -} + \text{R}-\text{S}-\text{S}-\text{R} \quad (3)$$

$$\text{R}-\text{S}^{\bullet} + \text{Q} \rightarrow \text{R}-\text{SQ}^{\bullet} \quad (4)$$

$$\text{R}-\text{SQ}^{\bullet} + \text{R}-\text{SH} \rightarrow \text{Q}^{\bullet -} + \text{R}-\text{S}-\text{S}-\text{R} \quad (5)$$

$$\text{Q}^{\bullet -} + \text{O}_2 \rightarrow \text{Q} + \text{O}_2^{\bullet -} \quad (6)$$

$$\text{O}_2^{\bullet -} + \text{O}_2^{\bullet -} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (7)$$

$$\text{Fe}^{3+} + \text{O}_2^{\bullet -} \rightarrow \text{Fe}^{2+} + \text{O}_2 \quad (8)$$

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + {\cdot}\text{OH} + \text{OH}^- \quad (9)$$

$$\text{Me}_2\text{SO} + {\cdot}\text{OH} \rightarrow {\cdot}\text{CH}_3 + \text{MeSO(OH)} \quad (10)$$

There is ample precendence for all of the above reactions. Reduction of quinones by thiolate (eq 1), reaction of thiyl radical with thiolate (*23*) (or quinones) to give the corresponding disulfide radical anion (*18*) (eq 2) and quinone−thiol radical, which can react with thiolate to form semiquinone radical anion and disulfides (eqs 4 and 5), reduction of quinones to these semiquinone radical anions by disulfide radical anion (eq 3), reduction of O_2 by a semiquinone radical anion (eq 6), and chemical disproportion of superoxide to hydrogen peroxide and molecular oxygen (eq 7) are all established processes. Hydrogen peroxide can undergo a one-electron reduction to hydroxyl radical in the presence of trace metals (e.g., iron contaminated in phosphate buffer) via a metal-catalyzed Haber−Weiss reaction (*24*) as shown in eqs 8 and 9. However, there were signals of methyl-DMPO instead of OH-DMPO (Figure 4). Because the concentration of Me_2SO, a potent scavenging agent for hydroxyl radical (*25*), in the reaction mixture was approximately 260 mM, a reasonable explanation is that the hydroxyl radical formed reacts better with Me_2SO than with DMPO, resulting in trapping the methyl radical (*18*, *21*, *26*). In ESR experiments, no signals of the semiquinone radical of phenanthraquinone (eqs 3 and 5) were detected. This discrepancy may result from instability of *o*-semiquinones (*27*) and/or rapid oxidation by molecular oxygen (eq 6).

Using synthetic thiol species, it was found that phenanthraquinone-mediated oxidation of sulfhydryls occurs specifically with proximal thiol systems (Table 1). Consistent with this idea, the reactive monothiol group (Cys34) of BSA (*28*) was not modified by reactions with phenanthraquinone. From these findings, we postulate that only proximal sulfhydryls in proteins will be oxidized by interaction with phenanthraquinone. However, it was found that phenanthraquinone-mediated oxidation of the protein sulfhydryls of rat lung microsomes was diminished by addition of catalase, but not Cu,Zn-SOD (Figure 6). Although hydrogen peroxide formed during reaction of phenanthraquinone with DTT (see Results) is shown to oxidize thiol oxidation of BSA (*29*), this quinone could not modify the monothiol group of BSA in the present

study. Indeed, it is indicated that interaction of phenanthraquinone with proximal thiols, but not monothiol, is essential to initiate sulfhydryl oxidation. Nevertheless, it should be noted that although reactions (eqs 1−5) caused by interaction of thiols with phenanthraquinone play a main role in the oxidation of protein sulfhydryls, hydrogen peroxide produced as a byproduct of the reaction with sulfhydryls may partially contribute to the sulfhydryl oxidation as reported by others (*29*). The reactivity of phenanthraquinone with the dithiol group in preference to the monothiol group may reflect the lower redox potential of the dithiol as compared to monothiols (*30*).

Intracellular thiol oxidation (i.e., conversion of thiols to disulfides) can be reversed by the thioredoxin/thioredoxin reductase system in the presence of NADPH (*31*). These proteins have vicinal dithiol functions at the active center (*31*). Interestingly, using a partially purified protein preparation, we have found that thioredoxin activity was completely inhibited by 10 μM phenanthraquinone (Taguchi, K., et al., unpublished observations), suggesting that oxidation of the active dithiol group is caused by this quinone. This implies that dysfunction of thioredoxin by phenanthraquinone may cause not only reduction of antioxidant status but also alteration in the redox-dependent signal transduction (*14, 32*).

We have recently shown that phenanthraquinone is a potent inhibitor of endothelial nitric oxide synthase, and thus suppresses endothelium-dependent relaxation of rat aortas by acetylcholine and increases blood pressure in rats (*33*). It was also found that the inhibitory action of phenanthraquinone on endothelial nitric oxide synthase activity was, in part, suppressed by addition of DTT (*34*). It was reported that sulfhydryl groups in endothelial cells play a critical role in nitric oxide formation (*35*−*38*). Patel et al. (*39*) reported that modification of the thiol groups in the transporter for L-arginine was associated with a down-regulation of overall L-arginine transport into endothelial cells. Taken together, our data suggest that phenanthraquinone may alter the sulfhydryl status for both the L-arginine transporter and endothelial nitric oxide synthase, both of which will result in a loss of nitric oxide biosynthesis.

Our previous results indicate that quinones in DEP are good substrates for NADPH−cytochrome P450 reductase and that superoxide and hydroxyl radical generated during redox cycling of the quinones by this flavin enzyme participate in the DEP-promoted oxidative stress (*5*). Nel and co-workers (*3, 6, 7*) have proposed that such an overproduction of reactive oxygen species is associated with allergic inflammation and induction of apoptosis by DEP exposure. However, the chemicals associated with this toxicity remain uncharacterized. The study indicated that phenanthraquinone (a relatively abundant quinone in DEP) can act as a catalytic oxidizing agent for proximal protein sulfhydryls in addition to its ability to interact with NADPH−cytochrome P450 reductase, leading to the overproduction of reactive oxygen species (*5, 40*). This chemical reactivity indicates that phenanthraquinone may be, at least partially, responsible for DEP-induced oxidative stress.

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