Oxidative Stress More Strongly Induced by *ortho*- Than *para*-quinoid Polycyclic Aromatic Hydrocarbons in A549 Cells

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The effect of four *ortho*-quinoid polycyclic aromatic hydrocarbons (PAHs) and seven *para*-quinoid PAHs on the viability of A549 cells were examined. The *ortho*-quinoid PAHs [1,2-naphthoquinone (1,2-NQ), 9,10-phenanthrenquinone (9,10-PQ), 5,6-chrysenequinone (5,6-CQ), and benzo[*c*]phenanthrene-5,6-quinone (B[*c*]P-5,6-Q)] overproduced hydrogen peroxide (H_2O_2) without being consumed themselves. These *ortho*-quinoid had strong cytotoxic effects except for 1,2-NQ, since of its tendency to covalently bind to thiol groups. The cytotoxicity appears to be due to the overproduction of H_2O_2 by *ortho*-quinoid PAHs in a redox cycle coupled with the consumption of thiol group. In contrast, the *para*-quinoid PAHs were not as strong cytotoxic and did not produce H_2O_2.

Key words —— *ortho*-quinoid polycyclic aromatic hydrocarbon, hydrogen peroxide, redox cycle, oxidative stress, cytotoxicity

INTRODUCTION

Diesel exhaust (DE) compounds are the major constituents of air pollutants. Among them, polycyclic aromatic hydrocarbons (PAHs) and their derivatives are suspected to have several adverse human health effects. Several quinoid PAHs exist not only in DE and the atmosphere[1,2] but also in the human body.[3] One of the principal metabolic routes of PAHs is the formation of quinoid PAHs via dihydrodiol dehydrogenases.[3] The toxicities of quinoid PAHs have been extensively studied and several reviews are available.[4–7] Quinones can act as artificial electron acceptors and two general mechanisms are responsible for the toxic effects: alkylation of vital elements in the cell and redox cycling which produces reactive oxygen species (ROS). The latter has been characterized to induce oxidative stress through enzymatic and nonenzymatic redox cycling coupled with their corresponding semiquinone radicals.[3,8] There is growing evidence that ROS have adverse effects on DNA and essential macromolecules.[8–11]

Kumagai *et al.* demonstrated that 9,10-phenanthrenequinone (9,10-PQ) is redox-active which can catalyze the transfer of electrons from dithiol to oxygen, generating superoxide. The consumption rate of thiol groups was proportional to the concentration of the catalytically active redox-active species in the sample.[5] Although several quinoid PAHs have been detected in the environment as well as in animal bodies, the toxicity of other quinoid PAHs has not been studied.

Recently, we found that *ortho*-quinoid PAHs such as 5,6-chrysenequinone (5,6-CQ) produced ROS as much as 9,10-PQ and that they reduced the viability of A549 cells more strongly than *para*-quinoid PAHs. The goals of the present study are to test whether *ortho*-quinoid PAHs are more cytotoxic than *para*-quinoid PAHs to A549 cells and determine whether the activity is related PAH structure.

MATERIALS AND METHODS

Chemicals and Reagents —— 1,4-Naphthoquinone (1,4-NQ), and 9,10-PQ were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). 1,4-Anthraquinone (1,4-AQ), 1,4-phenanthrenequinone (1,4-

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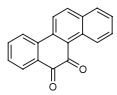
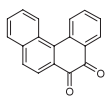
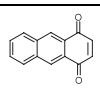
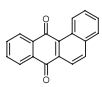
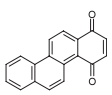
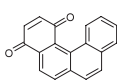
PQ), benzo[*c*]phenanthrene-1,4-quinone (B[*c*]P-1,4-Q), benzo[*c*]phenanthrene-5,6-quinone (B[*c*]P-5,6-Q) and 5,6-CQ were from Chiron AS (Trondheim, Norway). 9,10-Anthraquinone (AQ), 1,2-benzanthraquinone (1,2-BAQ) and 1,4-chrysenequinone (1,4-CQ) were from Wako Pure Chemical (Tokyo, Japan). 1,2-Naphthoquinone (1,2-NQ) was from Tokyo Chemical (Tokyo, Japan). The structures, configurations and molecular weights of quinoid PAHs tested in this study were indicated in Table 1 with their abbreviations. The stock solutions of the test compounds were maintained in dimethylsulfoxide (Wako Pure Chemical) solutions. All other chemicals used were obtained from commercial sources and were of the highest grade available.

Cell and Culture Conditions —— Human pulmonary epithelial A549 cells were obtained from Riken Gene Bank (Tsukuba, Japan). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% feral bovine serum (Sigma-Aldrich) in humidified 5% CO_2 at 37°C containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin (Wako Pure Chemical) and 2.5 mg/l amphotericin B (Sigma-Aldrich). For all experiments, cells were used at the exponential phase and plated on 100 mm culture dishes, and cultured at 70–80% confluence. Cells were allowed to adhere to the dish overnight, and then the culture medium was replaced with fresh DMEM (serum free or 1%) with or without test compounds as indicated.

Table 1. Structures of Quinoid PAHs Tested

Compound		M.W.	Structure
Form configuration	Abbreviation		
ortho-quinoid PAH	1,2-NQ	158.16	
	9,10-PQ	208.22	
	5,6-CQ	258.28	
	B[*c*]P-5,6-Q	258.28	
para-quinoid PAH	1,4-NQ	158.16	
	1,4-AQ	208.22	
	9,10-AQ	208.22	
	1,4-PQ	208.22	
	1,2-BAQ	258.28	
	1,4-CQ	258.28	
	B[*c*]P-1,4-Q	258.28	

Cell viability Assay —— Cell survival was quantified by the colorimetric assay based on the conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Wako Pure Chemical) to MTT-formazan derivative (crystal) by mitochondrial enzymes in viable cells as previously described.[12] At least three independent experiments were performed. The cytotoxic effect of quinoid PAHs, on the other hand, was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

Measurement of H_2O_2 —— The concentration of H_2O_2 was determined by means of ferrous iron oxidation in xylenol orange (FOX assay) according to the procedure of Watanabe and Forman.[13] Cells in 96 well plates, pretreated with 250 μM buthione-[*R*, *S*]-sulfoximine (BSO) for 24 hr, for glutathione depletion and 3-amino-1,2,4-triazole (ATZ) for 1 hr, for catalase inactivation. After preincubation, cells were treated with each quinoid PAH for 1 hr, then washed with phosphate buffered saline (PBS) and incubated at 37°C with 50 μl of Krebs-Ringer phosphate buffer (pH 7.4) containing 5 mM glucose. After 30 min, 20 μl of the buffer was removed, mixed with 180 μl of FOX working reagent [100 μM xylenol orange, 4.4 mM butylated hydroxytoluene, 250 μM $Fe(NH_4)_2(SO_4)_2$, 25 mM H_2SO_4 in 90% methanol], and incubated for 20 min at room temperature, and the absorbance at 540 nm was read in a plate reader. The H_2O_2 concentration was assessed from an authentic H_2O_2 standard curve (μM).

Measurement of Thiol Group [Dithiothreitol (DTT) Assay] —— An aliquot (0.5 ml) of the test solution was mixed with 1 ml of 0.4 M Tris-HCl (pH 8.9)/20 mM EDTA (ethylenediaminetetraacetic acid disodium salt) and 25 μl of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Wako Pure Chemical). Absorbance of each resulting mixture was measured at 412 nm against a blank solution to determine the content of the thiol group.

Measurement of Quinoid PAHs —— An aliquot (0.5 ml) of the incubation mixture was mixed with 10% trichloroacetic acid (Wako Pure Chemical, 0.5 ml), and then an aliquot (20 μl) of the solution was applied to a Shimadzu HPLC system equipped with a SPD-10AV UV-VIS spectrophotometric detector (Kyoto, Japan). Quinoid PAHs were separately eluted from an ODS-P column (4.6 × 250 mm i.d., 5 μm particle size, GL Science, Inertsil, Tokyo, Japan) using water/acetonitrile (3 : 7, v/v) as the mobile at a flow rate of 1 ml/min, and then detected at 254 nm.

RESULTS AND DISCUSSION

Airborne particulates have been shown to produce ROS such as H_2O_2.[1, 14–16] One possibility is that ROS are produced by quinoid PAHs, because quinoid PAHs, being electrophilic, attack nucleophiles, resulting in thiol adduct formation, and redox cycling, in which a rapid and sequential reduction of quinone leads to production of ROS.[5, 17] Two mechanisms have been proposed for the reaction of quinone with thiol groups. In one mechanism, the quinoe covalently binds to a protein thiol and in the other, redox cycling leads to overproduction of ROS.[5] It is essential to clarify which kinds of quinoid PAHs have such cytotoxic activities. This report compared the activity of four *ortho*-quinoid

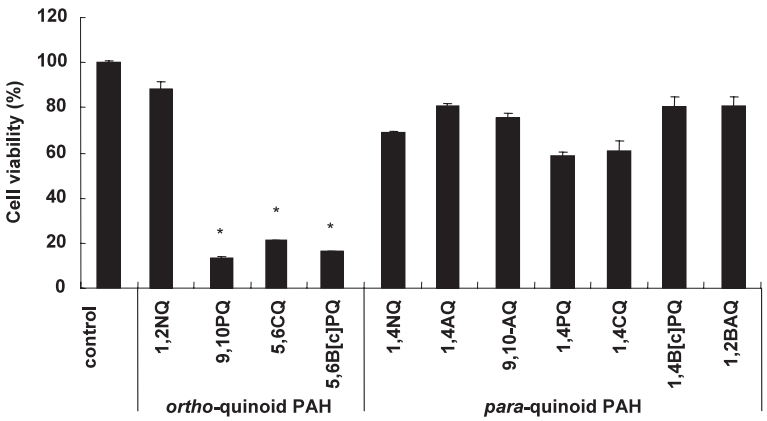


Fig. 1. Cellular Toxicity of Quinoid PAHs

A549 cells were incubated with 10 μm quinoid PAH for 60 min. The viability of the cells was determined by MTT assay. Each value is the mean ± S.D. of three determinations.

PAHs and seven *para*-quinoid PAHs.

A549 cells were treated with each of the quinoid PAHs at a concentration of 10 μM for 12 hr. Three of the *ortho*-quinoid PAHs (9,10-PQ, 5,6-CQ and B[*c*]P-5,6-Q) significantly reduced the viability of A549 cells to about 20% of the control, but *para*-quinoid PAHs had little effect on viability (Fig. 1). Although the toxicity of 1,2-NQ, which is an *ortho*-quinoid PAH, was weaker at 12 hr, the loss of viability was as large as the loss of viability caused by the other *ortho*-quinoid PAHs after the treatment for 24 hr (data not shown). This result suggests that *ortho*-quinoid PAHs are strongly cytotoxic.

The formation of H_2O_2 in the incubation mixture of A549 cells and 10 μM of each quinoid PAH was measured. Among quinoid PAHs tested, only the *ortho*-quinoid PAHs (1,2-NQ, 9,10-PQ, 5,6-CQ and B[*c*]P-5,6-Q) generated a significant amount of H_2O_2 (at least twice the amount generated by tthe control). The H_2O_2 levels in the presence of *para*-quinoid PAHs were at the same level as the control (Fig. 2). When A549 cells were treated with various doses of the four *ortho*-quinoid PAHs for 24 hr, the protein carbonyl content increased dose-dependently compared with untreated control (data not shown).

When the mixture of 10 nmol quinoid PAH and 100 nmol DTT in potassium phosphate buffer (pH 7.5) was incubated for 60 min, the four *ortho*-quinoid PAHs consumed almost 100% of the thiol groups. The *ortho*-quinoid PAHs consumed much more of the thiol groups than the by *para*-quinoid PAHs (Table 2). Even though the initial concentration of thiol groups (DTT) was ten times higher than that of quinoid PAHs, the *ortho*-quinoid PAHs were able to consume almost all of the thiol groups. Af-

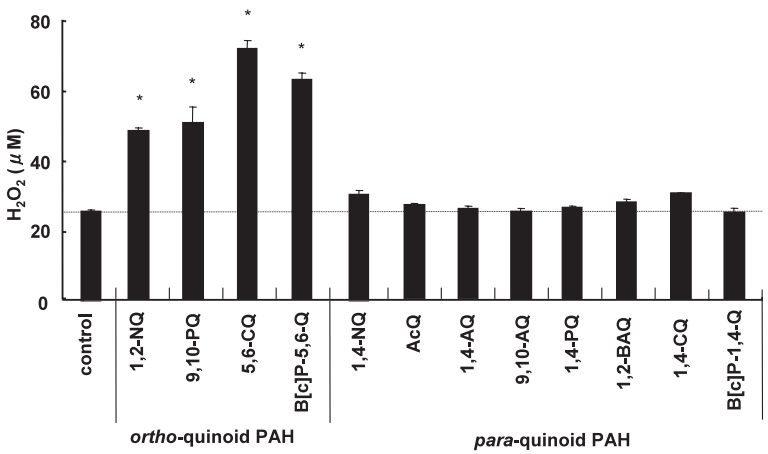


Fig. 2. Different Effect of *ortho*- and *para*-quinoid PAHs on H_2O_2 Production in A549 Cells

A549 cells pre-incubated with BSO and ATZ were incubated with 10 μM quinoid PAH at 37°C for 1 hr. H_2O_2 levels were determined by FOX assay. Each value is the mean ± S.D. of three determinations. Statistical significance, $^*p < 0.001$ *vs.* control.

Table 2. Consumption of thiol group and quinoid PAHs in DTT assay

Compound		Percent of -SH group	Percent of quinoid PAH
Form configuration	Abbreviation	remaining after 60 min	remaining after 60 min
ortho-quinoid PAH	1,2-NQ	0 ± 1.7	6.4 ± 3.0
	9,10-PQ	0 ± 1.0	98.7 ± 4.0
	5,6-CQ	0 ± 0.8	99.8 ± 0.3
	B[*c*]P-5,6-Q	0 ± 1.8	93.3 ± 9.6
para-quinoid PAH	1,4-NQ	2.1 ± 1.4	< 0.6
	1,4-AQ	33.5 ± 4.4	1.2 ± 0.3
	9,10-AQ	87.0 ± 5.6	90.8 ± 5.9
	1,4-PQ	60.1 ± 7.9	58.0 ± 8.5
	1,2-BAQ	73.5 ± 1.9	95.3 ± 4.4
	1,4-CQ	49.3 ± 6.9	35.4 ± 3.1
	B[*c*]P-1,4-Q	43.6 ± 6.9	27.7 ± 8.5

ter the incubation, the concentrations of all but one of the *ortho*-quinoid PAHs remained at more than 93% of the initial concentration, the exception being 1,2-NQ whoseconcentration dropped to 6.4% of the initial concentration. The large consumption of 1,2-NQ suggests that 1,2-NQ has a tendency to covalently bind to thiol groups. This may explain the weaker cytotoxicity of 1,2-NQ described in Fig. 2. On the contrary, *para*-quinoid PAHs, 1,4-NQ, 9,10-AQ, 1,4-AQ, 1,4-PQ, 1,2-BAQ, 1,4-CQ, and B[*c*]P-1,4-Q, did not consume thiol groups so much after the incubation (Table 2). Although the consumption of thiol groups was large in the presence of 1,4-NQ, the rate of consumption was much less than the rate of consumption in the presence of 1,2-NQ.

The above results suggest that *ortho*-quinoid PAHs form a redox cycle by consumption of thiol groups, and induce a cytotoxic effect through oxidative stress by overproduction of H_2O_2 as shown in Fig. 3. The thiol group on the side chain of cysteine residues is very sensitive to redox reactions and is a redox sensor.[11, 18] Thiol groups irreversibly change to oxidized forms such as disulfide bonds. Once thiol disulfides are formed, they interfere with biological functions. ROS have been thought to cause severe oxidative stress leading to aging, carcinogenesis, chronic inflammatory processes, and acute symptomatic responses in the respiratory tract.[2] Taken together, the above results suggest that ROS overproduced by *ortho*-quinoid PAHs modify protein structure through destruction of the sulfhydryl moiety.[19, 20]

Several *ortho*-qunoid PAHs such as 9,10-PQ and 9,10-AQ are present in the atmosphere at the

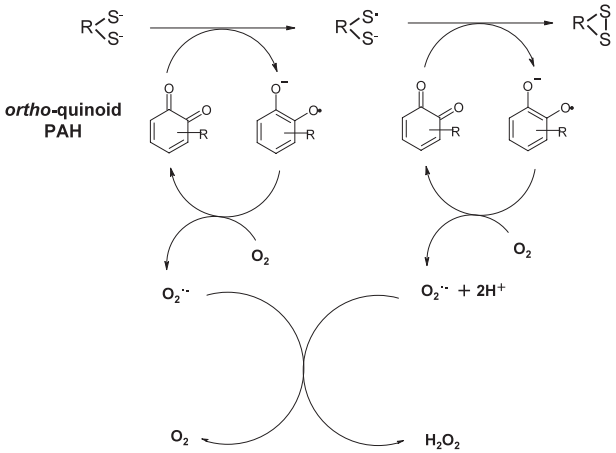


Fig. 3. Redox Cycle for Overproducing H_2O_2 by *ortho*-quinoid PAH

concentration range from 20 to 730 pg m^{-3}.[1, 2, 17] The concentration ratios of 1,2-NQ (*ortho*-type) to 1,4-NQ (*para*-type) in the atmosphere, gasoline- and diesel-engine exhausts were respectively 0.25, 1.17 and 0.09, although the concentration ratio of *ortho*-type to *para*-type was not reported for the other quinoid PAHs.[21] These facts suggest the other *ortho*-quinoid PAHs might also exist in the atmosphere.[22] Moreover, when PAHs are inhaled bt the human body, they are converted to their corresponding quinones by cytochrome P450 1A1, epoxide hydrolase and dihydrodiol dehydrogenase in the endoplasmic reticulum.[3, 8] These *ortho*-quinoid PAHs possibly act as oxidizing agents to cause pulmonary dysfunctional diseases.[23–25]

This is the first report that *ortho*-quinoid PAHs are more cytotoxic than *para*-quinoid PAHs and this effect is caused by overproduction of ROS through the selective formation of redox cycle. The detail mechanism underlying this difference is now studied.

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