— **Minireview** —

Polycyclic Aromatic Hydrocarbon Quinones as Redox and Electrophilic Chemicals Contaminated in the Atmosphere

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Polycyclic aromatic hydrocarbon quinones (PAHQs) produced by combustion of gasoline exhibit two chemical characteristics; one is their electron transfer abilitiy, transferring electrons from reducing agents to molecular oxygen to generate reactive oxygen species (ROS) associated with oxidative stress and the other is their ability to arylate cellular proteins, resulting in the disruption of signal transduction pathways. This review summarizes toxicological and pharmacological significances of such envirnmental chemicals through redox cycling and covalent modification.

Key words —— quinone, oxidative stress, redox cycling, electrophile, covalent modification, diesel exhaust particles

INTRODUCTION

Diesel exhaust particles (DEP) containing numerous compounds are a major component of particulate matter in ambient air. They contain aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), heterocyclics and a variety of metals. Their small size allows ready access to the lungs, which raises health concerns related to pulmonary cancer, allergy, asthma and cardiopulmonary diseases. There is little doubt that the deleterious health effects of DEP are associated with oxidative stress, characterized by the presence of unusually high concentrations of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radical. For example, Sagai *et al.*[1] reported that intratracheal injection of DEP into mice caused lung edema formation that was significantly blocked by pretreatment with polyethylene glycol-modified superoxide dismutase (SOD), an enzyme scavenging superoxide that readily enters cells. A subsequent study revealed that DEP exposure resulted in formation in murine lung of 8-hydroxydeoxyguanosine, which is produced by hydroxyl radical derived from superoxide.[2] Since antioxidant enzymes are extensively expressed in mammalian tissues, we thought that superoxide might be enzymatically and continuously generated from some of DEP components and therefore overwhelm defenses. Our rationale was that if redox active chemicals such as quinones contained in DEP are efficient substrate for NADPH cytochrome P450 reductase (P450R), there would be a reaction consisting of extensive NADPH oxidation and concomitant production of superoxide with a stoichiometric ratio of 1 : 2 without consumption of the quinoid chemicals during redox cycling catalyzed by this flavoprotein. In 1997, we proposed for the first time that polycyclic aromatic hydrocarbon quinones (PAHQs) are potential constituent responsible for catalytic generation of ROS, resulting in oxidative stress through P450R-catalyzed redox cycling.[3] As part of an extended collaboration study with Southern California Particle Center (SCPC) of air samples from sites in the Los Angeles Basin, we developed a method for quantitative determination of PAHQs as their diacetoxy derivatives in DEP and atmospheric paticulate matter (PM)2.5 collected in Riverside, California. Using this assay, we reported that 9,10-phenanthraquinone (9,10-PQ, 24 μg/g DEP) and 1,2-naphthoquinone (1,2-NQ, 14 μg/g DEP) are present in DEP and

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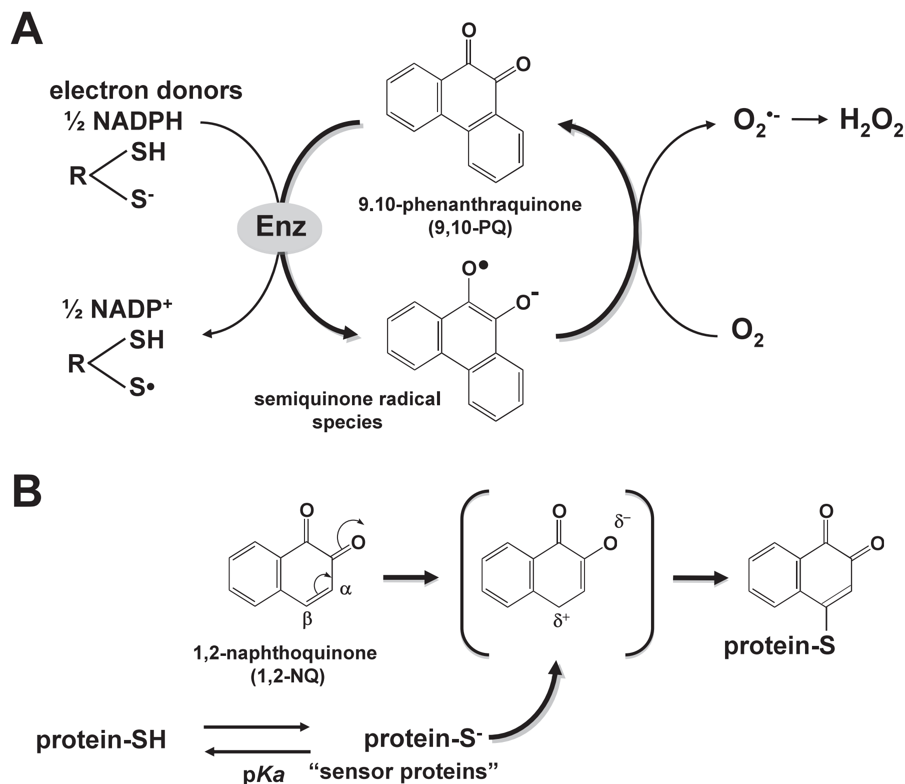

Fig. 1. Chemical Properties of PAHQs
A, redox cycling; B, covalent modification; Enz, flavoproteins such as P450R and NOS.

that these PAHQs were found at significant concentrations in the $PM_{2.5}$.[4] It is well recognized that PAHQs are capable of two reactions: one is electrophilic attack to nucleophiles such as protein thiols, resulting in protein adduct formation, and the other is redox cycling, in which the quinone undergoes reduction and oxidation with the generation of ROS.[5] Our findings have indicated that 9,10-PQ is classified as capable of redox cycle reaction and 1,2-NQ is capable of both protein moditication and redox cycle reaction.

9,10-PQ

Dysfunction of Nitric Oxide-dependent Vascular Tone

9,10-PQ is thought to be generated by photooxidation and metabolic activation of phananthrene. With purified cytochrome P450R, it was shown that 9,10-PQ readily undergoes one-electron reduction by this enzyme in the presence of NADPH and thus produces superoxide[3] as shown in Fig. 1A. Next, we hypothesized that 9,10-PQ could affect nitric oxide (NO) production catalyzed by NO synthase (NOS) because NOS isozymes all consist of an *N*-terminal oxygenase domain and a *C*-terminal reductase domain, which is highly homologous with P450R; this domain is capable of transferring electrons from NADPH to artificial acceptor molecules. Consistent with this notion, we found that 9,10-PQ, with a one-electron reduction potential value of −124 mV, interacts with the P450R domain on neuronal NOS, and thus inhibits NO formation by shunting electrons away from its normal catalytic pathway.[6]

Epidemiologic examinations suggested that exposure of humans to ambient particulate matter is associated with an increased risk of cardiopulmonary-related diseases and mortality. It was reported that incubation of rat aortic rings with suspension of DEP suppressed endothelium-dependent vasorelaxation caused by acetylcholine, suggesting that DEP component such as 9,10-PQ could contribute to an impairment of NO-dependent vasorelaxation. Experiments with an enzyme preparation of bovine aortic endothelial cells (BAEC) *in vitro*, arotic rings of rats *ex vivo*, and rats *in vivo*, we found that 9,10-PQ inhibits endothelial NOS activity through an uncoupling reaction on the

electron transport of this enzyme as demonstrated with purified neuronal NOS,[6] thereby disrupting NO-dependent vascular tones such as endothelium-dependent vasorelaxation by acetylcholine and blood pressure controlled by NO.[7] Taken together, our findings suggest that 9,10-PQ participates, at least in part, in the endothelial dysfunction caused by DEP.

Redox Cycling with Dithiol

Our preliminary study, showed that the concentration-dependent inhibition of endothelial NOS activity by 9,10-PQ was partially blocked by dithiothreitol (DTT), but not glutathione (GSH),[8] suggesting that there is an alternative mechanism of endothelial NOS inhibition exists whereby 9,10-PQ catalyzes the modification of presumably proximal thiols required to maintain the enzyme activity because thiol groups in endothelial NOS were reported to be essential for maximal catalytic activity. In fact, we found that 9,10-PQ selectively interacts with dithiols such as those on DTT, resulting in the oxidation of thiol group through a thiyl radical intermediate and the reduction of molecular oxygen to superoxide and hydrogen peroxide (see Fig. 1A), whereas no reaction of 9,10-PQ with monothiol such as GSH even at 10 mM was observed.[9] Although thiol group of DTT was completely consumed 30 min after the reaction during redox cycling of 9,10-PQ (1 nmol) with DTT (100 nmol) without flavoproteins such as P450R, no change in 9,10-PQ level in the reaction mixture, implying that 9,10-PQ can act as a pure redox active PAHQ without its covalent modification to thiol. Such a phenomenon was supported by recent observation that a redox cycling reaction of not only 9,10-PQ but also 1,2-NQ, 5,6-chrysenequinone and benzo[*c*]phenanthrene-5,6-quinone with DTT occurs;[10] interestingly, they have shown that little interaction of seven *para*-PAHQs with DTT was seen.

Biotransformation Associated with the Initiation and Termination of Oxidative Stress

Exposure of human pulmonary epithelial A549 cells to 9,10-PQ resulted in the cellular protein oxidation and subsequent apoptotic cell death.[11] In reductive activation of 9,10-PQ to generate ROS, there are two reduced 9,10-PQ species of biological importance, its semiquinone radical ($9,10\text{-PQ}^{\cdot-}$) and its hydroquinone (9,10-dihydroxyphenanthrene, $9,10\text{-PQH}_2$). Although detoxification of quinones is generally thought to be two-electron reduction forming hydroquinones, we found that 9,10-PQ form disproportionately with $9,10\text{-PQH}_2$ formed by aldo-keto reductase (AKR) isozymes, producing $9,10\text{-PQ}^{\cdot-}$ which reacts easily with molecular oxygen to yield superoxide.[12] Results of exposure of A549 cells to 9,10-diacetoxyphenanthrene as a precursor of $9,10\text{-PQH}_2$ indicated that $9,10\text{-PQH}_2$ plays an important role in the protein oxidation and cellular toxicity of 9,10-PQ, showing that two-electron reduction of 9,10-PQ can also initiate redox cycling to cause cellular oxidative stress.[12] However, we also found that $9,10\text{-PQH}_2$ undergoes glucuronidation by UDP-glucuronosyltransferase (UGT) isozymes to yield its monoglucuronide (PQHG) and thus is excreted into extracellular space during exposure of A549 cells to 9,10-PQ.[13] In cell-free systems, 9,10-PQ exhibited a rapid thiol oxidation and subsequent oxygen consumption in the presence of DTT, whereas PQHG did not. Unlike 9,10-PQ and $9,10\text{-PQH}_2$, PQHG completely lost the ability to oxidize cellular protein and cause cell death in A549 cells.[12,13] Overall, we conclude that PQHG is a metabolite of 9,10-PQ, produced through $9,10\text{-PQH}_2$ that terminates its redox cycling and transports it to extracellular space. Thus, although $9,10\text{-PQH}_2$ is still a redox-active metabolite, it is postulated that two-electron reduction of 9,10-PQ to $9,10\text{-PQH}_2$ is an obligatory pathway for biotransformation of PQHG to lose redox activity involving in the oxidative cell damage.

Cellular Protection by Nrf2

Nuclear factor erythroid 2-related factor 2 (Nrf2) which is negatively regulated by the Kelch-like ECH-associated protein 1 (Keap1) is known to be a transcription factor that coordinately regulates the down-stream genes (*e.g.*, phase II xenobiotic metabolizing enzymes and phase III transporters) through antioxidant-responsive element.[14] Because some of Nrf2 target proteins are responsible for metabolism and extracellular excretion of 9,10-PQ, we thought that deletion of Nrf2 would affect the biotransformation of $9,10\text{-PQH}_2$ and PQHG from 9,10-PQ and thus cytotoxicity of the PAHQ. With primary hepatocytes and enzyme preparations from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice, we found that Nrf2 does indeed play a role in cellular protection against 9,10-PQ through upregulation of some of down-stream genes such as AKRs, NADPH:quinone oxidoreductase 1 (NQO1), UGTs and multidrug

resistance-associated proteins (MRPs).[13)]

Induction of Inflammation *In vivo*

A single injection of 9,10-PQ (1 μg/body) into mice by intratracheal instillation caused a significant induction of the pulmonary expression of interleukin (IL)-5 and eotaxin.[15)]

Under these conditions, an increase in the numbers of neutrophiles and eosinophiles in bronchoalveolar lavage fluid (BALF) as compared to vehicle challenge, suggesting that intratracheal exposure of mice to 9,10-PQ induces recruitment of inflammatory cells, at least in part, via the local expression of these cytokines. Moreover, we found that in the presence of ovalbumin (OVA), repeated injection of 9,10-PQ (2.1 ng/body) into mice by intratracheal administration significantly increased the numbers of eosinophiles and mononuclear cells in BALF as compared with OVA alone.[16)] These observations suggest that 9,10-PQ appears to be partially associated with the DEP toxicity on the allergic airway inflammation.

1,2-NQ

Redox Cycle-catalyzed Suppression of No-dependent Vasorelaxation

Our previous observations indicated that like 9,10-PQ, 1,2-NQ is a potent inhibitor for neuronal NOS with an IC_{50} value of 12 μM as well[6)] and that 9,10-PQ disrupts NO-dependent vascular tone,[7)] suggesting that 12-NQ could affect vasorelaxation controlled by NO. We found that dissolved oxygen is rapidly consumed during interaction of DTT with 1,2-NQ, but not *trans*-1,2-dihydroxy-1,2-dihydronaphthalene, which is not redox active.[17)] Consistent with its redox capability, 1,2-NQ caused a significant inhibition of endothelial NOS activity by the membrane fraction of BAEC and suppression of acetylcholine-induced endothelium-dependent vasorelaxation in aortic rings of arts, whereas *trans*-1,2-dihydroxy-1,2-dihydronaphthalene had little effect on the NO biochemistry and pharmacology.[17)] Thus, these findings suggest that 1,2-NQ inhibits endothelial NOS activity through redox cycle-based uncoupling reaction, thereby disrupting NO-dependent vasorelaxation.

Disruption of Signal Transduction Through Covalent Modification

Naphthalene is reported to be biotransformed to 1,2-NQ by cytochrome P450, followed by epoxide hydrolase, AKR isozymes and autooxidation. Our preliminary study revealed that negligible levels of 1,2-NQ were detected in the supernatant of the reaction mixture after incubation of naphthalene with the $9000 \times g$ supernatant of rat liver in the presence of NAD(P) and NAD(P)H, suggesting that most of 1,2-NQ produced from naphthalene is rapidly bound to proteins in the enzyme preparation. To address this issue, we prepared a specific antibody against 1,2-NQ.[18)] This antibody can recognize 1,2-NQ, 1,2-dihydroxynaphthalene, 1,2-NQ-4-mercaptoethanol adduct and 1,2-NQ-4-sulfonate, but not 1,4-NQ, and found that there were cellular proteins modified by 1,2-NQ. This suggests that 1,2-NQ exhibits not only redox but also electrophilic characteristics. As shown in Fig. 1B, 1,2-NQ is a typical Micheal acceptor that reacts covalently with protein thiolate ions to form a stable protein adduct through a C-S bond. Jones[19)] has recently proposed that while there are about 214000 cysteine (Cys) unique residues encoded in the human genome, with perhaps 80–90% of protein thiols relatively inert to redox reaction; 1000–42000 Cys may be present as thiolate ions that readily undergo oxidation. We speculate, therefore, that such proteins are targets for 1,2-NQ.

During a screening study to determine whether PAHs and their quinones could affect tracheal tension of guinea pig ring preparation, we observed that PAHQs which have an ability to covalently bind to protein caused tracheal contraction of guinea pig rings. With 1,2-NQ as a model PAHQ, we found that 1,2-NQ activates a phospholipase A2/lipoxygenase/vaniloid receptor signaling pathway, resulting in increased intracellular calcium content in the smooth muscle cells associated with a contraction of guinea pig tracha.[20)] Interestingly, tracheal contraction of guinea pig caused by 1,2-NQ was significantly blocked by pretreatment with either genistein (protein tyrosine kinase inhibitor) or PD153035 [epidermal growth factor receptor (EGFR) inhibitor],[20)] suggesting a tracheal contraction coupled to EGFR activation during exposure to 1,2-NQ. As asthma is of one of the major adverse health effects of airborne particulate matter, trachea contraction caused by 1,2-NQ through EGFR phosphorylation is of relevance in the general toxicology of airborne particulate matter.

For understanding of mechanistic detail for 1,2-NQ-mediated phosphorylation of EGFR, we used with human epithelial A431 cells because of high expression of EGFR. Since the phosphorylation of protein tyrosine kinases such as EGFR is reported to be negatively regulated by protein tyrosine phosphatases (PTPs),[21] we thought that if reactive thiols of PTPs undergo covalent modification by 1,2-NQ, irreversible inactivation of PTPs, resulting in prolonged activation of EGFR, would occur. As expected, a concentration-dependent phosphorylation occurring during exposure of A431 cells to 1,2-NQ was found to be coupled to the reduction of PTP activity in the cells; under these conditions, 1,2-NQ did bind to PTP1B among cellular PTPs.[18] In cell-free systems, matrix-assisted laser desorption and ionization time-of flight mass spectrometry (MALDI-TOF/MS) analysis revealed that 1,2-NQ is covalently bound to Cys121, thereby causing reduction of the catalytic activity. Thus, we concluded that covalent modification of 1,2-NQ to PTP1B is at least partially responsible for the reduction of PTP activity, which leads to prolonged phosphorylation of EGFR in A431 cells.[18] Furthermore, we found that cAMP response element-binding protein (CREB), a transcription factor with conserved cysteine residues that regulate DNA binding is also a molecular target for 1,2-NQ and that covalent modification by 1,2-NQ results in suppression of the DNA binding activity and substantially down-regulates expression of CREB-regulated protein such as Bcl-2 in BAEC.[22] Thus, it seems likely that modification of kinases-regulated protein or transcription factor by 1,2-NQ is associated with alteration in signal transduction pathways controlled by these target proteins.

Initial Response and Cellular Protection by Nrf2

Because Nrf2 is known as a transcription factor for cellular response and adaptation against electrophiles,[14] we examined effect of Nrf2 deletion on chemical modification of proteins and cytotoxicity during exposure to 1,2-NQ (Miura *et al.*, unpublished observations). Studies with primary mouse hepatocytes from $Nrf2^{+/+}$ and $Nrf2^{-/-}$ mice suggested that deletion of Nrf2 enhanced 1,2-meditaed covalent attachment of the cellular proteins through thiolate ions and cellular damage. It is postulated that there are at least two detoxification pathways of 1,2-NQ; one is GSH conjugation and the other is two-reduction reduction, followed by glucuronidation as reported with 9,10-PQ.[12] Proteins [*e.g.*, GSH S-transferases (GSTs), NQO1, AKRs and UGTs] responsible for these reactions and MRP transporters for export of polar metabolites are regulated by Nrf2, their upregulation plays a crucial role in the decline of 1,2-NQ cytotoxicity. Recently, studies with zebrafish revealed that the Cys151 of Keap1, a negative regulator for Nrf2, is easily modified by 1,2-NQ, resulting in Nrf2 activation.[23] Similar results were obtained with purified mouse Keap1 and mouse primary hepatocytes (Miura *et al.*, unpublished observation). Thus, we speculate that activation of Nrf2 following exposure to exogenous electrophile such as 1,2-NQ provides an initial protective response to a reactive chemical.

Table 1. Biological and Pharmacological Effects of 9,10-PQ and 1,2-NQ as Redox Active and Electrophilic Chemicals

PAHQ examined	biological and pharmacological effects	ref.
9,10-PQ	Inhibition of NO formation catalyzed by NOS	6, 8)
	Disruption of NO-dependent vascular tone	7)
	Redox cycling with dithiols	9)
	Oxidative stress through one- and two-electron reductions	11, 12)
	Glucuronidation as a metabolic pathway to terminate a redox cycling to generate ROS	13)
	Cellular protection against oxidative stress through Nrf2/Keap1 system	13)
	Inflammation of mouse lung by intratracheal injection	15, 16)
1,2-NQ	Disruption of NO-dependent vascular tone	17)
	Tracheal contraction through EGFR phosphorylation	20)
	EGFR phosphorylation coupled to covalent binding to PTP1B through Cys121	18)
	Covalent modification of CREB, resulting in inhibition of the function of this transcription factor	22)
	Activation of Nrf2 through chemical modification of Keap1	23)
	Inflammation and up-regulation of MUC5AC in mouse Lung during intratracheal exposure	24, 25)

Inflammation and Involvment of Asthma *In vivo*

Pulmonary exposure of mice to 1,2-NQ (1.58–158 ng/body) dose-dependently aggravated antigen-related airway inflammation, as characterized by infiltration of eosinophils and lymphocytes around the airways and an increase in goblet cells in the bronchial epithelium.[24] It was also found that combined exposure to 1,2-NQ and OVA as antigen enhanced the local expression of a variety of cytokines, compared with intratracheal exposure of mice to OVA or 1,2-NQ alone.[25] Moreover, we found that 1,2-NQ is also able to enhance airway hyperresponsiveness in the absence or presence of OVA and that amplified lung expression of IL-13 and MUC5AC might partially participates in the deterioration of asthma feature caused by 1,2-NQ.[25]

Development of Assay for Determination of Redox Active and Electrophilic Component Present in Atmospheric Samples

On the basis of the redox reaction with 9,10-PQ, we developed an assay for ambient particulate matter (PM)-redox activity, utilizing the reduction of molecular oxygen by DTT, which serves as an electron donor.[26] Using this assay, it was found that PM fractions collected in different sites in the Los Angeles Basin catalyze the oxidation of DTT.

Accumulated findings with 1,2-NQ let us to a hypothesis that there are constituents with electrophilic properties other than 1,2-NQ in atmospheric samples. A collaborative study with the SCPC developed a procedure that provides a quantitative measure of electrophiles contaminated in samples of PM collected in the Los Angeles Basin and in DEP.[27] Recently, we have established a convenient assay consisting of modification of sensor proteins by electrophile as determined by MALDI-TOF/MS analysis and an initial response against ambient samples as evaluated by Nrf2/Keap1 system (Iwamoto *et al.*, unpublished observation).

CONCLUSION

Our findings, described here are summarized in Table 1. 9,10-PQ with redox activity causes oxidative stress as measured by generation of ROS, cellular protein oxidation, apoptotic cell death through redox cycling with cellular electron donors. Although 1,2-NQ is also a good electron acceptor for redox cycling, this quinone causes arylation of cellular protein with thiolate ions (termed as sensor proteins) though covalent attachment, resulting in disruption of signal transduction pathways. However, Nrf2/Keap1 system acts as a factor for cellular protection against such reactive chemicals promoting oxidative stress and covalent modification in the cells. It is no doubt that unidentified components with redox active and electrophilic properties are present in the atmosphere and thus may be associated with the adverse effects of PM.

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