— **Minireview** —

Induction of Oxidative Stress and Dysfunction of Nitric Oxide-Dependent Vascular Tone Caused by Quinones Contained in Diesel Exhaust Particles

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(Received June 19, 2001)

This article reviews studies of overproduction of reactive oxygen species during reductive activation of quinones contained in diesel exhaust particles. The resulting inhibition of nitric oxide synthase activity causing suppression of vasorelaxation by phenanthraquinone, one of the quinone in diesel exhaust particles, is also reviewed.

Key words —— diesel exhaust particles, quinone, nitric oxide, reactive oxygen species, endothelial dysfunction

Diesel exhaust particles (DEP) are a major constituent of ambient particulate matter. The small size of DEP makes them easily respirable, which raises health concerns regarding their effects on lung cancer, allergy, asthma *etc*.[1,2] DEP contain a large variety of compounds, such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons and heterocyclics.[3,4] It is well established that the metabolic activation of benzopyrene or nitropyrene, which are also contained in DEP, is associated with induction of lung cancer.[1] The current consensus is that reactive oxygen species (ROS) generated by DEP play an important role in these adverse effects.[5–9] For this reason, identification of the substances in DEP which are involved in the production of ROS is of interest for elucidating oxidative stress-dependent DEP toxicity.

Reductive Activation of Quinones in DEP, Leading to Overproduction of Reactive Oxygen Species

It was previously reported that formation of lung edema following intratracheal DEP injection into mice was markedly suppressed by pretreatment with polyethylene glycol-modified superoxide dismutase,[5] an enzyme which scavenge superoxide.[10] Nagashima *et al*.[11] reported that intratracheal exposure to DEP caused formation in the murine lung of 8-hydroxydeoxyguanosine, which is produced by hydroxyl radical,[12] in the lung. These observations strongly suggest that DEP exposure can generate ROS, leading to oxidative stress-dependent pulmonary damage. Although enhanced inflammation involving activation of alveolar macrophages following DEP exposure may also lead to generation of ROS indirectly,[13] a reasonable explanation is that DEP chemicals[5] and/or metals contaminating DEP[14] could directly produce ROS such as superoxide and hydroxyl radical. However, it is unlikely that the superoxide or hydroxyl radical, generated by DEP solely *via* chemical reactions, promotes 8-hydroxydeoxyguanosine production *in vivo* and induces lung toxicity because superoxide dismutase is extensively distributed in mammalian tissues.[10] Thus we explored the possibility that superoxide could be enzymatically and continuously generated from DEP and therefore overwhelm defenses.[15]

Most quinones can undergo one-electron reduction by NADPH-cytochrome P450 reductase (P450 reductase) in the presence of NADPH, resulting in overproduction of superoxide during the redox reaction.[16,17] Interestingly, a few reseach groups[3,4,7] including ours[15,18] have pointed out the presence of quinones with different structures in DEP. Our rationale was that if quinones contained

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in DEP are good substrate for P450 reductase, then there would be a reaction consisting of extensive NADPH oxidation and concomitant generation of superoxide with a stoichiometric ratio of 1 : 2 during redox cycling by this enzyme.

As expected, NADPH oxidation was stimulated during interaction of a methanol extract of DEP with a Triton N-101 treated microsomal preparation of mouse lung whereas the cytosolic fraction was less active. When purified P450 reductase was used as the source of enzyme, the turnover value was enhanced approximately 260-fold. P450 reductase effectively reduced DEP moieties (37.5 μg) extracted by methanol with a specific activity of 2348 nmol of NADPH oxidized/mg/min.[15] Under these conditions, superoxide was also generated with a specific activity of 3240 nmol/mg/min,[15] suggesting that one-electron reduction of presumably of quinones, does seem to occur. 9,10-Phenanthraquinone (PQ) was shown to be a relatively abundant quinone in DEP[4] and was found to be a good substrate for P450 reductase (specific activity, 14040 nmol/mg/min) in 20 mM hepes buffer (pH 7.6) at 25°C, whereas, 9,10-anthraquinone (AQ), another major quinone in DEP,[4] was a poor substrate for the enzyme.[19] This suggests that PQ, but not AQ, may be the main participant in the enzymatic superoxide formation caused by DEP. When 0.036 units of P450 reductase (corresponding 12% of the total activity of a mouse lung) were used, superoxide generation by methanol extract of DEP (37.5 μg) in the presence or absence of P450 reductase was 2.4 and 0.026 nmol, respectively.[15] This indicates that the contribution of chemical conversion to the DEP-mediated ROS production is negligible compared to P450 reductase-requiring reaction as indicated by superoxide assay. However, the DEP used did contain Fe (321 ppm) and Cu (321 ppm),[15] indicating that these metals (Fe^{2+}, Cu^{+}) reduced by superoxide could potentially react with hydrogen peroxide derived from superoxide, resulting in formation of a powerful oxidant hydroxyl radical. Consistent with this notion, electron spin resonance experiments revealed that, amongst other factors, hydroxyl radical was indeed formed during reduction of DEP substances by P450 reductase.[15] Taken together, we conclude that DEP components with quinoid structure, undergo one-electron reduction to yield semiquinone radical which, in turn, can generate hydroxyl radical species *via* a metal-catalyzed Haber-Weiss reaction[20] through the redox-based generation of superoxide. Therefore, it is likely that such an overproduction of ROS contributes to, at least in part, contribute to the lung edema formation and production of 8-hydroxydeoxyguanosine observed *in vivo* after exposure to DEP. A possible mechanism for P450 reductase-catalyzed metabolic activation of DEP moieties, presumably quinones, involved in the oxidative stress-dependent pulmonary toxixity is summarized in Fig. 1.

A further study indicated that chronic intratracheal administration of DEP to mice (once a week for 10 weeks) at a dose of 0.1 or 0.2 mg per animal caused a significant increase in P450 reductase ac-

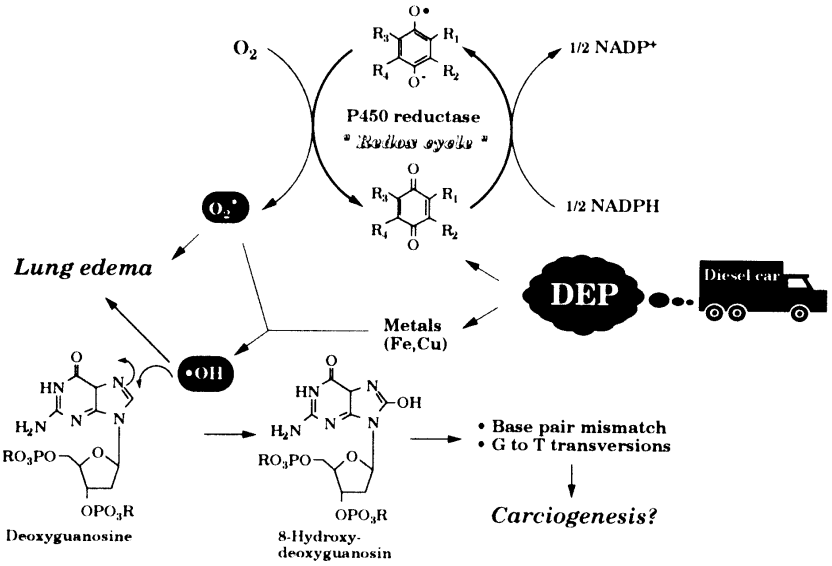

Fig. 1. P450 Reductase-Catalyzed Reductive Activation of DEP Quinones to Generate Reactive Oxygen Species

tivity and decrease in superoxide dismutase isozyme activity in the lung.[21] Thus the resulting overproduction of ROS may accelerate the development of the inflammation and hyperresponsiveness of the airway in mice following prolonged exposure to DEP.

Reductive Activation of PQ, Leading to Decreased Nitric Oxide Production

Nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS) and plays an important role in neurotransmission, vasorelaxation and immune response.[22] NOS isozymes all consist of an *N*-terminal oxygenase domain and a *C*-terminal reductase domain,[23] which is highly homologous with P450 reductase;[24] this domain is capable of transferring electrons from NADPH to artificial acceptor molecules.[23] Luo and Vincent[25] showed that the antineoplastic anthracyclines, doxorubicin and aclarubicin, which possess a quinone moiety in their structure, affected neuronal NOS (nNOS) activity in rat cerebellum. Thus, these findings led us to propose that quinoid compounds could interact with the P450 reductase domain on nNOS, resulting in a decrease in NO formation from L-arginine (because most quinones are able to accept electrons from NADPH during enzymatic reaction with P450 reductase).[16,17] Using rat cerebellar enzyme preparation, we demonstrated that the inhibition of NO formation by quinones which exhibit one-electron reduction potential ranging between –240 and –100 mV, increased at more positive one-electron reduction potential.[19] This suggested that quinone may act as an electron acceptor for nNOS. Among 22 quinones tested, PQ, corresponding to a one-electron reduction potential value of –124 mV, inhibited NO production most potently (IC_{50} value = 10 μM).[19] However, AQ with a one-electron reduction potential value of –348 mV had no effect on nNOS activity. A kinetic study revealed that PQ is a competitive inhibitor with respect to NADPH and a noncompetitive inhibitor with respect to L-arginine.[19] Purification of enzymes which are responsible for reducing PQ from 20000 × *g* supernatant of rat cerebellum by column chromatography indicated that one catalyst for PQ reduction was nNOS.[19] Specific activity of PQ reduction by purified nNOS was 6802 nmol of NADPH oxidized/mg/min, but this reaction required $CaCl_2$/calmodulin (CaM).[19] nNOS effectively reduced the 1,4-naphthoquinone and menadione as well as PQ, thereby causing a marked decrease in the production of NO from L-arginine. In contrast, 1,4-benzoquinone, AQ, mitomycin C and lapachol, which show negligible inhibitory effects on nNOS activity, were not reduced by the enzyme.[19] Taken together, we concluded that PQ interacts with the P450 reductase domain on nNOS, and thus inhibit NO formation by shunting electrons away from the normal catalytic pathway as shown in Fig. 2. The CaM-dependent one-electron reduction of quinones by nNOS has recently been supported by the results of experiments with the reductase domain.[26]

6-Anilino-5,8-quinolinedione is a benzoquinone derivative and has been widely used as an agent to reduce levels of NO-dependent intracellular cyclic GMP in tissues.[27] From our findings as described above, we postulated that the pharmacological action of 6-anilino-5,8-quinolinedione is, in part, due to inhibition of NOS, thereby reducing cyclic GMP levels. In fact, 6-anilino-5,8-quinolinedione inhibited NO formation by nNOS (IC_{50} value = 29 μM) while this quinone was effectively reduced by nNOS with a specific activity of 3419 nmol of NADPH

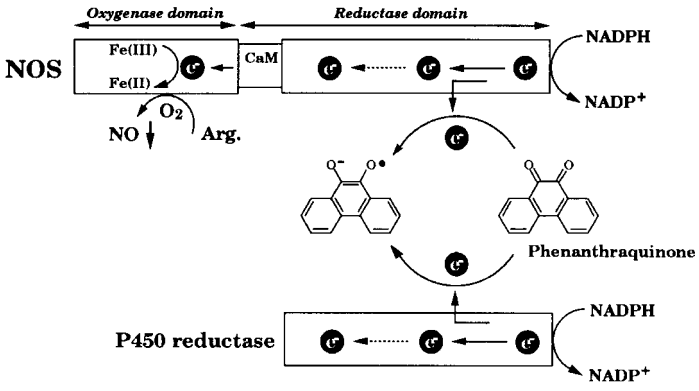

Fig. 2. One-Electron Reduction of Phenanthraquinone by NOS and P450 Reductase
Arg., L-arginine; CaM.

oxidized/mg/min (in the presence of $CaCl_2$/CaM) and P450 reductase with a specific activity of 831 nmol of NADPH oxidized/mg/min.[28] Under these conditions, enzymatic generation of superoxide during reduction of 6-Anilino-5,8-quinolinedione by nNOS and P450 reductase was confirmed by electron spin resonance experiments.[28] Superoxide reacts readily with NO, resulting in decreased cyclic GMP content.[22] Hence, we speculate that these flavin enzymes may also play an important role in the inactivation of NO by substances such as 6-anilino-5,8-quinolinedione, DEP quinones after these are reduced.

Epidemiologic studies have shown that exposure of humans to ambient particulate matter ia associated with an increased risk of cardiopulmonary-related diseases and mortality.[29–31] Bioassay studies *in vitro* also indicated that incubation of rat aortic rings with suspensions of DEP resulted in a suppression of endothelium-dependent vasorelaxation caused by acetylcholine.[32] These findings suggest that DEP components in urban air contribute to an impairment of vasorelaxation.

NO produced in endothelial cells is involved in the regulation of blood pressure, inhibition of platelet aggregation, inhibition of smooth muscle migration, and ischemic protection.[33] Reduction of NO formation by NOS inhibitors or disruption of the gene encoding endothelial NOS (eNOS) results in vasoconstriction and increase in blood pressure.[34,35] Impairment of NO production in the endothelium is implicated in the pathophysiology of vascular diseases.[36,37] Thus we hypothesized that PQ would inhibit the enzymatic activity not only of nNOS but also of eNOS, thereby altering NO formation, which could lead to suppression of eNOS-dependent vasorelaxation and increased blood pressure.

Using the total membrane fraction of bovine aortic endothelial cells as an eNOS enzyme preparation, it was found that PQ was a more potent inhibitor of eNOS than of nNOS,with an IC_{50} value of 0.6 μM (Fig. 3).[38] PQ (1 μM) inhibited eNOS activity by more than 80%. The inhibition characteristics of PQ on eNOS activity with respect to L-arginine and NADPH were almost the same as with nNOS enzyme preparations.[38] Alterations in the titration curve for acetylcholine-mediated vasodilation of rat aortic rings by PQ (5 μM) revealed significant suppression of the maximum response without changing the EC_{50} value (Fig. 3).[38] This suggests that the pharmacological action of PQ does not involve competition at the level of the rat aortic acetylcholine-receptor. In contrast, PQ did not affect the endothelium-independent relaxation caused by the NO-releasing agent nitroglycerine.[38] Therefore, we conclude that PQ binds to the P450 reductase domain of eNOS as well as nNOS, thereby inhibiting NO production by shunting the electron flow from NADPH. As a consequence, PQ appears to act as a modulators in the vasorelaxation caused by NO generated from constitutive NOS isozymes, but not by affecting NO itself. After an intraperitoneal injection of phenanthraquinone (0.36 mmol/kg) into rats, the blood pressure was quickly elevated (1.4-fold compared to the control level) to reach plateau levels (Fig. 3), and sustained for more than 30 min.[38] Interestingly, under these conditions, plasma levels of NO_2^-/NO_3^- in PQ-treated rats, which are indica-

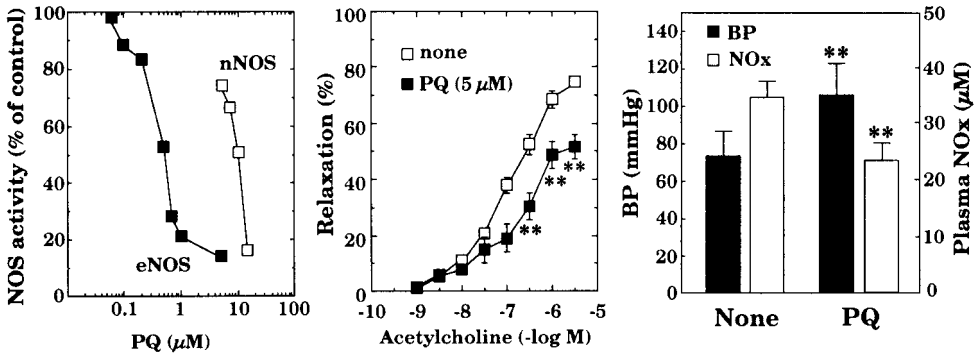

Fig. 3. Alterations in NOS Activities, Endothelium-Dependent Vasorelaxation of Rat Aorta by Acetylcholine, Mean Blood Pressure in Rat and Plasma NO_2^-/NO_3^- Levels in Rat by Phenanthraquinone

PQ, phenanthraquinone; BP, mean blood pressure; NOx, NO_2^-/NO_3^-. Left panel: total membrane fraction of bovine aortic endothelial cells and 20000 × *g* rat cerebellar supernatant fraction were used as enzyme preparations of eNOS and nNOS, respectively. Center panel: relaxations caused by acetylcholine in the absence and presence of PQ are expressed as percent decreases in tention from the concentration evoked by phenylephrine. **, $p < 0.01$ *vs.* control. Right panel: PQ (0.36 mmol/kg) was intraperitoneally administered to rats and then blood pressure was measured for 30 min. **, $p < 0.01$ compared with control.

tors of NO production *in vivo*,[39] were reduced to 68% of control levels (Fig. 3).[38] This further supports the possibility that the significant increase in blood pressure of rats following PQ exposure may be attributable to decreased production of NO, which regulates basal vascular tone, by inhibiting eNOS activity.

Our recent studies have demonstrated that PQ interacts readily with dithiol groups, but not monothiol groups, thereby oxidizing proximal protein sulfhydryls (*e.g.*, pulmonary microsomes from rats and total membrane preparations isolated from bovine aortic endothelial cells) (S. Koide *et al.*, unpublished observation). In this situation, formation of thiyl radical intermediates and generation of ROS such as superoxide and hydrogen peroxide is also detected even in the absence of NOS or P450 reductase. It should therefore be noted that PQ is an environmental chemical that can induce oxidative stress by shunting electrons not only from NADPH (in the presence of flavin enzymes) but also from proximal thiol groups.

CONCLUSION

Davidge *et al.*[40] have suggested that under conditions of oxidative stress, altered vascular function may be due to increased destruction of NO by superoxide. P450 reductase-catalyzed reductive activation of DEP quinones, leading to overproduction of superoxide, may contribute to DEP-mediated vascular dysfunction as well as lung edema formation and inflammation. Decreased eNOS-catalyzed NO production by PQ may provide useful information on the etiology of cardiopulmonary-related diseases and mortality on exposure to DEP because impairment of NO production in endothelium is thought to be implicated in the pathophysiology of vascular diseases.[36,37] We hypothesize that endothelial dysfunction caused by exposure to DEP is mediated by PQ.

Acknowledgements We wish to thank Dr. Toyoko Arimoto, Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences, and Mrs. Hiromi Nakajima-Tsubota, Ms. Kazumi Midorikawa and Ms. Akiko Endo, Master's Program in Environmental Sciences, University of Tsukuba, for their excellent contribution to our work. This research was supported in part by a grant-in-aid (#11877398 and #13672340, YK) for scientific research from the Ministry of Education, Science and Culture of Japan, by the Naito Foundation (YK).

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