Possible Mechanisms for Induction of Oxidative Stress and Suppression of Systemic Nitric Oxide Production Caused by Exposure to Environmental Chemicals

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Abstract

The cytotoxic effects evoked by exposure to environmental chemicals having electrophilic properties are often attributable to covalent attachment to intracellular macromolecules through sulfhydryl groups or enzyme-mediated redox cycling, leading to the generation of reactive oxygen species (ROS). When huge amounts of ROS form they overwhelm antioxidant defenses resulting in the induction of oxidative stress. Nitric oxide (NO) which plays a crucial role in vascular tone, is formed by endothelial NO synthase (eNOS). Since a decrease in systemic NO production is implicated in the pathophysiological actions of vascular diseases, dysfunction of eNOS by environmental chemicals is associated with cardiopulmonary-related diseases and mortality. In this review, we introduce the mechanism-based toxicities (covalent attachment and redox cycling) of electrophiles. Therefore, this review will focus on the possible mechanisms for the induction of oxidative stress and impairment of NO production caused by environmental chemicals.

Key words: oxidative stress, nitric oxide, cardiovascular dysfunction, redox cycling, metabolic activation

Introduction

Chemicals with an α,β -unsaturated carbonyl group are electrophiles that react readily with nucleophiles such as protein SH functions to cause alkylation of crucial cellular proteins. Alternatively, electrophiles such as quinones are highly redox active molecules that can be involved in the redox cycle with their semi-quinone radical anions, resulting in the formation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and ultimately the hydroxyl radical. It is recognized that numerous mechanisms of quinone toxicity (i.e., covalent attachment to modify protein sulfhydryls, redox cycling to generate ROS) can be correlated with known pathologies of the parent compound(s) (1).

It is suggested that ROS may have direct protein targets

wherein exposure of these proteins to an altered redox state alters the function of the target protein (2). Fig. 1 shows alterations in protein thiol status caused by ROS and electrophilic chemicals. Reactive cysteine residues (-SH) in proteins easily undergo oxidation to yield sulfenic groups (-SOH), disulfides (-S-S) and/or mixed disulfides (-S-SG). However, the antioxidant glutathione (GSH) can reversibly reduce these modified sulfhydryls to the reactive thiols. Under a situation that GSH is depleted by exposure

Fig. 1 Reversible and irreversible changes in the redox state of protein thiols caused by exposure to environmental chemicals. GSH, glutathione; GRX, glutaredoxin; TRX, thioredoxin; Enz, enzyme(s); ROS, reactive oxygen species; H_2O_2 , hydrogen peroxide.

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Abbreviations used: reactive oxygen species, ROS; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NO synthase; M6DH, morphine 6-dehydrogenase; MDMA, 3,4-methylenedioxymethamphetamine; CYP, cytochrome P450; DHMA, 3,4-dihydroxymethamphetamine; P450R, cytochrome P450 reductase; TNT, 2,4,6-trinitrotoluene; DEP, diesel exhaust particles; 8-OHdG, 8-hydroxydeoxyguanosine; $E_1^{\cdot}$, one-electron reduction potential; CAM, calmodulin; BAEC, bovine aortic endothelial cells; NOx, nitrite and nitrate; DTT, dithiothreitol; LPO, lipid peroxidation; iAsV, inorganic pentavalent arsenic; BH_4 , (6R)-5,6,7,8-tetrahydrobiopterin.

to some environmental chemicals, oxidized cysteine residues in proteins remain unchanged, and thus redox regulation of the proteins may be disrupted. On the other hand, continued high levels of ROS would lead to further and most likely irreversible oxidation of protein thiols to sulfinic groups ($-\text{SO}_2\text{H}$) and sulfonic groups ($-\text{SO}_3\text{H}$). Since electrophiles can act as Michael acceptors, these materials covalently bind to GSH to form the GSH adducts. Under conditions such as high exposure to electrophiles or decreased GSH level, the reactive chemicals indeed attack cysteine residues in proteins to form the protein thiol adducts (3, 4). As a consequence, the alkylation of proteins is associated with necrosis and dysfunction of enzymes. It is also suggested that covalent attachment of proteins to xenobiotics through thiol functions may be associated with an alteration in the redox state of proteins.

Covalent attachment

Morphine is a potent analgesic that is widely used in the clinical management of severe acute and chronic pain. The clinical usefulness of this natural occurring compound is limited because of the development of both tolerance and dependence after repeated administration. Misra and Woods (5) previously proposed the involvement of an unknown metabolite (e.g., morphinone) of morphine which can react readily with sulfhydryls in the development of tolerance because this postulated product has an α,β -unsaturated carbonyl group. It was reported that synthetic morphinone is able to covalently bind to the sulfhydryl group of opioid receptors, and inactivates irreversibly opioid binding sites, thereby blocking the analgesic effect of morphine (6). They (7) also reported that subcutaneous administration of morphinone produced a reduction in hepatic GSH content and that pretreatments of mice with GSH significantly increased the survival rate of mice administered a lethal dose of morphinone, whereas morphinone lethality was markedly potentiated by the thiol-modifying agent diethyl maleate. Subsequently, Yamano et al. (8) purified and characterized an NAD(P)-dependent dehydrogenase (M6DH) catalyzing oxidation of morphine to morphinone from guinea-pig liver cytosol. Kumagai et al. developed a method for simultaneous determination of morphinone and its related metabolites of morphine by high-performance liquid chromatography (9), and isolated an unidentified metabolite from the bile of guinea-pig administered morphine (10, 11). The isolated metabolite was identified as (8S)-(glutathion-S-yl)dihydromorphinone in comparison with the synthetic morphinone-GSH adduct. Interestingly, it was shown that morphinone-GSH adduct was excreted into bile as a major metabolite of morphine and that formation of morphinone-GSH adduct in vivo was influenced by pretreatment with diethyl maleate and inhibitors for M6DH (12, 13). Finally, a postulated mechanism for the metabolic activation of morphine was as follows: at low doses, the morphinone formed is trapped by conjugation with GSH. At high doses, however, GSH is rapidly depleted and morphinone could react with protein thiol functions including opioid receptor to initiate a toxic reaction sequence as shown in Fig. 2. This metabolic pathway has also been observed in humans (S. Yamano, personal communication), and since the addition reaction can proceed nonenzymatically, the same covalent attachment must occur in humans.

3,4-Methylenedioxymethamphetamine (MDMA), which is commonly known as "Ecstasy", is a popular recreational amphetamine analog that produces a unique set of effects in humans and animals. However, MDMA has serious toxic effects, both acute and chronic (14). Neurotoxicity to the serotonergic system in the

brain caused by exposure to MDMA causes permanent physical and psychiatric problems (15). It was suggested that the neurotoxic action of MDMA appears to be related to metabolism (16), so extensive studies of the metabolism and the potential toxicity of the metabolites have been performed. With microsomal enzyme preparation from rat liver and brain, it was shown that the methylenedioxy group of MDMA was oxidized by cytochrome P450 (CYP) isozymes to yield 3,4-dihydroxymethamphetamine (DHMA) and that the catechol metabolite formed was further oxidized to *o*-quinone by superoxide generated during microsomal NADPH-dependent electron transfer (17–19). An extensive study elucidated that CYP isozymes belonging to CYP2D subfamily catalyze demethylenation of MDMA to DHMA with low K_m values and that the reaction occurring with high K_m values is mediated by members of CYP2B family (20). Experiments with NG108-15 cells revealed that the DHMA analog α -methyldopamine undergoes oxidation to form a quinone that reacts easily with intracellular GSH to form its GSH adduct; α -methyldopamine toxicity was significantly accelerated by GSH depletion (21). These findings suggest that toxicity of α -methyldopamine appears to occur through oxidation to a reactive quinone, such as morphinone, followed by reaction with vital thiol functions (see Fig. 2). Recent observations have suggested that DHMA and its conjugated form(s) are found to be a major metabolite of MDMA in human urine and

I. Covalent attachment

II. Redox cycling

Fig. 2 Metabolic activation of chemicals involving covalent attachment and redox cycling.

M6DH, morphine 6-dehydrogenase; TNT, 2,4,6-trinitrotoluene. MDMA, 3,4-methylenedioxymethamphetamine; CYP2D (2B), cytochrome P450 2D (2B) isozyme; DHMA, 3,4-dihydroxymethamphetamine; GSH, glutathione; protein-SH, protein sulfhydryls; $\text{O}_2^{\bullet-}$, superoxide.

plasma (22). Therefore, it appears likely that oxidation of DHMA to a quinone species plays an important role in MDMA neurotoxicity.

Redox cycling

2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (lapachol), occurs in the wood of several species of and was tested extensively as an antimalarial agent in the 1940's. Lapachol is also known to show antitumor activity (23). Bachur et al. (24) reported that microsomal enzyme catalyzed the metabolism of lapachol in the presence of NADPH. However, the enzyme responsible for bioactivation of lapachol, leading to generation of ROS that can cause DNA damage was unknown. An immunoinhibition study with antibody against cytochrome P450 reductase (P450R) revealed that P450R was a predominant enzyme catalyzing one-electron reduction of lapachol (25). We also showed that, ROS generated during P450R-mediated redox cycling of lapachol can cause DNA damage as evaluated by conversion of the closed circular, supercoiled pBR322 DNA to the open circular form (Fig. 2) (25).

2,4,6-Trinitrotoluene (TNT) is an industrial chemical used as an explosive; chronic human exposure to TNT can result in aplastic anemia, liver damage and cataracts (26, 27). Although numerous factors can cause induction of cataracts, oxidative stress is an initiating factor in the development of cataracts (28, 29). Zitting et al. (30) argued previously that TNT was a substrate for nitroreductase(s), leading to production of ROS. The lens appears to be vulnerable to peroxidation and thus any cumulative effect of ROS may be important because the lifetime of a lens's constituents is extremely long. Thus, it was postulated that TNT-induced cataract formation may result from the presence of protein(s) in the lens capable of reducing TNT that may produce ROS. We purified to homogeneity and identified an enzyme that catalyzes the reduction of TNT, resulting in the production of ROS from bovine lenses (31). The final preparation of TNT reductase showed a single band with a subunit molecular weight of 38 kDa on SDS-PAGE. Sequence data from peptides obtained by digestion with lysylendopeptidase *Achromobacter* protease I revealed that TNT reductase is identical to zeta-crystallin. Superoxide was produced during zeta-crystallin mediated reduction of TNT, while minimal enzyme activity or protein content for superoxide dismutase (SOD), a superoxide-scavenging enzyme, was found in the lens (31). From these findings it was suggested that the induction of cataracts by TNT may be associated with increased oxidative stress, as a result of reductive activation of TNT generating superoxide (Fig. 2), since there was minimal antioxidant enzyme activity for defense against ROS exogenously produced in the lens.

Overproduction of ROS caused by diesel exhaust particles (DEP) chemicals

Diesel exhaust particles (DEP) are a major constituent of ambient particulate matter. A large variety of compounds, such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons and heterocyclics are found to be contained in DEP (32, 33). The small size of DEP makes them easily respirable, which raises health concerns regarding their effects on lung cancer, allergy, asthma etc. (34, 35). Several lines of evidence indicated that ROS generated by DEP play an important role in these adverse effects (36–40). 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.

In 1993, Sagai et al. (36) reported that formation of lung edema following intratracheal DEP injection into mice was markedly suppressed by pretreatment with polyethylene glycol-modified SOD. They (41) subsequently reported that intratracheal exposure to DEP resulted in the formation, in the murine lung of 8-hydroxydeoxyguanosine (8-OHdG), which is produced by hydroxyl radical (42). A proposed mechanism was that chemicals contained in DEP can generate ROS directly, resulting in oxidative stress-dependent lung damage (36, 43). However, it is unlikely that the superoxide or hydroxyl radical, generated by DEP solely through chemical reactions, promotes 8-OHdG production in vivo and induces lung toxicity because SOD is extensively distributed in mammalian tissues. Thus, we explored the possibility that superoxide could be enzymatically and continuously generated from DEP and therefore overwhelm defenses (44).

A variety of quinones can undergo enzymatic one-electron reduction with P450R, establishing futile redox cycles that amplify the generation of ROS (3, 4). Thus, our rationale was that if quinones contained in DEP are good substrates for P450R, 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. It was shown that NADPH oxidation was stimulated during the 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 (44). When purified P450R was used as the source of the enzyme, the turnover value was enhanced approximately 260-fold (44), suggesting the function of P450R as a dominant enzyme to reduce DEP chemicals. P450 reductase effectively reduced chemicals in methanol extract of DEP (37.5 µg) with a specific activity of 2,348 nmol of NADPH oxidized/mg/min (44). Under these conditions, superoxide was also generated with a specific activity of 3,240 nmol/mg/min. These findings suggest that one-electron reduction of presumably quinones, does appear to occur. When 0.036 units of P450 reductase (corresponding to 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 (44). This finding further supported our hypothesis that the contribution of chemical conversion to the DEP-mediated ROS production is negligible compared with the P450R-mediated reaction. We also detected that the DEP used did contain Fe (321 ppm) and Cu (321 ppm) (44), suggesting that these metals (Fe^{2+} , Cu^{+}) reduced by superoxide could potentially react with hydrogen peroxide derived from superoxide, resulting in the formation of a powerful oxidant hydroxyl radical. Consistent with this notion, electron spin resonance experiments indicated that, amongst other factors, hydroxyl radical was indeed formed during P450R-catalyzed reduction of DEP substances. Taken together, it is suggested that DEP components with a quinoid structure, undergo one-electron reduction to yield semiquinone radical which, in turn, can generate hydroxyl radical through a metal-catalyzed Haber-Weiss reaction through the redox-based generation of superoxide. Therefore, it is likely that such an overproduction of ROS contributes to, at least in part, the lung edema formation and formation of 8-OHdG observed in vivo after exposure to DEP. Fig. 3 summarizes a possible mechanism for P450R-mediated reductive activation of DEP chemicals, presumably quinones, involved in the oxidative stress-dependent pulmonary toxicity.

Fig. 3 Cytochrome P450 reductase-catalyzed reduction of DEP quinones to generate ROS.

DEP, diesel exhaust particles; $O_2^{\bullet -}$, superoxide; $\bullet OH$, hydroxyl radical.

Decreased nitric oxide (NO) production caused by phenanthraquinone (PQ), a component of DEP

Nitric oxide (NO) is a biosignal molecule that plays an important role in neurotransmission, vasorelaxation and immune response (45). NO is synthesized from L-arginine by NO synthase (NOS). NOS isozymes all consist of an *N*-terminal oxygenase domain and a *C*-terminal reductase domain, which is highly homologous with P450R (see Fig. 4); this domain is capable of transferring electrons from NADPH to artificial acceptor molecules (46, 47). It was reported that the antineoplastic anthracyclines, doxorubicin and aclarubicin, which possess a quinone moiety in their structure, affected neuronal NOS (nNOS) activity in the rat cerebellum (48). Our hypothesis was that quinoid compounds could interact with the reductase domain on nNOS, leading to a decrease in NO formation from L-arginine because most quinones are able to accept electrons from NADPH during enzymatic reaction with P450R (3, 4). Using rat cerebellar enzyme preparations, it was reported that the inhibition of NO formation by quinones which exhibit a one-electron reduction potential ($E_1^{\bullet}$) ranging between -240 and -100 mV, increased to a more positive one-electron reduction potential (49). This suggested that quinone may act as an electron acceptor for nNOS. Among 22 quinones tested, PQ ($E_1^{\bullet} = -124$ mV), which is contained in DEP (33), inhibited NO production most potently (IC_{50} value = $10 \mu M$). However, AQ ($E_1^{\bullet} = -348$ mV), a component of DEP (33), showed no effect on nNOS activity (49). A kinetic study revealed that PQ is a competitive inhibitor with respect to NADPH and a noncompetitive inhibitor with respect to L-arginine (49). Purification of enzymes that are responsible for reducing PQ from $20,000\times g$ supernatant of rat cerebellum by column chromatography suggested that one catalyst for PQ reduction was nNOS (49). The specific activity of PQ reduction by purified nNOS was $6,802$ nmol of NADPH oxidized/mg/min, but this reaction required calcium/calmodulin (CaM) (49) that stimulates electron-transfer between the reductase and oxygenase domains of nNOS (50, 51). It was reported that NOS-catalyzed reduction of cytochrome *c* was calcium/CaM-dependent (52), whereas that of adriamycin (53) and nitroblue tetrazolium (54) were unaffected by calcium/CaM. Thus, these results suggest a differential role of calcium/CaM in the NOS-catalyzed electron transfer to each acceptor.

nNOS effectively reduced the 1,4-naphthoquinone and menadione in addition to PQ, thereby causing a marked decrease in the production of NO from L-arginine (49). In contrast, 1,4-benzoquinone, AQ, mitomycin C and lapachol, which show negli-

Fig. 4 A proposed mechanism for inhibition by phenanthraquinone of nitric oxide synthase-mediated production of nitric oxide.

CaM, calmodulin; e^- , the electron donated from NADPH. Fe(II) and Fe(III) indicate the reduced form and oxidized form of the heme iron, respectively.

gible inhibitory effects on nNOS activity, were not reduced by the enzyme (49). Taken together, it is suggested that PQ interacts with the P450 reductase domain on nNOS, and thus inhibit NO formation by shunting electrons away from the normal catalytic pathway (Fig. 4). The CaM-dependent one-electron reduction of quinones by nNOS has recently been supported by the results of experiments with the reductase domain (55).

Epidemiologic studies have suggested that exposure of humans to ambient particulate matter is associated with an increased risk of cardiopulmonary-related diseases and mortality (56–58). It was reported that incubation of rat aortic rings with suspensions of DEP resulted in a suppression of endothelium-dependent vasorelaxation caused by acetylcholine (59). These findings strongly 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 (60). Reduction of NO formation by NOS inhibitors or disruption of the gene encoding endothelial NOS (eNOS) results in vasoconstriction and increase in blood pressure (61, 62). Impairment of NO production in the endothelium is implicated in the pathophysiology of vascular diseases (63, 64). 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 (BAEC) 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 $0.6 \mu M$ (Fig. 5) (65). PQ ($1 \mu M$) inhibited eNOS activity by more than 80%. A kinetic study suggested that the inhibition characteristics of PQ on eNOS activity with respect to L-arginine and NADPH were similar to those with nNOS enzyme preparations (65). Alterations in the titration curve for acetylcholine-mediated vasodilation of rat aortic rings by PQ ($5 \mu M$) revealed significant suppression of the maximum response without changing the EC_{50} value (Fig. 5) (65). 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 (65). Therefore, it is suggested that PQ binds to the P450 reductase domain of eNOS similar to nNOS, thereby inhibiting NO production by shunting the electron flow from

Fig. 5 Changes in constitutive nitric oxide synthase activity, endothelium-dependent relaxation of rat aorta, mean blood pressure in rats and plasma NOx levels in rats by phenanthraquinone.

BP, blood pressure; NOx, nitric oxide metabolites, nitrite and nitrate; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase. Left panel: total membrane fraction of bovine aortic endothelial cells and 20,000×g rat cerebellar supernatant fractions were used as enzyme preparations of eNOS and nNOS, respectively. Center panel: relaxations caused by acetylcholine in the absence and presence of phenanthraquinone are expressed as percent decreases in tension from the concentration evoked by phenylephrine. **, $P < 0.01$ vs. control. Right panel: phenanthraquinone (0.36 mmol/kg) was intraperitoneally administered to rats and then blood pressure was measured for 30 min. **, $P < 0.01$ compared with controls. Reprinted from Kumagai and Shimojo by permission of the Journal of Health Sciences.

NADPH. As a consequence, PQ appears to act as a modulator 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 with the control level) to reach plateau levels (Fig. 5), and sustained for more than 30 minutes (65). Interestingly, under these conditions, the plasma levels of NO metabolites, nitrite and nitrate (NOx), which are indicators of NO production in vivo (66), in PQ-treated rats were reduced to 68% of control levels (Fig. 5) (65). 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 reacted readily with dithiol compounds such as dithiothreitol (DTT), 2,3-dimercapto-1-propanol and 2,3-dimercapto-1-propane sulfonic acid, resulting in modification of the thiol groups, whereas negligible reactivities of this quinone with monothiol compounds such as GSH, 2-mercaptoethanol, and *N*-acetyl-L-cysteine were observed (67). These results suggest that the reactivity of PQ with the dithiol group in preference to the monothiol group may reflect the lower redox potential of the dithiol compared with monothiol (68). 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 P450R (67). We also reported that PQ was capable of modifying thiol groups in pulmonary microsomes from rats and total membrane preparation isolated from BAEC, but not bovine serum albumin, which has a Cys34 as a reactive monothiol group (67). During interaction of PQ with DTT or the BAEC preparation, PQ-mediated modification of thiols was catalytic since thiol destruction occurs without loss of PQ, whereas thiol loss via the thiol alkylating agent *N*-ethylmaleimide results in simultaneous loss of NEM (67). As mentioned earlier, quinones have two chemical properties consisting of 1) electrophilic attack to nucleophiles, resulting in thiol adduct formation and/or 2) redox cycling, wherein the rapid and sequential reduction and return to the quinone leads to production of ROS (3, 4). Therefore, it is obvious that the reactivity of PQ toward dithiols is classified into the latter case. Taken together it is suggested that

PQ-mediated destruction of protein sulfhydryls appears to involve the oxidation of proximal thiols and the reduction of molecular oxygen (Fig. 6).

Several studies suggested that thiol groups in eNOS are essential for maximal enzyme activity (69–72). Since PQ can modify protein thiols (67), we examined the possibility that PQ may also modify critical thiol residues on eNOS, leading to a disruption of catalytic activity (73). PQ and the thiol-modifying agent *N*-ethylmaleimide suppressed NO formation from L-arginine by the total membrane fraction of BAEC in a concentration-dependent manner. DTT completely blocked *N*-ethylmaleimide-mediated inhibition of eNOS activity. PQ-inhibited eNOS activity was partially reduced by DTT, but not by the monothiol agent GSH. These results suggest that PQ-mediated suppression of eNOS activity involves not only uncoupling of the electron transport of this enzyme, but also modification of presumably the proximal protein sulfhydryls that play an important role in the maximal catalytic activity.

Davidge et al. (74) suggested that under conditions of oxidative

Fig. 6 A proposed mechanism for phenanthraquinone-catalyzed oxidation of proximal protein sulfhydryls, leading to reactive oxygen species generation.

$O_2^{\bullet -}$, superoxide; H_2O_2 , hydrogen peroxide; $S^{\bullet}$, thiyl radical.

stress, altered vascular function may be due to increased destruction of NO by superoxide. P450R-catalyzed reductive activation of DEP quinones, leading to overproduction of superoxide, may contribute to DEP-mediated vascular dysfunction in addition to 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 was suggested to be implicated in the pathophysiology of vascular diseases (63, 64). It is hypothesized that endothelial dysfunction caused by exposure to DEP is mediated by PQ.

Chronic exposure of humans to arsenic decreased systemic NO production

Arsenic is ubiquitously distributed in nature throughout the earth's crust, and particularly in Asia, millions of people are chronically exposed to arsenic. The major source of exposure to arsenic for the general population is naturally-polluted drinking water (75). In vitro studies have suggested that endothelial cells undergo damage by arsenic (76). It was also reported that exposure of experimental animals or cultured cells to arsenic induces oxidative stress (77, 78). There are, at least, two possibilities for arsenic-mediated oxidative stress; one is increased ROS levels (77, 79, 80) and the other is modification of intracellular sulfhydryls (81–83). Most interestingly, prolonged exposure to arsenic results in peripheral and cardiovascular manifestations (e.g., peripheral arterial occlusive disease, hypertension and atherosclerosis), as does impaired production of endothelial NO (61–63, 84). However, no information has been available on the relation among NO synthesis, oxidative stress and chronic arsenic poisoning in humans. From these findings it was hypothesized that systemic NO formation may be reduced through the exposure to arsenic. To confirm our hypothesis, we conducted a cross-sectional study in an endemic area of chronic arsenic poisoning in Inner Mongolia, China (85, 86).

Fig. 7 Levels of arsenic and NO metabolites in tube well-water and blood. C, control; As, arsenic exposure. Reprinted from Pi et al. (85) by permission of Free Radical Biology & Medicine.

For determinations of systemic NO production and induction of oxidative stress in humans, we measured serum levels of nitrite and nitrate and lipid peroxidation (LPO), respectively.

The subjects were thirty-three inhabitants who continued to drink well-water containing high concentrations of inorganic arsenic (mean value=0.41 mg/l) for about 18 years in Inner Mongolia, China, and 10 other people who lived in this area, but were exposed to minimal concentrations of arsenic (mean value=0.02 mg/l) were employed as controls. The mean blood concentration of total arsenic was six-fold higher in exposed subjects than controls; 42.1 vs 7.3 µg/ml, $P<0.001$ (Fig. 7) (85). Eighty-two percent of arsenic in the well-water was inorganic pentavalent arsenic (iAsV) (86). The mean serum concentration of NOx was lower in arsenic exposed subjects than in controls: 24.7 vs 51.6 µM, $P<0.001$ (Fig. 7) (85). In contrast, the mean serum LPO level was higher in arsenic exposed subjects than in controls: 8.8 vs 7.1 µM, $P<0.001$ (86). In total samples, an inverse correlation with serum NOx levels was strong for blood inorganic arsenic ($r=-0.52$, $P<0.001$), and less strong for its metabolites, monomethyl arsenic ($r=-0.45$, $p<0.005$) and dimethyl arsenic ($r=-0.37$, $P<0.05$) (85). However, serum LPO levels were significantly correlated with the concentrations of inorganic arsenic ($r=0.34$, $P<0.05$), monomethyl arsenic ($r=0.34$, $P<0.05$) and dimethyl arsenic ($r=0.42$, $P<0.01$) in blood, respectively (86). The serum concentrations of LPO were inversely correlated with the levels of NOx in serum ($r=-0.33$, $P<0.05$) (86). Furthermore, an in vitro study revealed that inorganic arsenite or arsenate suppresses the activity of human eNOS (85).

Taken together it is suggested that long-term exposure of humans to arsenic by drinking well-water possibly reduces NO production in endothelial cells due to, in part, inhibition of eNOS activity in endothelial cells. Thus, peripheral vascular disorders caused by arsenic may be attributable in part to impairment of NO production in vivo. It was found that the decreased systemic NO production caused by prolonged exposure to arsenic is associated with increased oxidative stress as evaluated by LPO.

As shown in Fig. 8A, under normal conditions, eNOS is capable of producing abundant NO and minimal superoxide from L-arginine, but this reaction requires a variety of cofactors such as NADPH, CaM, calcium, and (6R)-5,6,7,8-tetrahydrobiopterin (BH₄) (46). However, under unusual situations (e.g., decreased levels of

Fig. 8 Nitric oxide synthase-mediated reactive oxygen species formation.

Arg., L-arginine; O₂⁻, superoxide; H₂O₂, hydrogen peroxide.

the substrate L-arginine and BH₄, which is a cofactor essential for the catalytic activity of NOS), activation of NOS leads to “uncoupling of NOS” with subsequent enhanced production of ROS such as superoxide and hydrogen peroxide without efficient NO formation (Figs. 8B and 8C) (87, 88). Thus, it is hypothesized that prolonged exposure to arsenic may affect levels of BH₄ and L-arginine, thereby reducing systemic NO production and concomitant generation of ROS. To address this possibility, rabbits were exposed to iAsV by daily drinking water intake, and then we examined 1) changes in biomarkers for dysfunction of NOS in biological fluids; 2) alteration in vascular tone of the endothelium-intact aorta (89). During prolonged exposure to iAsV (5 mg/l) for 18 weeks, there were no significant differences in the drinking water intake, body weight or food consumption in control and arsenic-exposed groups. Again, few appreciable signs of toxicity were seen. Nevertheless, the exposure to iAsV caused a significant decrease in plasma NOx levels (76% of control, $P < 0.05$) as observed in humans exposed to arsenic (89). Taken together with the result of humans exposed chronically to arsenic it is suggested that serum (or plasma) level of NOx may be a useful biomarker for prolonged arsenic poisoning by drinking water intake. Under the same conditions, increased induction of oxidative stress *in vivo*, as assessed by urinary hydrogen peroxide levels (90, 91), was also observed (120% of control, $P < 0.05$) (89). The hepatic BH₄ levels of rabbits exposed to iAsV declined to 62% of controls ($P < 0.01$) without altering gene expression of GTP-CH I, a rate-limiting enzyme for BH₄ biosynthesis (89). The levels of L-arginine in the ventricle of rabbit hearts was unaffected by iAsV exposure (89). More interestingly, the calcium ionophore A23187 induced a transient contraction of the aortas of rabbits exposed to iAsV, whereas no such phenomenon was seen in controls (89). Pretreatment with L-nitroarginine, an inhibitor for NOS, and BH₄ blocked the contraction effect of iAsV (89). These findings suggest that an “uncou-

pling” of NOS evoked by decreased levels of BH₄, but not L-arginine, promotes, at least in part, increased formation of superoxide, which was shown to act as an endothelium-derived contracting factor (92, 93). Therefore, our cross-sectional study and experiments with rabbits suggest the possibility that the activation of “uncoupled” NOS caused by iAsV may play an important role in the reduction of systemic NO production and increased oxidative stress in humans who live in an endemic area of chronic arsenic poisoning.

Conclusion

Quinones and arsenicals are ubiquitous chemicals found in environment. The findings of the present study demonstrate that these environmental chemicals can disrupt NO biosynthesis and up-regulate oxidative stress. Polycyclic aromatic hydrocarbons, which are a ubiquitous class of environmental contaminants, undergo biotransformation to ultimately form metabolite quinones via multienzyme systems consisting of CYP, epoxide hydrolase and dihydrodiol dehydrogenase. Metal ions, and in some cases molecular oxygen can also catalyze quinone production. Thus, it is postulated that a variety of unidentified quinones, which potentially cause cardiovascular dysfunction or oxidative damage, may exist in the environment. Further work is required to identify environmental quinones with adverse effects and then to elucidate their molecular targets and mechanism(s) of action.

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