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Mechanisms of oxidative DNA damage induced by carcinogenic arylamines

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1. ABSTRACT

Most arylamines are pro-carcinogens, and require metabolic activation to yield ultimate carcinogen metabolites. *O*-Acetylation of the *N*-hydroxy form of an arylamine yields an acetoxyarylamine, which can form a highly reactive arylnitrenium ion, the ultimate metabolite responsible for DNA adduct formation. However, we demonstrate here that the *N*-hydroxy and nitroso forms of arylamines can also induce DNA damage, including 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG) lesions, via reactive oxygen species formation. The *N*-hydroxy and nitroso derivatives of carcinogenic arylamines may contribute to the carcinogenic process through H_2O_2 formation. *N*-Hydroxy derivatives induce metal-mediated DNA damage, with remarkable enhancement by NADH. Nitroso derivatives induce NADH-dependent DNA damage in the presence of metal ions. Hydroxy derivatives of arylamines formed by enzymatic hydroxylation or as *o*- or *p*-aminophenols can also induce DNA damage in the presence of metal ions. The autoxidation of *o*-phenylenediamine and several arylamine metabolites is accelerated in the presence of SOD or manganese, resulting in the enhancement of metal-mediated DNA damage. The oxidative DNA damage induced by arylamine compounds may participate in chemical carcinogenesis, in addition to DNA adduct formation.

2. INTRODUCTION

Arylamines are used in the chemical, dye, and rubber industries, as well as in hair dyes, fungicides, cigarette smoke, diet, and automobile exhaust (1). A recent population-based study demonstrated potential environmental exposure to arylamines (2). Environmental pollutants have genotoxic and cytotoxic effects in most living organisms (3). Phenotyping, by the use of probe substrates of cytochrome P450 (CYP) and other enzymes, is widely used to assess genetic effects on the metabolism of environmental chemicals (4). The first and obligatory step in the activation of arylamines is *N*-hydroxylation, and this pathway is mediated primarily by cytochrome P450 enzymes (5). CYP1A1, CYP1A2, CYP1B1, CYP2E1, and CYP4B1 are among the P450 enzymes most responsible for the biotransformation of chemicals, especially for the metabolic activation of pre-carcinogens (5, 6). Acetylation is an important step in the metabolic activation and deactivation of arylamines, and *N*-acetylation forms the amide derivative, which is often nontoxic. However, when *O*-acetylation of the *N*-hydroxyarylamine (following oxidation) yields an acetoxy arylamine derivative, it breaks down spontaneously to a highly reactive arylnitrenium ion, the ultimate metabolite responsible for DNA adduct formation, resulting in mutagenic and carcinogenic lesions (7). Associations of the NAT1 genetic polymorphism with

incidence of myeloma, lung, and bladder cancers, as well as associations of the NAT2 polymorphism with non-Hodgkin lymphoma, liver, colorectal, and bladder cancers have been reported (8).

Multiple factors - including dose of arylamine, production of reactive metabolites, DNA adduct formation, promotion, tissue growth and differentiation, and genetic susceptibility - are important determinants of arylamine carcinogenesis (1). DNA adduct formation is usually assumed to be the major mechanism of arylamine-induced carcinogenesis. In addition, our work indicates the importance of reactive oxygen species (ROS) generation and resulting oxidative DNA damage.

Chemical carcinogenesis follows a multistep process involving both mutation and increased cell proliferation, and oxidative mechanisms have a potential role at each step (9). High levels of ROS generated by carcinogenic chemicals can cause DNA damage and affect the transcription of genes, which may lead to carcinogenesis. Numerous reports demonstrate the induction of oxidative DNA damage by known carcinogens, and the presence of elevated levels of ROS in a wide range of cancers (10-12). Oxidatively modified DNA lesions found in many tumors strongly implicate such damage in the etiology of cancer. Although many base- and sugar-derived oxidative DNA lesions having been identified, most studies have focused upon the guanine modification, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxodG), and its potential role in pathogenesis (13). We suggest, in this review, that metabolic oxidations of the amino group, including *N*-hydroxylation and nitrosation, activate arylamines to generate ROS and induce 8-oxodG formation.

The genotoxicity of carcinogens can be evaluated using the bacterial reverse mutation assay (Ames test). Monocyclic aromatic hydrocarbons are mostly found to be negative in the Ames test, while polycyclic aromatic hydrocarbons frequently show positive results, probably acting by DNA adduct formation. We have demonstrated that many Salmonella-negative carcinogens, such as metals (14, 15), benzene (16, 17), and *ortho*-phenylphenol (18), induce oxidative DNA damage (19). In addition, polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene (20) and benzo[*a*]anthracene (21, 22) induce not only DNA adduct formation but also oxidative DNA damage. It is known that both monocyclic and polycyclic arylamines form DNA adducts in relation to their mutagenicity (23-26). Based on our experimental results, we propose possible mechanisms of oxidative DNA damage to account for the chemical structural requirements of carcinogenic arylamines.

3. OXIDATIVE DNA DAMAGE MEASUREMENT

Damage to ^{32}P-labeled DNA is tested by the method described below (27, 28). Ligated DNA fragments containing exons of the relevant human tumor genes were amplified from plasmid by the polymerase chain reaction (PCR) method. ^{32}P-5'-end-labeled fragment was obtained by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase. The 5'-end-labeled double-stranded fragments were further digested with a restriction enzyme to obtain the singly-labeled fragments. The standard reaction mixture (in a microtube; 1.5 ml) for detection of DNA damage by the test chemicals contained a reagent, a transition metal ion (Cu(II), Co(II), Mn(II), or Fe(III)), 5'-end labeled DNA fragments, and calf thymus DNA in 10 mM sodium phosphate buffer (pH 7.8) containing 2.5 µM DTPA, and was incubated for the indicated times at 37 °C. In several experiments, test chemicals were pretreated with enzymes such as cytochrome P450 and cytochrome P450 reductase. After ethanol precipitation, the DNA was treated with 1 M piperidine for 20 min at 90 °C or 10 units of formamidopyrimidine-DNA glycosylase (Fpg) protein in the reaction buffer (10 mM HEPES-KOH (pH 7.4), 100 mM KCl, 10 mM EDTA and 0.1 mg/mL BSA) for 2 h at 37 °C. The recovered and denatured DNA fragments were electrophoresed on an 8 % polyacrylamide 8 M urea gel (12 x 16 cm), and autoradiograms were obtained by exposing X-ray film to the gel. Oligonucleotides were detected on the autoradiogram as a result of DNA damage. When DNA strand breakage occurs, oligonucleotides can be detected without any additional treatment. Hot alkaline piperidine treatment detects DNA base modification and liberation. Fpg protein catalyzes the excision of 8-oxodG as well as Fapy residues. To clarify the reactive species involved in oxidative DNA damage by the test chemical, radical scavengers can be added to the reaction mixture before incubation. Catalase, superoxide dismutase (SOD, CuZn-SOD or Mn-SOD), metal chelators, and •OH scavengers such as ethanol, mannitol, sodium formate were used as scavengers. To measure the amount of 8-oxodG in calf thymus DNA by test chemicals, calf thymus DNA fragments were incubated at 37 °C with the reagent in the presence of a metal ion and /or a reductant for the indicated times. After ethanol precipitation, DNA was digested to nucleosides using nuclease P_1 and calf intestine phosphatase and analyzed by HPLC with an electrochemical detector (ECD). When cultured human cells were treated with the reagent, DNA was extracted under anaerobic conditions, and treated with nuclease P_1 and bacterial alkaline phosphatase, followed by HPLC-ECD analysis (29). This experimental system is a convenient and useful method to detect oxidative DNA damage.

4. DNA DAMAGE INDUCED BY ARYLAMINES

We have investigated mechanisms of oxidative DNA damage induced by many carcinogens, including arylamines, using the above-mentioned experimental system. Here, we discuss the mechanisms of action with reference to three chemical structural properties of carcinogenic arylamines.

4. 1. DNA damage by *N*-hydroxy and nitroso forms of arylamines and the corresponding nitro derivatives

Figure 1 shows chemical structures of arylamines and their *N*-hydroxy, nitroso and nitro derivatives, for which we demonstrated oxidative DNA damage.

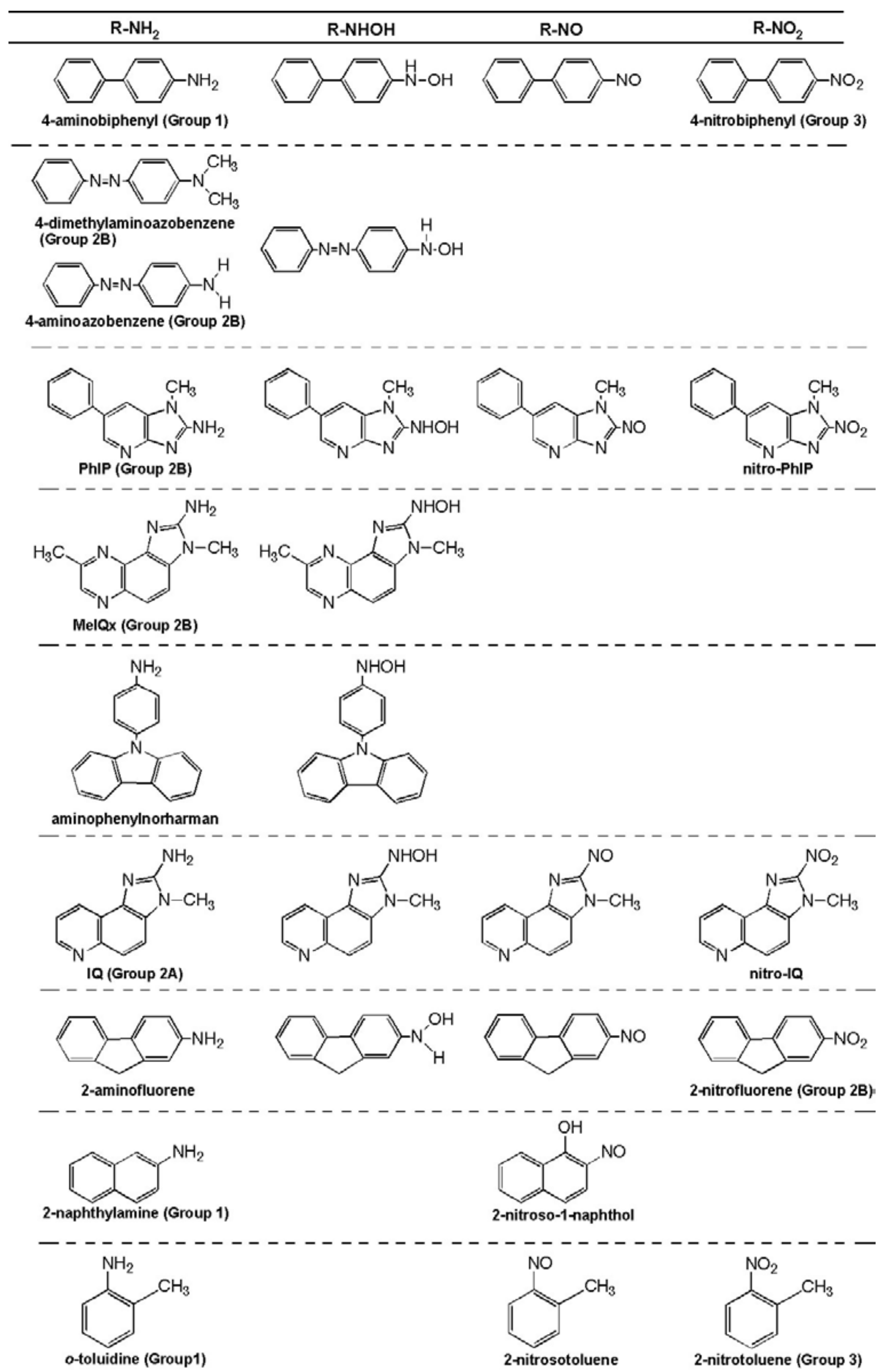

Figure 1. Chemical structures of arylamines and their *N*-hydroxy, nitroso and nitro derivatives.

Epidemiologic studies have revealed increased risks for development of urinary bladder cancer among dye workers occupationally exposed to aromatic amines such as 4-aminobiphenyl (4-ABP) and benzidine (4,4'-diaminobiphenyl) (30). A representative carcinogenic arylamine, 4-ABP is a bicyclic aromatic amine formerly

used as a highly efficient rubber antioxidant and dyestuff intermediate (31). 4-ABP is classified by the International Agency for Research on Cancer (IARC) as Group 1 (carcinogenic to humans), prompting discontinuation of its production, but 4-ABP exposure continues via tobacco smoke (32). DNA adduct formation has been thought to be a major cause of DNA damage by carcinogenic arylamines (33). The well-established bioactivation route leading to covalent DNA adduct formation and possible mechanism of oxidative DNA damage by arylamines are shown in Figure 2, with 4-ABP as example. The amino group of the arylamine is oxidized to an *N*-hydroxy derivative [*N*-hydroxy-4-ABP] in the liver, followed by *N*-acetyltransferase (NAT)-catalyzed estrrification, giving the *N*-acetoxy derivative [*N*-acetoxy-4-ABP] or sulfate conjugation catalyzed by sulfotransferase (SULT). These metabolites give rise to arylnitrenium ions, which then react with DNA to form adducts, such as the deoxyguanosine adduct of 4-ABP (dG-C8-4-ABP) (34).

We investigated the ability of *N*-hydroxy-4-ABP to cause DNA damage by an alternative oxidative mechanism. *N*-Hydroxy-4-ABP caused Cu(II)-mediated DNA damage. Addition of the endogenous reductant NADH dramatically enhanced the level of DNA damage. It is suggested that metal-mediated autoxidation of the *N*-hydroxy-4-ABP to the hydronitroxide radical is followed by further autoxidation to 4-nitrosobiphenyl. 4-Nitrosobiphenyl is reduced to the hydronitroxide radical and/or *N*-hydroxy-4-ABP to form a redox cycle, resulting in enhanced formation of Cu(I)-hydroperoxo complex (Cu(I)OOH) and oxidative DNA damage (35). Thus, Cu(II)-mediated oxidative DNA damage may play an important role in the carcinogenic process of 4-ABP in addition to DNA adduct formation. 4-Nitrobiphenyl was the first nitroaromatic compound shown to be a bladder carcinogen (36). 4-Nitrobiphenyl can be reduced metabolically to 4-nitrosobiphenyl, and then further to *N*-hydroxy-4-ABP (37), which is a common metabolite leading to DNA damage. Both 4-ABP and 4-nitrobiphenyl are prohibited in many countries, although 4-nitrobiphenyl is classified as IARC Group 3 (not classifiable as to its carcinogenicity to humans) (38). Benzidine (4,4'-diaminobiphenyl) is a component in the production of dyes, and has been linked to human bladder and pancreatic cancer. Benzidine is thought to initiate carcinogenesis by metabolic pathways similar to those of 4-ABP, involving *N*-acetylation and reactive oxygen species (39).

A carcinogenic amino azo dye, 4-dimethylaminoazobenzene (DAB) (40) had been used as a food additive, “butter yellow” before its toxicity was recognized. DAB is metabolically demethylated to give 4-aminoazobenzene (AAB), after which *N*-hydroxylation may occur. Both DAB and AAB are classified as Group 2B (possibly carcinogenic to humans) by IARC. We demonstrated oxidative DNA damage induced by *N*-hydroxy-AAB at low (nano-molar order) concentrations in the presence of NADH and Cu(II) (41). Therefore, the *N*-hydroxy form may be a key metabolite in both oxidative DNA damage and DNA adduct formation. In the case of azo dyes bearing exocyclic amino groups, such as DAB and AAB, the mechanism of carcinogenic action has been attributed principally to formation of DNA adducts following metabolic oxidation of the amino group. In contrast, azo dyes without exocyclic amino groups, such as azobenzene, have no obvious reactive functional group capable of forming DNA adducts. Nevertheless, both hydrazobenzene and azobenzene are carcinogenic to rodents (42). We demonstrated the oxidative DNA-damaging ability of hydrazobenzene, a metabolite of azobenzene formed by azo reduction (43). Hydrazobenzene causes DNA damage in the presence of Cu(II). However, azobenzene and its metabolite aniline (formed through cleavage of the azo linkage) did not cause DNA damage, even in the presence of Cu(II). Oxygen consumption and UV-visible spectroscopic measurements have shown that hydrazobenzene is autoxidized to azobenzene with H_2O_2 formation, resulting in Cu(II)-mediated oxidative DNA damage.

Heterocyclic aromatic amines are suspected colorectal cancer carcinogens, which arise from cooking meat and fish at high temperatures and are found in well-done meat (44). A European cohort study showed that heterocyclic aromatic amines may increase the risk of colorectal adenomas (45). 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*] pyridine (PhIP), 2-amino-3-methylimidazo [4,5-*f*] quinoline (IQ), and 2-amino-3,8-dimethylimidazo [4,5-*f*]quinoxaline (MeIQx), are representative members of this class, isolated not only from cooked meat but also found in cigarette smoke condensate (46). Norharman (9H-pyrido[3,4-*b*]indole), a heterocyclic amine found in cigarette smoke or cooked foodstuffs, is not mutagenic itself, but reacts with non-mutagenic aniline to form mutagenic aminophenylnorharman (APNH), with carcinogenic potential in the colon and liver (47). *N*-Hydroxy metabolites of PhIP (48), MeIQx (49), and APNH (50), cause oxidative DNA damage, and the addition of NADH greatly enhances damage by establishing an NADH-dependent redox cycle in a manner similar to the 4-ABP metabolite.

Many nitroaromatic compounds yield arylamines via nitroso and *N*-hydroxy forms (51). Amino, *N*-hydroxy, nitroso and nitro derivatives may be interconverted by electron transfer, either enzymatically or non-enzymatically. Most nitroaromatic compounds require the action of nitroreductase enzyme to be reduced. However, interestingly, we found that a heterocyclic nitro compound, nitro-IQ, can be reduced to the nitro anion radical by NADH. Nonenzymatic reduction of nitro-IQ and subsequent autooxidation of the radical lead to ROS generation and DNA damage (52). Relevantly, we demonstrated oxidative DNA damage induced by dinitropyrenes (DNPs) in the presence of cytochrome P450 reductase; the positional congeners gave varying intensities of DNA damage, 1,8-DNP, 1,6-DNP > 1,3-DNP (53). Determining factors such as electronic and structural features of the molecule are important for generation of radicals and ROS through redox reactions. Nitrofluorene (NF) is commonly found in diesel emissions, and *N*-acetylaminofluorene (AAF) had been developed as a pesticide. Both NF and AAF are carcinogens acting via a

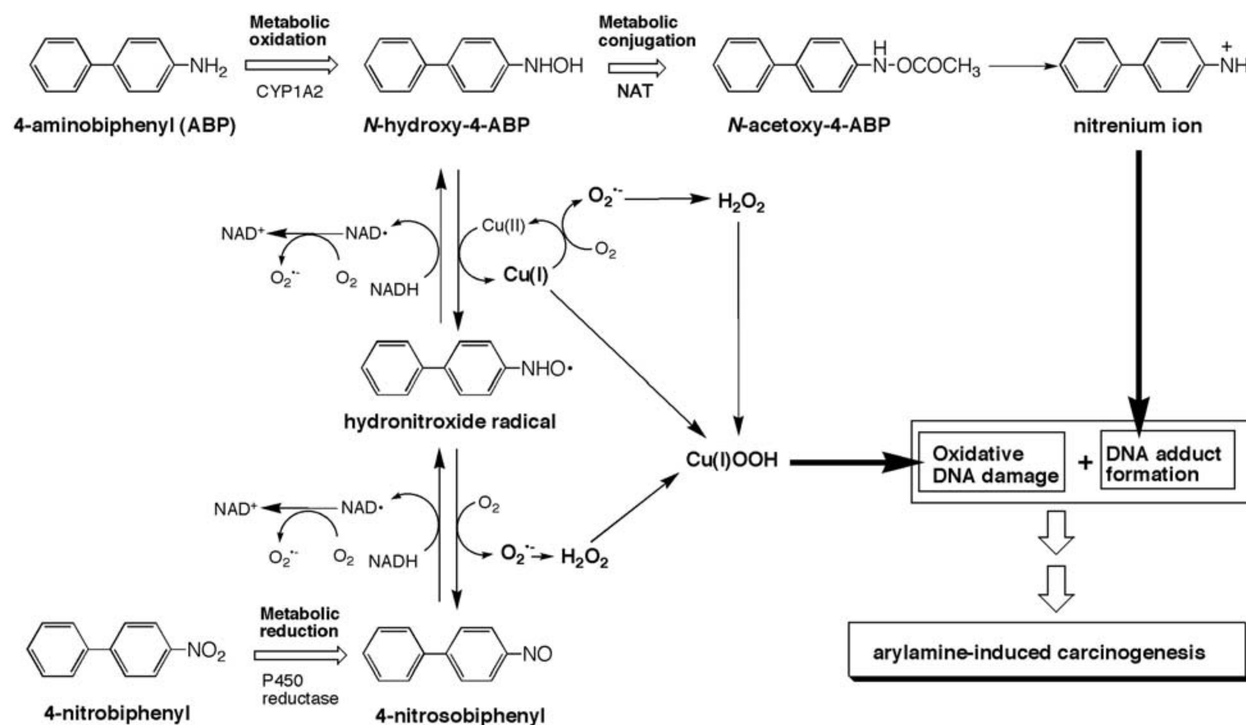

Figure 2. Possible mechanism of carcinogenicity induced by 4-aminobiphenyl and 4-nitrobiphenyl through *N*-hydroxy and nitroso metabolites.

common metabolite, *N*-hydroxy-aminofluorene (*N*-OH-AF). We demonstrated oxidative DNA damage by *N*-OH-AF through H_2O_2, in both a cell-free system and in cultured human cells (54). DNA adducts may be formed from enzymatic nitroreduction of certain nitro aromatic compounds via the *N*-hydroxy form (51, 55). However, the formation of *N*-hydroxy metabolites of nitro aromatic compounds also suggests the occurrence of oxidative DNA damage.

2-Naphthylamine was formerly used for synthesis of azo dyes (38), and is also contained in tobacco smoke (32). It is a known human carcinogen (Group 1), which contributes to the development of bladder cancer (38). We found that a nitroso form metabolites of 2-naphthylamine (2-nitroso-1-naphthol) induced DNA damage in the presence of NADH and Cu(II) (56). *o*-Nitrosotoluene, a minor metabolite of *o*-toluidine (Group 1 carcinogen), induced DNA damage in the presence of Cu(II) and NADH through *o*-toluolhydronitroxide radical and ROS formation (57). The nitroso forms of arylamines require NADH to generate hydronitroxide radicals. When the hydronitroxide radical reacts with Cu(II) to yield Cu(I), O_2 reduced to O_2^- by the interaction with Cu(I), and O_2^- is dismutated to give H_2O_2. H_2O_2 reacts with Cu(I) to form reactive species, such as Cu(I)OOH, capable of causing DNA damage. Therefore, it is reasonable to consider that the *N*-hydroxy and nitroso derivatives of carcinogenic arylamines such as 4-ABP, amino azo dyes, 2-naphthylamine and heterocyclic amines, play important roles in the carcinogenic process through H_2O_2 formation.

4.2. DNA damage by aminophenol forms of arylamines

Figure 3 shows chemical structures of monocyclic arylamines that we examined for possible oxidative DNA damage. Anisidines are used as solvents, dye intermediates, and in pharmaceutical chemistry. The three isomers of anisidine have different carcinogenic potentials, according to the IARC classification (*o*-anisidine: group 2B, *p*-anisidine: group 3, *m*-anisidine, which may have no or little carcinogenicity, no report available) (58). No anisidine isomers (*o*-, *m*-, *p*-) induced DNA damage in our system. Anisidines are metabolized to their aminophenol forms by microsomal dealkylation. We examined whether hydroxylated metabolites of anisidine isomers (*o*-, *m*-, and *p*-aminophenol) cause oxidative DNA damage in the presence of metal ions, using ^{32}P-5'-end-labeled DNA fragments obtained from the c-Ha-*ras*-1 and *p53* genes (59). Both carcinogenic anisidine metabolites, *o*-aminophenol and *p*-aminophenol, caused DNA damage in the presence of Cu(II), with *o*-aminophenol being more efficient. When SOD or Mn(II) was added, DNA damage was enhanced. *m*-Aminophenol, metabolite of the non-carcinogenic *m*-anisidine, did not cause DNA damage under the same conditions. Figure 4 (A and B) shows the mechanism of oxidative DNA damage induced by aminophenol form of arylamines, through aminophenoxyl radicals and ROS formation. These results suggest that the carcinogenicity of anisidine isomers may be related to the extent of oxidative DNA damage caused by their metabolites (59).

We showed that metabolites of *o*-toluidine induced oxidative DNA damage (57). *o*-Toluidine itself

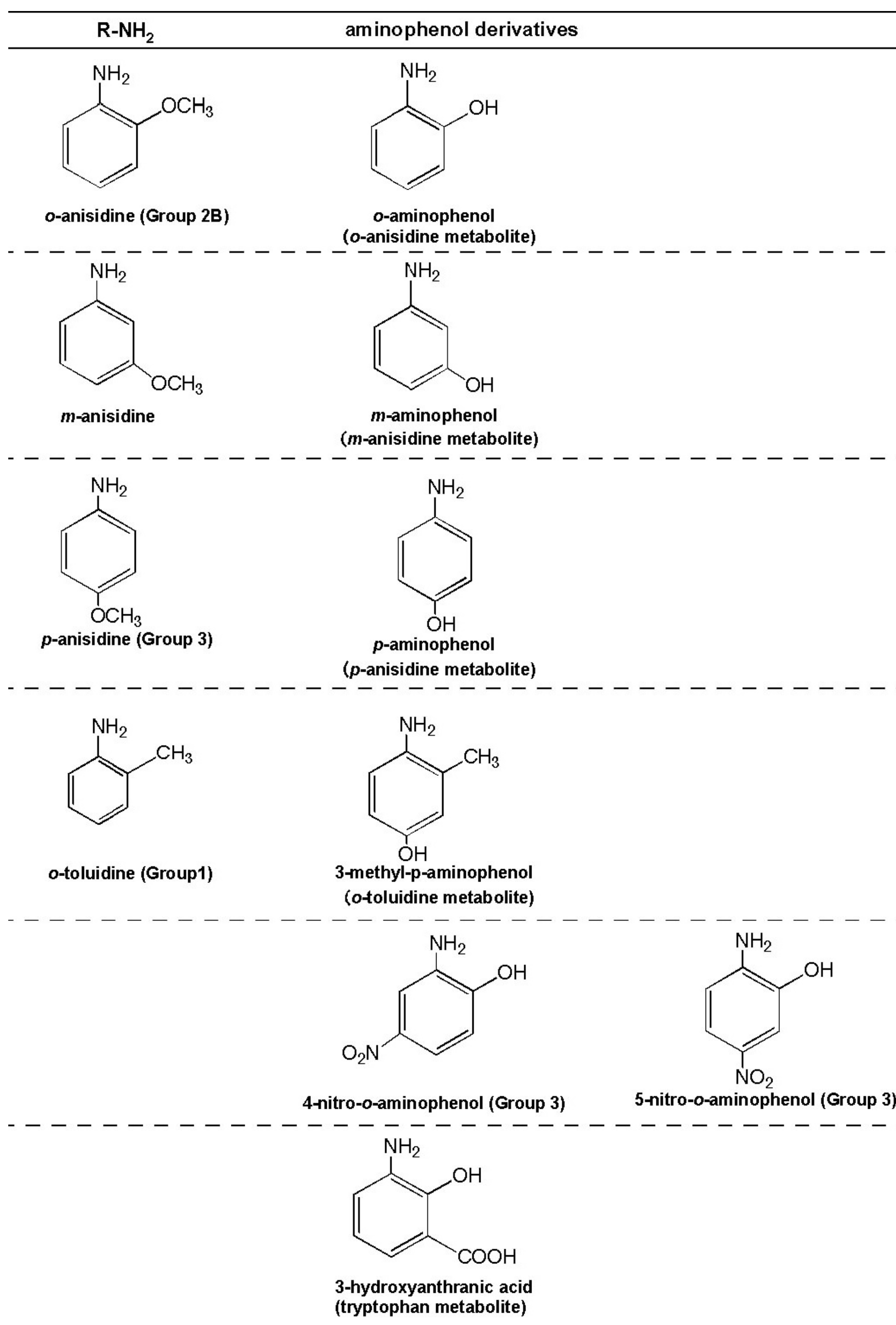

Figure 3. Chemical structures of monocyclic arylamines and their aminophenol derivatives.

did not induce DNA damage, but 3-methyl-*p*-aminophenol, a major metabolite of *o*-toluidine, caused DNA damage in the presence of Cu(II). Our UV-visible and ESR spectroscopic studies have demonstrated that 3-methyl-p-aminophenol is autoxidized to form the aminomethylphenoxyl radical. A minor metabolite, nitrosotoluene, also induced oxidative DNA damage. Consequently, it is considered that the radical reacts with O_2 to form O_2^- and subsequently H_2O_2, and that the reactive species generated by the reaction of H_2O_2 with Cu(I) participate in the DNA damage.

There is an association between occupational exposure to hair dyes and incidence of cancers (60, 61).

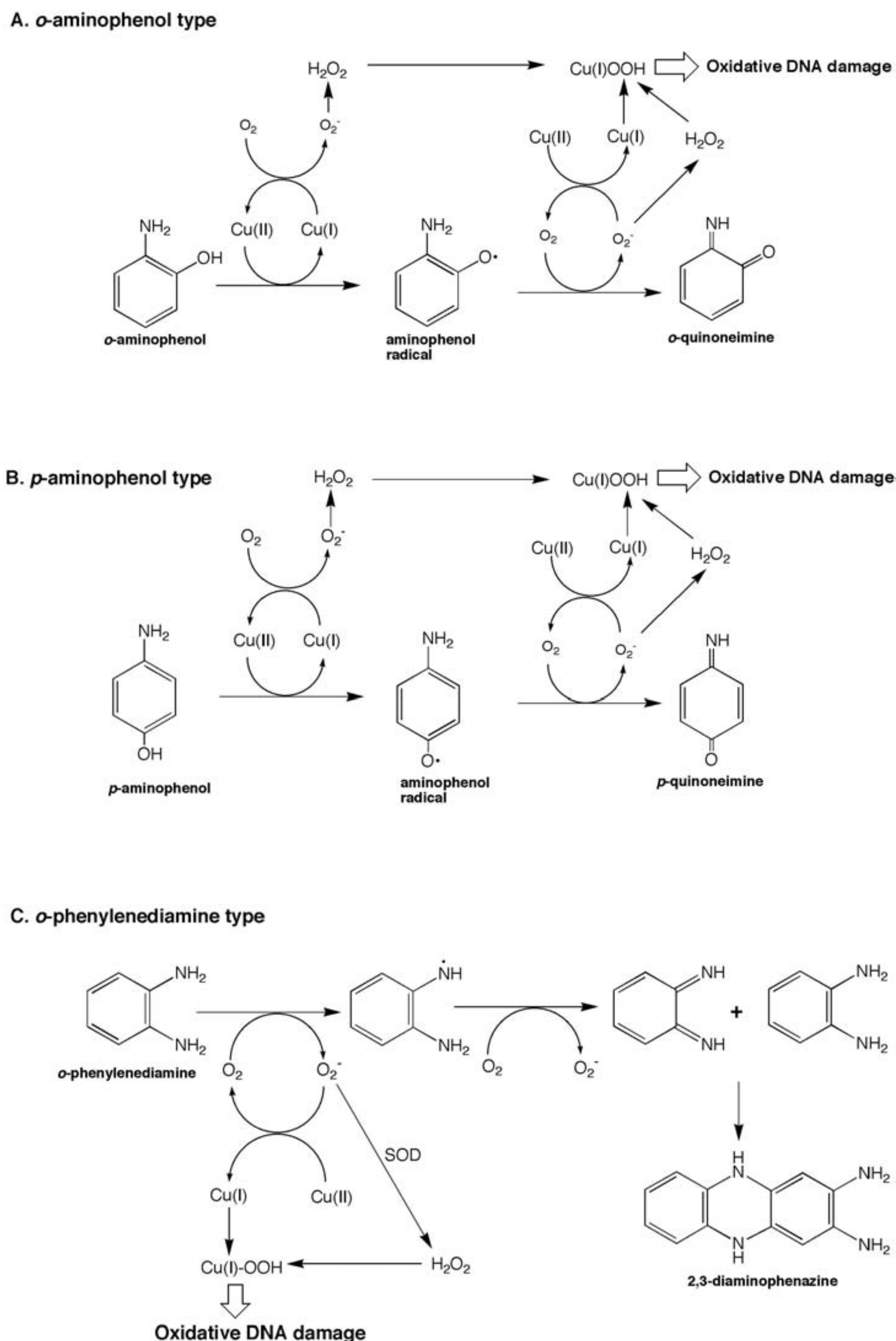

Figure 4. Possible mechanisms of oxidative DNA damage induced by aminophenols and phenylenediamines.

The National Toxicology Program, USA, demonstrated the carcinogenicity of 4-nitro-*o*-aminophenol and 5-nitro-*o*-aminophenol in rodents (62, 63). We examined the induction of DNA damage by several hair dye components. Two components, carcinogenic 4-nitro-*o*-aminophenol and 5-nitro-*o*-aminophenol, induced Cu(II)-dependent DNA damage in the presence of Cu(II) (64). 4-Nitro-*o*-aminophenol induces DNA damage more efficiently than 5-nitro-*o*-aminophenol. The mechanism shown in Figure 4A was postulated. Nitro-*o*-aminophenols undergo Cu(II)-mediated autoxidation to aminophenoxyl radicals with generation of ROS, causing DNA damage. The tendency to donate electrons to molecular oxygen and become radicals may correlate with the position of nitro group, as supported by the voltammetric data (65). We compared the extent of DNA damage induced by the hair dye components *m*-phenylenediamine and 4-methoxy-*m*-phenylenediamine (66). Carcinogenic 4-methoxy-*m*-

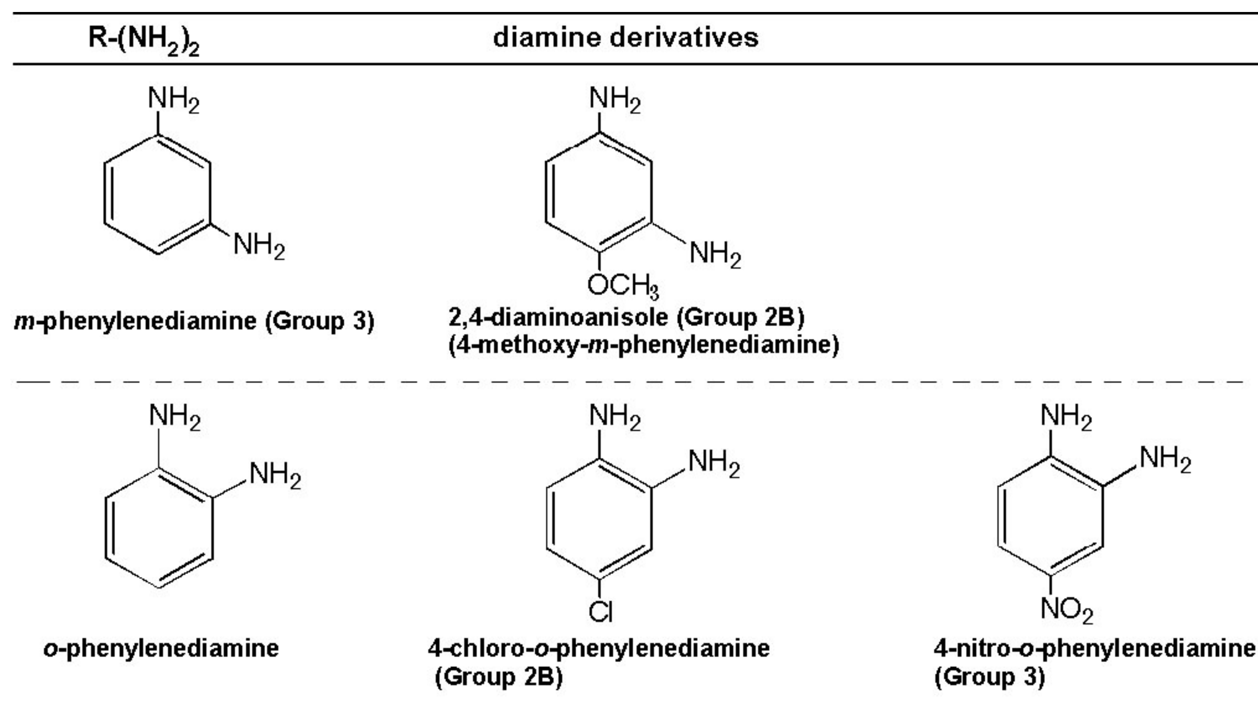

Figure 5. Chemical structures of phenylenediamines and their derivatives.

phenylenediamine caused Cu(II)-mediated DNA damage, including 8-oxodG formation, and SOD enhanced the DNA damage. UV-visible spectroscopic studies have shown that Cu(II)-mediated autoxidation of 4-methoxy-*m*-phenylenediamine and SOD accelerated the autoxidation. On the other hand, the non-carcinogenic but mutagenic *m*-phenylenediamine did not cause clear DNA damage and significant autoxidation, even in the presence of Cu(II), under the condition used. These results suggest that carcinogenicity of *m*-phenylenediamines is associated more with the ability to cause oxidative DNA damage than with bacterial mutagenicity. From the point of view of chemical structure, addition of a methoxy group to *o*- or *p*-position, with respect to the amino group, leads to some potential for autooxidation. Several arylamines, in their *o*- or *p*-aminophenol metabolite forms, can induce oxidative DNA damage through autoxidation, with or without enhancement by SOD and manganese.

4.3. DNA damage by diamine forms of arylamines

Permanent oxidant hair dyes are consisted of many chemical components, including phenylenediamines (Figure 5). We examined DNA damage induced by the mutagenic *ortho*-phenylenediamine (*o*-PD) and its derivatives, 4-chloro-*ortho*-phenylenediamine (Cl-PD) and 4-nitro-*ortho*-phenylenediamine (NO_2-PD). Carcinogenic compounds, *o*-PD and Cl-PD, caused Cu(II)-mediated DNA damage, and SOD enhanced this DNA damage (67). UV-vis spectroscopic studies showed that the spectral change of o-PD and Cl-PD required Cu(II), and addition of SOD enhanced it. This suggested that SOD enhanced the rate of Cu(II)-mediated autoxidation of *o*-PD and Cl-PD, to form 2,3-diaminophenazine (68), leading to enhancement of DNA damage (Figure 4C). On the other hand, the mutagenic but non-carcinogenic NO_2-PD induced no DNA damage. These results are consistent with the view that the carcinogenicity of *ortho*-phenylenediamine compounds is a consequence of oxidative DNA damage, rather than the formation of bulky DNA adducts of the type detected by standard bacterial mutagenicity assays.

5. PERSPECTIVE

We have demonstrated oxidative DNA damage induced by monocyclic and polycyclic aromatic amines and their metabolites. The amino group of arylamines is the key to their carcinogenicity. When the amino group undergoes biochemical *N*-oxidation, further conversion can induce DNA adduct formation, via covalent binding, or oxidative DNA damage, via electron transfer reactions. In addition, the amino group in the *o*- or *p*-position in a phenol structure, and two amino groups in the *o*-position in the benzene structure, can autoxidize with generation of ROS. Here we have reviewed our previous studies and proposed possible mechanisms of metal-mediated oxidative DNA damage via redox reaction through NADH-dependent, NADH-enhanced, and SOD or manganese-enhanced processes. Nitroso derivatives induced NADH-dependent DNA damage, and *N*-hydroxy derivatives induced DNA damage with remarkable enhancement by NADH. SOD or manganese enhanced DNA damage by *o*-phenylendiamine and several aminophenols.

3-Hydroxyanthranilic acid (3-HAA), a tryptophan metabolite, is carcinogenic in the bladders of mice (69). 3-HAA is an *o*-aminophenol derivative. High concentrations of 3-HAA were found in the urine of patients with bladder cancer, suggesting the abnormal

metabolism of tryptophan (70). We showed that 3-HAA induced Cu(II)-mediated DNA damage, with enhancement by preincubation of 3-HAA with Mn(II) (71). UV-visible spectroscopy showed that Mn(II) and Cu(II) enhanced the rate of autoxidation of 3-HAA, forming the aminophenoxyl radical (anthranilyl radical) and ROS. Attention should be paid not only to environmental arylamines but also to endogenous arylamines.

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