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Mechanism of oxidative DNA damage induced by metabolites of carcinogenic naphthalene

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ABSTRACT

Naphthalene is a carcinogenic polycyclic aromatic hydrocarbon, to which humans are exposed as an air pollutant. Naphthalene is metabolized in humans to reactive intermediates such as 1,2-hydroxynaphthalene (1,2-NQH_2), 1,4-NQH_2, 1,2-naphthoquinone (1,2-NQ), and 1,4-NQ. We examined oxidative DNA damage by these naphthalene metabolites using ^{32}P-labeled DNA fragments from human cancer-relevant genes. 1,2-NQH_2 and 1,4-NQH_2 induced DNA damage in the presence of Cu(II). The DNA-damaging activity of 1,2-NQH_2 was significantly increased in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH), whereas that of 1,4-NQH_2 was not. In the presence of NADH, 1,2-NQ induced Cu(II)-dependent DNA damage, whereas 1,4-NQ did not. The calculated energy of the lowest unoccupied molecular orbital (LUMO), which corresponds to the reduction potential, was estimated to be −0.67 eV for 1,2-NQ and −0.75 eV for 1,4-NQ. These results suggest that 1,2-NQ was reduced more easily than 1,4-NQ. Furthermore, 1,2-NQH_2, 1,4-NQH_2, and 1,2-NQ plus NADH formed 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) as an oxidative DNA marker. Catalase and bathocuproine inhibited DNA damage, suggesting that H_2O_2 and Cu(I) were involved. These results indicate that NQH_2s are oxidized to the corresponding NQs via semiquinone radicals, and that H_2O_2 and Cu(I) are generated during oxidation. 1,2-NQ is reduced by NADH to form the redox cycle, resulting in enhanced DNA damage. The formation of the corresponding semiquinone radicals was supported by an electron paramagnetic resonance (EPR) study. In conclusion, the redox cycle of 1,2-NQ/1,2-NQH_2 may play a more important role in the carcinogenicity of naphthalene than that of 1,4-NQ/1,4-NQH_2.

1. Introduction

Naphthalene is a commercially important polycyclic aromatic hydrocarbon that is produced from coal tar and petroleum [1]. It is widely used in industrial materials and moth-proofing. More than 6500 tons of naphthalene were used worldwide in 2000. The greatest occupational exposure to naphthalene was previously estimated to be creosote impregnation [1]. It is also found in bitumen fumes at various concentrations, depending on the temperature at which the fumes are generated [2]. Human exposure to naphthalene may occur during the inhalation of air pollutants such as diesel and gasoline engine exhaust, and also during cigarette smoking, in addition to occupational exposure during its production and use as an industrial intermediate [1,3]. The daily intake of naphthalene from ambient air was previously estimated to be 19 μg based on a median concentration of 0.95 μg/m^3 in urban/suburban air samples collected from US cities [4]. The National Toxicology Program (NTP) demonstrated the carcinogenic activity of naphthalene in female B6C3F$_1$ mice based on an increase in the

Abbreviations: IARC, The International Agency for Research on Cancer; NQ, naphthoquinone; NQH_2, hydroxynaphthalene; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; HPLC, high-performance liquid chromatography; HPLC-ECD, HPLC with an electrochemical detector; ROS, reactive oxygen species; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; DTPA, diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; SOD, superoxide dismutase; NADH, reduced form of nicotinamide adenine dinucleotide; NTP, National Toxicology Program; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; TBARS, thiobarbituric acid reactive substance; Cu(I)OOH, a copper-hydroperoxo complex

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incidence of pulmonary alveolar/bronchiolar adenomas [5]. Furthermore, a previous study clearly demonstrated the carcinogenic activity of naphthalene in male and female F344/N rats due to an increase in the incidence of respiratory epithelial adenomas and olfactory epithelial neuroblastomas of the nose [6]. Epidemiological studies are inadequate for evaluating the cancer risk associated with naphthalene for humans. Thus, the International Agency for Research on Cancer (IARC) reported that naphthalene is "possibly carcinogenic to humans (Group2B)" [1].

Naphthalene is metabolized in humans and rodents to reactive intermediates. It is metabolized by CYP enzymes to its 1,2-epoxide, which may be non-enzymatically isomerized to 1- and 2- hydroxynaphthalene (1- and 2- naphthol), and further oxidized to 1,2- and 1,4-naphthoquinone (1,2- and 1,4-NQ) [2,7,8]. When ^{14}C-labeled naphthalene was applied dermally to rats, primary urinary metabolites, such as 1,2-NQH_2 (17.2%) and 1,2-NQ (11.4%), were identified [9]. A previous study reported adducts of serum albumin with 1,2-NQ (76.6 pmol/g) and 1,4-NQ (48.6 pmol/g) in coke oven workers [10]. Furthermore, 1,2- and 1,4-NQ have been found in the environment as products of fuel combustion, tobacco smoke, and plants. Ambient concentrations of quinones in the particulate and gas phases collected for five days from a trafficked site in Birmingham, were reported to be 1,2-NQ (3374 pg m^{-3}), 1,4-NQ (1798 pg m^{-3}), and benzoquinone (1652 pg m^{-3}) [11].

Naphthalene and 1-naphthol become cytotoxic after metabolic activation by human liver microsomes. 1,2-NQ was found to be directly toxic to mononuclear leukocytes and depleted glutathione, whereas 1,2-epoxide was not, suggesting that NQ is responsible for the cytotoxicity of naphthalene rather than the epoxide [1]. Quinones may catalyze redox cycling among their reduced forms, oxidized forms, and/or their semiquinone radicals to produce reactive oxygen species (ROS). ROS, which have been implicated in the pathogenesis of cancer, are produced from catechol [12], hydroquinone [13], and their derivatives [14–17] during autoxidation into the corresponding benzoquinones to induce oxidative DNA damage. However, the mechanisms responsible for the carcinogenicity of naphthalene and its metabolites have yet to be elucidated in detail.

In the present study, in an attempt to clarify the mechanisms underlying naphthalene carcinogenicity, we investigated the mechanisms and site specificity of DNA damage induced by the naphthalene metabolites, 1,2-NQH_2, 1,4-NQH_2, 1,2-NQ, and 1,4-NQ, in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH) and metal ions using ^{32}P-labeled DNA fragments of human tumor-relevant genes. The formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), an oxidative DNA marker, generated by these naphthalene metabolites in calf thymus DNA was analyzed using high-performance liquid chromatography (HPLC) with an electrochemical detector (HPLC-ECD). We examined the redox properties of these naphthalene metabolites by electron paramagnetic resonance (EPR) and *ab initio* molecular orbital calculations.

2. Materials and methods

2.1. Materials

Restriction enzymes (*Ava* I, *Hin*d III, and *Pst* I) and T_4 polynucleotide kinase were purchased from New England Biolabs (Beverly, MA). Restriction enzymes (*Apa* I, *BssH* II, and *Eco*R I) and calf intestine phosphatase were from Boehringer Mannheim GmbH (Mannheim, Germany). [γ-^{32}P]ATP (222 TBq/mmol) was obtained from DuPont New England Nuclear (Boston, MA). Diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid (DTPA) and bathocuproinedisulfonic acid were purchased from Dojin Chemical Co. (Kumamoto, Japan). Superoxide dismutase (SOD, 3000 units/mg from bovine erythrocytes) and catalase (45,000 units/mg from the bovine liver) were obtained from Sigma Chemical Co. (St. Louis, MO). 1,2-NQH_2 and 1,4-NQH_2 were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 1,2-NQ and 1,4-NQ were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Copper chloride ($CuCl_2$, $2H_2O$) and NADH were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

2.2. Preparation of ^{32}P-labeled DNA fragments

DNA fragments were obtained from human *TP53* tumor suppressor genes [18], the human *p16* tumor suppressor gene (*CDKN2A*) [19], and human *HRAS* protooncogene [20], as described previously [21]. The DNA fragment containing exons 7 and 8 of the *TP53* gene was amplified from human genomic DNA by the polymerase chain reaction (PCR) method using Human p53 Amplimer Panel (CLONTECH). The PCR product was digested with *Sma* I and ligated into the *Sma* I-cleaved pUC 18 plasmid, and was then transferred to *Escherichia coli* JM 109. The plasmid pUC 18 was digested with *Eco*R I and *Hin*d III, and the resulting DNA fragment was fractionated by electrophoresis on 2% agarose gels. The fragment (*Hin*d III 13972 – *Eco*R I 14621) was labeled at the 5′ termini by dephosphorylation with calf intestine phosphatase and rephosphorylation with [γ-^{32}P]ATP and T_4 polynucleotide kinase to obtain the 5′-end-labeled 650-bp fragment (*Hin*d III* 13972 – *Eco*R I* 14621) (*, ^{32}P-label). The 5′-labeled 650-bp fragment was further digested with *Apa* I to obtain a singly labeled double-stranded 211-bp fragment (*Hin*d III* 13972 – *Apa* I 14182). The DNA fragment containing exon 2 of the *CDKN2A* gene was obtained by the PCR amplification of human genomic DNA, ligated into the plasmid pGEM®-T Easy Vector, and amplified by *Escherichia coli* XL1 blue, MRF'. The plasmid was digested with *Eco*R I, and fractionated by electrophoresis on 2% agarose gels. Dephosphorylation with calf intestine phosphatase and phosphorylation with [γ-^{32}P] ATP and T_4 polynucleotide kinase yielded the 5′-end-labeled 460-bp fragment (*Eco*R I *9481 – *Eco*R I *9940). The 460-bp fragment was further digested with *Bss*H II to obtain the singly labeled 309-bp fragment (*Eco*R I *9481 – *Bss*H II 9789). The DNA fragment containing exon 4 of the *HRAS* protooncogene was amplified by PCR from the plasmid pbcN-1 (American Type Culture Collection), which carries a 6.6-kb *Bam*HI chromosomal DNA segment containing the *HRAS* gene. Nucleotide numbering started with the *Bam*HI site. The PCR product was ligated into the pUC19 plasmid vector and amplified by *Escherichia coli* MC1061. A singly labeled 337-bp fragment (*Pst* I 2345 – *Ava* I* 2681) was obtained by *Pst* I digestion of the 5′-end-labeled 435-bp fragment (*Ava* I* 2247 – *Ava* I* 2681) after dephosphorylation and phosphorylation with [γ-^{32}P] ATP and T_4 polynucleotide kinase.

2.3. Detection of DNA damage using ^{32}P-labeled DNA fragments

The standard reaction mixture (1.5-mL Eppendorf) contained the ^{32}P-labeled DNA fragments, naphthalene metabolites (1,2-NQH_2, 1,4-NQH_2, 1,2-NQ 1,4-NQ), calf thymus DNA (20 μM/base), and $CuCl_2$ in 200 μL of 10 mM sodium phosphate buffer (pH 7.8) containing 5 μM DTPA in a microtube. After a 1 h incubation at 37 °C, DNA fragments were treated at 90 °C for 20 min in 1 M piperidine, then electrophoresed on an 8% polyacrylamide/8 M urea gel, as previously described [22]. The preferred cleavage sites were identified by direct comparisons of the positions of the oligonucleotides with those produced by the chemical reactions of the Maxam-Gilbert procedure [23] using a DNA-sequencing system (LKB 2010 Macrophor). A laser densitometer (LKB 2222 UltroScan XL) was used to measure the relative amounts of oligonucleotides, as previously described [24].

2.4. Analysis of 8-oxodG formation in calf thymus DNA

DNA fragments (100 μM/base) from the calf thymus were incubated with the naphthalene metabolites, NADH and $CuCl_2$ in 4 mM phosphate buffer (pH 7.8) containing 5 μM DTPA for the indicated times at 37 °C. After ethanol precipitation, DNA was digested to nucleosides with