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Depurinating naphthalene–DNA adducts in mouse skin related to cancer initiation

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Abstract

Naphthalene has been shown to be a weak carcinogen in rats. To investigate its mechanism of metabolic activation and cancer initiation, mice were topically treated with naphthalene or one of its metabolites, 1-naphthol, 1,2-dihydrodiolnaphthalene (1,2-DDN), 1,2-dihydroxynaphthalene (1,2-DHN), and 1,2-naphthoquinone (1,2-NQ). After 4 h, the mice were sacrificed, the treated skin was excised, and the depurinating and stable DNA adducts were analyzed. The depurinating adducts were identified and quantified by ultraperformance liquid chromatography/tandem mass spectrometry, whereas the stable adducts were quantified by ³²P-postlabeling. For comparison, the stable adducts formed when a mixture of the four deoxyribonucleoside monophosphates was treated with 1,2-NQ or enzyme-activated naphthalene were also analyzed. The depurinating adducts 1,2-DHN-1-N3Ade and 1,2-DHN-1-N7Gua arise from reaction of 1,2-NQ with DNA. Similarly, the major stable adducts appear to derive from the 1,2-NQ. The depurinating DNA adducts are, in general, the most abundant. Therefore, naphthalene undergoes metabolic activation to the electrophilic *ortho*-quinone, 1,2-NQ, which reacts with DNA to form depurinating adducts. This is the same mechanism as other weak carcinogens, such as the natural and synthetic estrogens, and benzene.

Keywords

Naphthalene; Depurinating naphthalene–DNA adducts; Stable naphthalene–DNA adducts; Metabolic activation of naphthalene; 1,2-Naphthalene quinone ultimate carcinogenic metabolite

Introduction

Naphthalene is a component of coal tar products and moth repellents. It is used extensively in the production of plasticizers, resins, insecticides, and surface-active agents [1]. It is a ubiquitous pollutant found mainly in ambient air and to a minor extent in effluent water. The major contributor of naphthalene in air is fossil fuel combustion, but a significant amount is also released as a pyrolytic product of mainstream and side-stream tobacco smoke. Naphthalene was found in nearly 40% of human fat samples [2] and 75% of human breast

milk samples [3]. These facts indicate that the population of the United States is exposed to naphthalene, which is included as one of 189 hazardous air pollutants under the Clean Air Act Amendments of 1990 (Title III) of the Environmental Protection Agency [4].

Chronic inhalation of naphthalene (10 or 30 ppm) by mice led to diverse effects, including inflammation of the nose, metaplasia of the olfactory epithelium, and hyperplasia of the respiratory epithelium [5–7]. No neoplastic effects in male mice were found, but female mice showed a slight increase in alveolar/bronchiolar adenomas and carcinomas at the highest exposure level. More recently, the U.S. National Toxicology Program conducted a 2-year bioassay study with rats exposed to doses of 10, 30, or 60 ppm naphthalene [8,9], showing a concentration-dependent increase in adenomas of the respiratory epithelium of the nose and neuroblastomas of the olfactory epithelium. These results in rodent studies have raised concerns about naphthalene as a potential human carcinogen [1,10].

The toxicity of naphthalene depends on its metabolic activation (Fig. 1). Studies conducted *in vitro* and *in vivo* demonstrated that the first step in the metabolic conversion of naphthalene is the cytochrome P450-dependent formation of the 1,2-epoxide (Fig. 1) [5,11–16]. This compound is unstable at physiological pH [13,14] and can either react with glutathione to form glutathione conjugates or convert to the metabolites 1-naphthol by chemical isomerization or naphthalene 1,2-dihydrodiol (1,2-DDN) by epoxide hydrolase [15,16]. Conversion to 2-naphthol can occur after β -elimination of the sulfated/glucuronidated conjugate of naphthalene 1,2-dihydrodiol (not shown in Fig. 1). 1,2-DDN [17–19] or 1-naphthol [20] can be further metabolically oxidized to 1,2-dihydroxynaphthalene (1,2-DHN) or its oxidized product, 1,2-naphthoquinone (1,2-NQ). 1,2-NQ is thought to be the metabolite that binds covalently to proteins [20,21]. In a recent publication, we reported the predominant formation of depurinating adducts (Fig. 1) after reaction of 1,2-NQ or enzyme-activated 1,2-DHN with DNA [22].

In this article we report our results from topical treatment of SENCAR mice with naphthalene at two dose levels. In addition, mice were treated with metabolites of naphthalene, 1-naphthol, 1,2-DDN, 1,2-DHN, and 1,2-NQ, and formation of naphthalene–DNA adducts was measured. The depurinating adducts were identified and quantified by ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) by using the standard synthesized adducts, 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua, whereas the unknown stable adducts were measured by the ^{32}P -postlabeling technique. These findings are critical for understanding the metabolic activation of naphthalene in the initiation of cancer.

Materials and methods

Chemicals, reagents, and enzymes

1,2-NQ, 1-naphthol, DMSO, boric acid, and NaBH_4 were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,2-DHN and 1,2-DDN were prepared by reacting 1,2-NQ with NaBH_4 as described previously [23]. Proteinase K, Tris (Sigma 7–9), EDTA, 2'-deoxyguanosine 3-monophosphate (dG3p), 2'-adenosine 3-monophosphate (dA3p), 2'-cytidine 3-monophosphate (dC3p), thymidine 3-monophosphate (dT3p), NADPH, and SDS

were purchased from Sigma (St. Louis, MO). Histological grade acetone (Fisher Scientific) was used to prepare solutions for treatment of mouse skin.

SENCAR mouse skin treatment

Forty-nine female SENCAR mice (NCI) at 6 weeks of age were housed in the Eppley Animal Facility. When the mice were 8 weeks old, a dorsal area of the skin was shaved and groups of 4 or 5 mice were treated with the compounds. Two groups were treated with each compound and dose. Naphthalene (1200 or 500 nmol) or its metabolite (500 nmol) was dissolved in acetone to deliver a 50- μ l application on the mouse skin. The treated areas were outlined immediately after the application. Four hours later, the mice were euthanized according to IACUC guidelines, and the treated areas of the skins were excised, placed together for each treatment group in 50-ml tubes, and stored at -20°C . For each set of skins, the epidermis of the mouse skin was isolated, minced, and ground together in liquid N_2 . Approximately 10% of the ground epidermis was used for analyzing chromosome-bound stable DNA adducts by the ^{32}P -postlabeling technique, as described below. The remaining epidermis was processed for analyzing depurinating adducts, as described in the following section.

Sample preparation for analysis of depurinating adducts

Samples of ground epidermis were weighed and suspended in 15 ml of Tris buffer composed of 20 ml of 1 M Tris (pH 8.0), 20 ml of 0.5 M EDTA, and 1 ml of 10% SDS plus distilled water to a total volume of 100 ml. The suspension was homogenized by using a Tissue Tearor (Model 587370, BioSpec Product, Bartlesville, OK). The homogenate was incubated with proteinase K (10 mg dissolved in 1 ml of 1 M Tris, pH 8.0) at 37°C for 5 h. The resulting viscous liquid was cooled on ice, extracted with hexane to remove fat, and finally treated with 2 vol of ethanol. The precipitated material was removed by centrifuging at high speed for 5 min, and the supernatant was removed and evaporated in a Jouan RC1010 centrifuge evaporator (Jouan Inc.). The residue was suspended in 1 ml of $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (1/1), filtered through a 0.2- μm acrodisc syringe filter (Fisher Scientific) directly into a 2-ml Eppendorf tube, and evaporated to about 200 μl . The solution was diluted with 1 ml of distilled water and passed through a Certify II solid phase extraction cartridge (200 mg, Varian Inc, Palo Alto, CA), which had been preequilibrated by passing successively 1 ml each of CH_3OH , H_2O , and 0.1 M potassium phosphate buffer (pH 7.8) through it. The cartridge was then washed with 0.1 M potassium phosphate buffer (pH 7.8) and eluted with 1 ml of elution buffer composed of $\text{CH}_3\text{OH}/\text{CH}_3\text{CN}/\text{H}_2\text{O}$ /trifluoroacetic acid (8/1/1/0.1). The eluate was evaporated to dryness, dissolved in 150 μl of $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (1/1), and analyzed by UPLC-MS/MS, as described below.

Ultraperformance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS)

Duplicate samples for each treatment group were analyzed by using a Waters Acquity Ultra Performance LC equipped with a MicroMass QuattroMicro triple stage quadrupole mass spectrometer (Waters, Milford, MA). The 10- μl injections were carried out on a Waters ACQUITY UPLC BEHC₁₈ column (1.7 μm , 1 $\times$ 100 mm). The instrument was operated in an electrospray–positive ionization mode. All aspects of system operation, data acquisition, and processing were controlled using QuanLynx v4.0 software (Waters). The column was eluted

starting with 5% CH₃CN in H₂O (0.1% formic acid) for 1 min at a flow rate of 150 µl/min and then changed linearly up to 55% CH₃CN in 10 min. Ionization was achieved using the following settings: capillary voltage 3 kV; cone voltage 15–40 V; source block temperature 100°C; desolvation temperature 200°C with a nitrogen flow of 400 L/h. Nitrogen was used as both the desolvation and the auxiliary gas. MS/MS conditions were optimized prior to analysis using pure standards of 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua. Argon was used as the collision gas. Three-point calibration curves covering the range of experimental values were run for each standard; *R* values close to 1.00 were obtained.

Reaction of 2'-deoxyribonucleoside-3-monophosphates with 1,2-NQ or cytochrome P450-activated naphthalene

To a final 1 ml volume composed of a 3 mM solution of each of the four nucleoside 3-monophosphates (dN3p=dAp, dGp, dTp, dCp) in 0.067 M Na-K phosphate, pH 7.0, was added 0.87 mM 1,2-NQ in 20 µl of DMSO. The mixture was incubated at 37 °C for 10 h and then quenched by removal of unreacted 1,2-NQ with chloroform extractions. The aqueous layer was evaporated and the residue was suspended in 25 µl of 0.5 M Tris buffer, pH 7, and then was ³²P-postlabeled as described previously [24]. For the reaction of dN3p with in situ-formed naphthalene-1,2-oxide, the mixture comprised of 0.87 mM naphthalene, 250 units of cytochrome P450 1A1, 0.6 mM NADPH, and 3 mM each of the four dN3p was incubated at 37 °C for 10 h and processed as described previously. The postlabeled adducts were analyzed by polyacrylamide gel electrophoresis (PAGE) [25], as described below.

Polyacrylamide gel electrophoresis analysis of ³²P-postlabeled adducts

The DNA digestion and ³²P-postlabeling were accomplished by following the reported method [24]. Duplicate labeled samples were electrophoresed by following the method reported by Terashima [25], with a slight modification. A nondenaturing 30% polyacrylamide gel (35×42×0.04 cm) was prepared by mixing 60 ml of 40% polyacrylamide solution, 10 ml of 10X TBE buffer, pH 7.0, 10 ml distilled water, 100 µl of 25% ammonium persulfate, and 50 µl of TEMED. The running buffer (1X TBE) was prepared from 10X stock TBE, and Na-pyrophosphate (1 mM final concentration) was added. A 10X TBE stock solution was prepared as reported previously [25]. The position of labeled adducts was established by developing an X-ray film exposed to the gel, which was later scanned to obtain a digital image. To measure the radioactivity of ³²P-labeled products, developed photographic film was superimposed on the gel and areas showing the bands were marked on the gel. Each marked area on the gel was cut out and placed in a scintillation vial and radioactivity (cpm) was counted in a liquid scintillation counter with EcoLume fluor (ICN, Irvine, CA).

Results

Eight-week-old SENCAR mice were treated in duplicate groups on the dorsal skin with naphthalene or one of its metabolites, 1-naphthol, 1,2-DDN, 1,2-DHN, or 1,2-NQ (Fig. 1). After 4 h, the mice were sacrificed, and the skin was removed and processed for analysis of DNA adducts. The raw data obtained from UPLC-MS/MS were normalized with respect to

the total weight of skin used for analysis, and the reported value of 8.5 µg of DNA per gram of skin [26] was used to calculate the results per mole DNA-P.

Depurinating adducts

Treatment of the skin with 500 nmol of naphthalene, dissolved in acetone, resulted in the formation of 0.87 pmol of the depurinating adduct 1,2-DHN-4-N3Ade/g epidermis or 0.1 µmol/mol DNA-P, when normalized with the DNA-P present in the sample (Table 1). Formation of the corresponding N7Gua adduct was not observed at this dose. By increasing the treatment dose to 1200 nmol, the amount of the N3Ade adduct observed was 2.57 pmol/g epidermis (0.3 µmol/mol DNA-P) (Fig. 2, Table 1) and, interestingly, the N7Gua adduct was also observed and quantified at 2.06 pmol/g epidermis (0.2 µmol/mol DNA-P). It is quite possible that the N7Gua adduct was formed in the low dose treatment; however, its amount was under the limit of detection.

Formation of both depurinating adducts, 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua, was also observed by treating the mouse skin with metabolites of naphthalene, namely 1-naphthol, 1,2-DDN, 1,2-DHN, and 1,2-NQ. Treatment with the *ortho*-quinone, 1,2-NQ, produced the depurinating 1,2-DHN-4-N7Gua adduct in almost fivefold higher amounts than that of the corresponding N3Ade adduct (Fig. 2, Table 1). An approximately similar result was obtained when the mouse skin was treated with the catechol metabolite, 1,2-DHN; however, the overall level of the depurinating adducts was slightly lower than that obtained with the quinone metabolite. Treatment with 1,2-DDN produced much lower amounts of the depurinating adducts compared to those obtained with 1-naphthol, 1,2-DHN, or 1,2-NQ (Fig. 2, Table 1). This can be tentatively explained by the absence of the enzyme dihydrodiol dehydrogenase in mouse skin (Fig. 1). In that case, 1,2-DDN would conjugate with sulfate or glucuronide and be converted to 2-naphthol by β-elimination (not shown in Fig. 1) [17–19]. The 2-naphthol can be metabolically oxidized to 1,2-DHN and then further oxidized to 1,2-NQ.

Stable adducts

Formation of stable adducts was measured by using a recently reported method, under slightly modified conditions, to resolve the ³²P-postlabeled adducts by electrophoresis on polyacrylamide gel [25]. Samples 1–4 in Fig. 3 show the standard nucleoside-3-monophosphates after postlabeling. To address the qualitative nature of the bands obtained after treatment of skin with naphthalene or its metabolites, we performed a standard reaction in which a mixture of nucleoside-3-monophosphates reacted with 1,2-NQ (sample 6) or naphthalene 1,2-oxide, produced in situ by incubating naphthalene with cytochrome P450 1A1 [27] (lane 5). Under similar conditions of ³²P-postlabeling, a number of bands (a–i) were observed for different mouse skin treatments (samples 7–12). Bands a and g were present in all of the treatments, whereas other spots were seen with various treatments. Most importantly, bands h and i were present only following treatment with naphthalene, and they corresponded to the bands obtained from naphthalene 1,2-oxide (lanes 5, 11, and 12).

The level of formation of stable adducts was found to be in the range of 0.99–1.09 µmol/mol DNA-P. A dose-dependent increase in formation of stable adducts was not observed when

the dose of naphthalene was changed from 500 to 1200 nmol, as shown in Fig. 4 and Table 1. In treatments with the metabolites, the highest level of adducts was seen with 1,2-NQ (Fig. 4) and, unexpectedly, 1,2-DHN made relatively lower amounts of stable adducts. The overall trend of formation of stable adducts with metabolites was found in the order: 1,2-NQ > 1,2-DDN > 1-naphthol > 1,2-DHN.

Discussion

Naphthalene is the most abundant member of the polycyclic aromatic hydrocarbons, which are produced primarily from incomplete combustion of hydrocarbon fuels. Although hydrocarbons with the greatest carcinogenic potency tend to have four to seven condensed aromatic rings, naphthalene, with two rings, has produced respiratory tract tumors in rats and mice of both sexes [1,7–10]. The apparent carcinogenicity of naphthalene in two mammalian species, coupled with the abundance of naphthalene in indoor and outdoor air, motivated the International Agency for Research on Cancer [1] and the U.S. Environmental Protection Agency [4] to reclassify naphthalene as a possible human carcinogen.

The mechanism of cancer initiation by naphthalene cannot be explained by either of the two major mechanisms of activation of polycyclic aromatic hydrocarbons. In fact, carcinogenic activation of naphthalene cannot occur by formation of diol epoxides [28] because it lacks a bay region or by radical cations because its ionization potential is too high [28]. It can be metabolized, however, to the electrophilic 1,2-NQ, which has the potential to react with DNA. Our previous studies on natural estrogens [29–32], synthetic estrogens [32–34], benzene [35], and dopamine [35] demonstrate the involvement of electrophilic *ortho*-quinones in the predominant formation of depurinating adducts. Therefore, we propose, analogously to the natural estrogens, the metabolic activation of naphthalene to ultimate carcinogenic 1,2-NQ, which reacts with DNA to form adducts (Fig. 5).

The major initiating pathway for estradiol (E_2) is illustrated in Fig. 5. E_2 can be metabolically converted to 4-hydroxy E_2 (4-OHE₂) by cytochrome P450. Oxidation of this catechol estrogen leads to the corresponding E_2 -3,4-quinone, which can react with DNA to form a small amount of stable adducts and predominant amounts of depurinating adducts, 4-OHE₂-1-N3Ade and 4-OHE₂-1-N7Gua (Fig. 5) [29–31]. These adducts detach from DNA, leaving behind apurinic sites, which can generate the critical mutations that initiate cancer [36–39]. Analogously, the conversion of naphthalene to 1-naphthol and further metabolic oxidation to 1,2-DHN and then to 1,2-NQ can lead to reaction of the latter with DNA and generation of stable adducts and depurinating 1,2-DHN-4-N3Ade and 1,2-DHN-4-N7Gua adducts (Fig. 5). As discussed below, we think the apurinic sites formed by loss of the depurinating adducts play a major role in the initiation of cancer by naphthalene.

Depurinating adducts

The depurinating and stable adducts formed in mouse skin after treatment with naphthalene at two dose levels or with naphthalene metabolites produced fairly similar results, as shown in Fig. 4. For the depurinating adducts (Fig. 2), the N3Ade adducts are more abundant than the N7Gua adducts when the skin is treated with naphthalene. Following treatment with the metabolites, the N7Gua adducts are the more abundant ones. Since the depurinating adducts

were analyzed after a 4-h treatment of the skin and the conversion of naphthalene to 1,2-NQ required 3 metabolic steps, we speculate that the N3Ade adducts predominate because they are released from DNA instantaneously. In contrast, when the skins were treated with the metabolites, the conversion to 1,2-NQ required at most two steps. In this case it can be assumed that the instantaneously released N3Ade adducts have the time to diffuse out of the tissue, while the slowly released N7Gua adducts are still accumulated in the skin. This has been previously observed with depurinating polycyclic aromatic hydrocarbon adducts in mouse skin [40].

Stable adducts

A variety of stable adducts were detected when mouse skin was treated with naphthalene or one of its metabolites (Fig. 3). When enzyme-activated naphthalene was reacted with a mixture of the four deoxyribonucleoside monophosphates (lane 5), one major adduct spot was observed (spot h + i). In contrast, when 1,2-NQ was reacted with the dN3ps (lane 6), the major spots were e and f. Adduct spots h and i were also observed when mouse skin was treated with naphthalene (lanes 11 and 12). These results suggest that the adducts in spots h and i arise from the naphthalene 1,2-oxide metabolite.

By far the major adduct spot observed in mouse skin preparations is spot a (lanes 7–12). Because this spot was never observed with the dN3ps (lanes 1–6), we think it is an artifact spot containing partially digested DNA. Therefore, we have calculated the total stable adducts both with and without spot a (Fig. 4, Table 1). When spot a is removed from consideration, the major adducts observed in the skin are e and f, which arise from reaction of 1,2-NQ with the DNA (lanes 6–12).

In addition, the depurinating adducts become the predominant ones with naphthalene and its metabolites, except at the low dose of naphthalene. Although formation of depurinating naphthalene–DNA adducts appears to predominate, the proportion of stable naphthalene–DNA adducts is higher than with the other carcinogens activated through formation of an *ortho*-quinone, which typically generates ca 1% stable adducts [29–35]. The abasic sites formed by loss of the depurinating adducts can generate mutations that may lead to the initiation of cancer.

Conclusions

1,2-NQ and enzymically activated naphthalene, 1-naphthol, 1,2-DDN, and 1,2-DHN react with DNA by 1,4-Michael addition to form specifically the depurinating 1,2-DHN-1-N3Ade and 1,2-DHN-1-N7Gua adducts. The 1,2-NQ also forms the major stable adducts (if spot a is not considered in Fig. 3). These chemical and biochemical properties are similar to those of the catechol-3,4-quinones of the natural estrogens [29–31], the catechol quinones of the synthetic estrogens diethylstilbestrol [34], and its hydrogenated derivative hexestrol [32,33], the catechol quinone of the leukemogen benzene [35], and the neurotransmitter dopamine [35]. The apurinic sites formed by the depurinating DNA adducts may be converted by error-prone base-excision repair into mutations that can initiate cancer [36–39,41]. Therefore, the formation of catechol quinones that can react by 1,4-Michael addition with

DNA to form the depurinating N3Ade and N7Gua adducts constitutes the mechanism of cancer initiation for weak carcinogens.

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Abbreviations

Ade	adenine
1,2-DDN	1,2-dihydro-1,2-dihydroxynaphthalene
1,2-DHN	1,2-dihydroxynaphthalene
dN3p	2'-deoxynucleoside 3-monophosphate
EDTA	ethylenediamine tetraacetic acid disodium salt
Gua	guanine
IACUC	Institutional Animal Care and Use Committee
1,2-NQ	1,2-naphthoquinon
4-OHE₂	4-hydroxyestradiol
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TBE	Tris-boric acid-EDTA buffer
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
UPLC-MS/MS	ultraperformance liquid chromatography–tandem mass spectrometry

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Fig. 1.
Pathways of naphthalene metabolism.

Fig. 2. Formation of depurinating adducts in mouse skin treated with naphthalene or one of its metabolites. The values are the average of two determinations that differed by <20%.

Fig. 3.

PAGE analysis of ^{32}P -postlabeled stable DNA adducts obtained from naphthalene and its metabolites *in vitro* and *in vivo*. For the *in vivo* study, naphthalene (1200 or 500 nmol) or its metabolite (500 nmol) was delivered in 50 μl acetone to mouse skin.

Fig. 4. Quantitative comparison of depurinating and stable adducts formed in mouse skin treated with naphthalene (500 or 1200 nmol) or one of its metabolites (500 nmol). The values are an average of two determinations that differed by <20%.

Fig. 5.
Comparable mechanisms of cancer initiation by natural estrogens and naphthalene.

Formation of depurinating and stable adducts in the mouse skin after treatment with naphthalene or its metabolites

Table 1

Treatments	Depurinating adducts ^a		Stable adducts ^a	
	$\mu\text{mol/mol DNA-P}$		$\mu\text{mol/mol DNA-P}$	
	1,2-DHN-4-N3Ade	1,2-DHN-4-N7Gua	With spot a	Without spot a
Naphthalene				
Low dose (500 nmol)	0.1	ND ^b	0.1	0.99
High dose (1200 nmol)	0.32	0.19	0.51	1.03
Naphthalene metabolites				
1-Naphthol (500 nmol)	0.07	0.43	0.50	0.80
1,2-DDN (500 nmol)	0.01	0.17	0.18	0.90
1,2-DHN (500 nmol)	0.09	0.51	0.60	0.62
1,2-NQ (500 nmol)	0.12	0.59	0.71	1.09
				0.23

^aValues are the average of two determinations that differed by <20%.

^bNot detected.