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Naphthalene DNA Adduct Formation and Tolerance in the Lung

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Abstract

Naphthalene (NA) is a respiratory toxicant and possible human carcinogen. NA is a ubiquitous combustion product and significant component of jet fuel. The National Toxicology Program found that NA forms tumors in two species, in rats (nose) and mice (lung). However, it has been argued that NA does not pose a cancer risk to humans because NA is bioactivated by cytochrome P450 monooxygenase enzymes that have very high efficiency in the lung tissue of rodents but low efficiency in the lung tissue of humans. It is thought that NA carcinogenesis in rodents is related to repeated cycles of lung epithelial injury and repair, an indirect mechanism. Repeated *in vivo* exposure to NA leads to development of tolerance, with the emergence of cells more resistant to NA insult. We tested the hypothesis that tolerance involves reduced susceptibility to the formation of NA-DNA adducts. NA-DNA adduct formation in tolerant mice was examined in individual, metabolically-active mouse airways exposed *ex vivo* to 250 μM ^{14}C-NA. *Ex vivo* dosing was used since it had been done previously and the act of creating a radioactive aerosol of a potential carcinogen posed too many safety and regulatory obstacles. Following extensive rinsing to remove unbound ^{14}C-NA, DNA was extracted and ^{14}C-NA-DNA adducts were quantified by AMS. The tolerant mice appeared to have slightly lower NA-DNA adduct levels than non-tolerant controls, but intra-group variations were large and the difference was statistically insignificant. It appears the tolerance may be more related to other mechanisms, such as NA-protein interactions in the airway, than DNA-adduct formation.

Keywords

naphthalene; DNA adducts; carcinogen; tolerance test

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Introduction

Naphthalene (NA) is a common volatile combustion product found throughout the environment. It is the smallest poly-aromatic hydrocarbon (PAH) and is found both outdoors and indoors [1–4]. Common sources of NA to the general population include vehicle emissions, biomass burning, cigarette smoke, mothballs, and house-hold block deodorizers [5]. NA is also widely used as a precursor or building block of other chemicals in industrial applications. It is used in the manufacture of plasticizers, dyes, resins, tanning agents and pesticides, can be used as a surfactant, and constitutes 1–3% of JP8 jet fuel [5]. NA metabolites have been found in the urine of most adults and children tested, regardless of occupation or locale [4,6–11]. NA is an established animal carcinogen producing elevations in bronchiolar alveolar carcinomas in female mice and dose dependent increases in neuroblastomas in the nasal epithelium of rats [12–16].

Chemicals that form stable DNA adducts in target tissues are generally considered carcinogens. These compounds are treated as though high dose can be linearly extrapolated to zero without a threshold for natural clearance of the adducted region. This approach of extrapolating high dose to environmental exposure probably over-estimates risk [17–19]. In this study, we used the sensitivity of AMS to measure adducts of ^{14}C-NA at environmentally relevant doses, the lung tissue concentration of NA from an aerosol NA exposure of 10 ppm, the occupational limit for NA exposure set by the U.S. Occupational Safety and Health Administration (OSHA).

The goal of the study was to determine if mice made tolerant to naphthalene would have fewer DNA adducts than naive animals. Tolerance is the resistance to further insult in susceptible cells after exposure to a repeated insult. Previous studies have demonstrated that the mechanism of NA tolerance and NA acute injury is the same regardless of route of exposure [20–23]. We expect that the glutathione adaptive response to NA exposure, with subsequent glutathione-mediated detoxification in these animals will result in them being less susceptible to formation of DNA adducts.

AMS has long been used to assess natural and anthropogenic contributions to particulate matter (PM) mass collected on filters [24–27]. In traditional source apportionment studies there are often no attempts to separate chemicals such as PAHs in PM. The studies are used to attribute anthropogenic fossil or biogenic sources of PM. More recently, specific large non-volatile PAHs commonly found in PM have been labeled with ^{14}C to ascertain bioavailabililty of ingested PAHs, metabolism, and elimination [28,29].

Conducting an exposure study with a radiolabeled aerosol is a challenging safety design and engineering problem. When the test compound is a potential carcinogen, it is nearly impossible to satisfy safety concerns within the budget limitations of a typical toxicology study, even when using low levels of radioactivity enabled by AMS measurement. Creating, controlling, and confirming control of an aerosol is difficult. *Ex vivo* dosing of fresh, metabolically active tissue is an alternative dosing procedure that mitigates safety and containment issues of an intentional aerosol. The concentration of NA used in culture (250 μM) for *ex vivo* exposure was comparable to the tissue NA concentration in airway cells

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when live mice were exposed to 10 ppm NA aerosol for 1 h [30]. The *ex vivo* exposure in culture produced a similar concentration of NA in the tissue by liquid diffusion as was produced by aerosol exposure [30].

Materials and Methods

Radiochemicals.

^{14}C-NA (98% pure) was diluted to a specific activity of 0.39 mCi/mmol (25 mM) with non-radiolabeled NA (Sigma, cat# 84679) in acetonitrile from a stock solution of ^{14}C-NA (1,4,5,8-^{14}C; 98% pure) in methanol (MC 2147; 500 μCi, 58 mCi/mmol; Moravek Biochemicals, Brea, CA).

Mouse Tissue.

Adult female NIH Swiss mice were purchased from ENVIGO. Mice were kept in a barrier facility with filtered air in Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved conditions on a 12h light/dark cycle with food and water *ad libitum* (always available). All animal experiments were performed under protocols approved by the Lawrence Livermore National Laboratory or University of California Davis Institutional Animal Care and Use Committee (IACUC) in accordance with National Institutes of Health guidelines. Each dosed group contained six mice.

Mice were made tolerant using 7 daily intraperitoneal injections of unlabeled NA at 200 mg/kg given in ~200 μL of corn oil (20 mg NA/mL corn oil). A tolerance control group received intraperitoneal injections of corn oil only on the same schedule. On day 8 mice were euthanized with an overdose of Fatal-Plus® C IIN (Vortech Pharmaceuticals, pentobarbital sodium). Lungs were removed as described [31–34] and airways were microdissected to isolate the metabolically active part of the lung for *ex vivo* exposure to ^{14}C-NA [33,34]. Each mouse lung was split in half with one side serving as the ^{14}C-NA exposure group and the other side serving as undosed control. Figure 1 shows an example of a microdissected mouse airway.

Tissue incubation.

Freshly dissected, metabolically active tissue was placed in a 20-ml scintillation vial with 990 μL of media and kept on ice until the start of the exposure. Radiolabeled ^{14}C-NA (10 μL of 25 mM NA) was added to each vial to yield final concentration of 250 μM NA. Vials were sealed and incubated in a water bath for 1 hour at 37°C. The water bath was kept in a fume hood and the vials were agitated by hand for ~10 s every 5 min during exposure. Parallel incubations were conducted with tissue in 990 μL media with 10 μL acetonitrile, and used as an indicator of undosed background level of ^{14}C. At the end of the exposure, all vials were cooled and promptly transferred to screw-top microfuge tubes. Tissue was washed 20–25 times with 1 mL of 100% ethanol (EtOH). The microfuge tubes were changed every 3 rinses to remove the tissue from residual unbound NA. Washes were checked using a liquid scintillation counter (PerkinElmer Liquid Scintillation Analyzer, Tri-Carb 2910R, Waltham, MA) and considered clean when the rinse ethanol exhibited background levels of ^{14}C as

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from 1 mL of fresh EtOH. At the completion of rinsing, all liquid was removed and each tissue sample was placed in new screw top microcentrifuge tube and stored frozen.

DNA isolation and quantification.

Frozen tissue was placed in 2-mL FastPrep Lysing Matrix Z tubes (2-mm zirconia beads; MP Biomedicals, Santa Ana, CA) with 500 μL ATL buffer (Qiagen, Hilden, Germany) in preparation for tissue destruction and homogenization. Tissue was homogenized using a mini bead beater mill (Cole-Parmer; Vernon Hills, IL) and employing 8–10 cycles of 40-second agitations, placed on ice between bead beating steps to prevent excessive heating. DNA was isolated from homogenized tissue using DNeasy Blood & Tissue kits (Cat No./ID: 69504; Qiagen, Hilden, Germany) following manufacturer instructions with the modification of extra protein digestion. Since NA metabolites are known to form plentiful protein adducts [35–37], an additional proteinase K lysis step was added to the base Qiagen protocol (Purification of Total DNA from Animal Tissues (Spin-Column Protocol)). The final proteinase K incubation time was 4 hours, with 25 μL of proteinase K (20 mg/mL) added at the beginning and 25 μL additional proteinase K added at 2 hours. Then 4 μl RNase A (100 mg/ml) was added to each sample after the proteinase K lysis step, and samples were incubated with RNase A for 2 minutes at room temperature. To maximize DNA yield from microdissected tissue samples, each sample was divided and purified through two DNAeasy columns and then recombined after elution. DNA was eluted twice, first with 200 μL and then with 100 μL of Buffer AE. DNA concentration and purity was monitored using a NanoDrop™ spectrophotometer.

AMS sample preparation and analysis.

All glass or quartz was baked prior to use following the standard practices at LLNL [38,39]. Each DNA sample suspended in water was transferred into a quartz combustion vial (6mm o.d. x 30 mm length) and dried overnight in a vacuum centrifuge. In the morning, combustion vials were removed from the centrifuge and 1-μL tributyrin (ICN Pharmaceuticals, Inc.; Costa Mesa, CA) was added as carbon carrier (0.59 mg C) to each vial using a volumetric capillary tube (Drummond Scientific Company, Broomall, PA) [35,40]. The carrier carbon is added to each purified DNA sample to ensure robust graphite formation. The use of 1-μL capillary tubes to deliver carrier improved the consistency of carrier addition over the previous technique of diluting tributyrin in methanol and adding 20–40 μL of tributyrin-methanol suspension [41–43]. Each vial received ≈40 mg of CuO as it was transferred to a quartz combustion tube that was evacuated and sealed with a torch. The sealed tubes were combusted at 900 °C for 3.5 h to oxidize all organic carbon to CO2 and then reduced to filamentous carbon using Ognibene's method [39]. Graphite samples were measured on a National Electrostatics Corporation (Middleton, WI) 250 kV single stage AMS spectrometer at the Lawrence Livermore National Laboratory [44]. AMS measurement times for these carrier-added graphite samples with $F^{14}C$ = 2–6 modern were typically 5–10 min/sample with a counting precision (relative standard deviation, RSD) of 0.5 % to 3 % and a standard deviation among 3 to 10 measurements of 1 % to 3 % [45]. The $^{14}C/^{13}C$ ratios of the samples were normalized to measurements of four identically prepared IAEA C-6 isotopic standards.

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Statistics.

Measurement Data are reported as mean ± standard deviation. Microsoft Excel™ was used to perform statistical analysis. Values of $p < 0.05$ were considered statistically significant when performing a Student's t-test with two-tailed, homoscedastic data.

Results and Discussion

All DNA samples were assayed for total yield and purity prior to AMS analyses. The results are shown in Table 1. The UV absorbance ratio (260 nm/280 nm) was consistent with protein free preparations. A 260/280 ratio > 1.80 is considered pure DNA. If the DNA concentration is <10 ng/ μL the spectrophotometer results are suspect. All samples were well above this limit. All DNA samples were relatively large considering the small amount of mouse lung tissue source material.

The AMS measurements of carrier added DNA presented no unexpected challenges. The tributyrin carbon carrier blanks and the undosed DNA background controls that did not receive ^{14}C-NA both had $F^{14}C \approx 0.1$. The $F^{14}C$ of the undosed DNA were slightly lower than tributyrin blanks as seen historically with the column DNA prep used, although the difference was not significant (p=0.16, Student's T-test). The dead carbon retained in the DNA prep is probably a combination of retained solvent and column bleed. We use the undosed controls for background subtraction rather than the tributyrin carrier blanks to include this small amount of dead carbon when doing carrier added DNA analyses. The samples dosed with ^{14}C-NA were all well elevated above the controls with measured $F^{14}C>2$.

Measured isotope ratios were converted to biologically relevant units using the carbon concentration, isotope ratio and mass of carbon carrier, the specific activity of the labelled compound, the formula weight (FW) of NA, and the DNA mass. Details on the procedure can be found in the literature [45–47]. The net ^{14}C above control average in each sample was converted into mass or atoms of NA and normalized by the mass of DNA in the sample.

$$\frac{\text{NA(fg)}}{\text{DNA}(\mu\text{g})} = \frac{\text{Net}^{14}\text{C(fmole)}}{\text{fraction NA labeled}} \times \text{FW}\left(\frac{\text{fg}}{\text{fmole}}\right) \times \frac{1}{\text{DNA mass}(\mu\text{g})} \quad (1)$$

$$\frac{\text{NA adducts}}{\text{DNA(pg)}} = \frac{\text{Net}^{14}\text{C(fmole)}}{\text{fraction NA labeled}} \times \frac{\text{atoms}}{\text{fmole}} \times \frac{1}{\text{DNA mass (pg)}} \quad (2)$$

The NA adduct levels normalized to DNA mass as calculated in Eq. 2 are depicted in Fig. 2. Individual samples were measured precisely but the scatter between samples yielded a large standard deviation for both NA tolerance (Fig. 2A) and control tolerance (Fig. 2B) groups. Although the NA tolerance mice had fewer adducts on average, the difference compared to controls was not significant (p=0.45, Student's T-test) due to variability between animals (Fig. 2C).

The reduction in NA-DNA adducts in the tolerant mice was consistent with previous tolerance studies of mice airways that examined Club cell toxicity, reductions in enzyme bioactivation to reactive metabolites, and detoxification mechanisms of NA [20–23]. Since NA metabolites form the DNA adducts, any mechanisms that reduce the number of reactive NA metabolites also reduce the number of NA-DNA adducts. Reducing the number of Club cells from acute toxicity response directly depletes the amount of enzyme available to produce reactive metabolites. This may be the most important factor since NA-DNA adducts are rare. Repeated exposures of NA to club cells produced an increase in glutathione (GSH) resynthesis [20–23]. Since GSH conjugates are the primary mechanism of detoxification, tolerance exposures increase the ability of Club cells to detoxify NA metabolites before adducts form. Nevertheless, NA-protein adducts are much more plentiful than NA-DNA adducts [35,37,48]. While a decrease in the concentrations of reactive metabolites available could lead to reduced DNA- and protein adducts, the effects may be more pronounced for protein adducts than for DNA adducts, as the present study suggests.

Conclusion

NA-DNA adduct formation in tolerance condition was examined in individual, metabolically-active mouse airways exposed *ex vivo* to 250 μM ^{14}C-NA. DNA was extracted and ^{14}C-NA-DNA adducts were quantified by AMS. The tolerant mice had only slightly lower NA-DNA adduct levels than controls. Thus, while the effects of tolerance - reduced numbers of Club cells and consequently reduced bioactivation enzyme activity along with enhanced detoxification from enhanced GSH - likely reduce the total amount of reactive NA metabolites available to form adducts, the impact on the number of NA-DNA adducts formed was negligible.

The AMS measurements enabled quantitation of NA derived adducts but did not identify which metabolites of NA form the adducts. Identification of specific NA metabolite adducts requires quantitative digestion of the DNA into individual nucleotides followed by a molecular separation and then quantitation. The HPLC liquid sample interface coupled to MS and AMS with a variable flow splitter could be employed to identify specific adducts [49,50].

Ex vivo dosing was used since it avoided creation of a radioactive aerosol of a potential carcinogen. This *ex vivo* dosing approach can be used to quantify adduct formation or receptor binding of volatile chemicals or aerosols in respiratory tissue. When working with acute toxins or potential infectious agents, keeping the test compounds in the liquid phase is much safer.

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Fig. 1.
Microdissected mouse airway.

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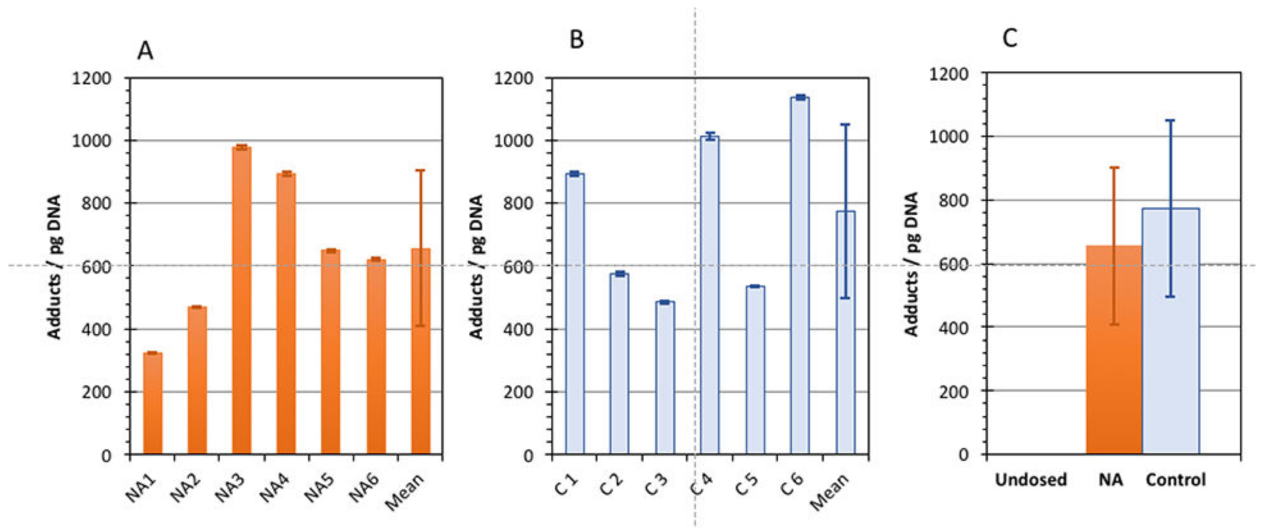

Fig. 2.

Adduct levels of individual samples and means for NA tolerance (A) and control tolerance (B) groups. The averages are compared to each other and the undosed controls in (C). The undosed control average was −0.7±2.1 adducts/pg DNA. Each mouse cell contains 5.6 pg of nuclear DNA.

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Table 1.

DNA purity and mass measurements. Samples NA1–6 received NA for seven days prior to *ex vivo* ^{14}C-NA dosing. Samples C1–6 received control corn oil but no NA for seven days prior to *ex vivo* ^{14}C-NA dosing. Samples B1–4 were background controls that did not receive ^{14}C-NA.

Sample	Absorbance Ratio (260/280)	DNA Concentration (ng/μl)	Total Mass (μg)
NA1	1.89	91.6	54.0
NA2	1.87	103.1	60.8
NA3	1.82	45.0	26.8
NA4	1.87	62.5	36.9
NA5	1.84	58.6	34.0
NA6	1.84	54.4	32.1
C1	1.92	42.1	24.8
C2	1.93	38.7	22.4
C3	1.89	45.7	26.7
C4	1.88	48.0	28.6
C5	1.85	66.1	39.3
C6	1.83	42.6	25.6
B1	1.87	96.3	54.9
B2	1.88	69.9	41.2
B3	1.80	37.5	21.9
B4	1.80	41.0	24.2