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Naphthalene toxicity in mice and aryl hydrocarbon receptor-mediated CYPs

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Abstract

Naphthalene (NP) has been designated a 'reasonably anticipated human carcinogen' because of positive responses in carcinogenicity bioassays in rodents. Whereas CYP2F enzymes are widely regarded as responsible for NP bioactivation, other metabolic enzymes—including CYP1A1 and CYP1A2—produce NP-1,2-oxide *in vitro*. We investigated the role of these aryl hydrocarbon receptor (AHR)-mediated enzymes in NP toxicity in two ways. First, NP was assessed for the ability to activate transcription via the AHR in an *in vitro* luciferase reporter assay and was found to have no activity. Second, mice deficient in AHR, CYP1A1 or CYP1A2 were dosed with NP alone, or following pretreatment with the CYP2F inhibitor 5-phenyl-1-pentyne. None of the knockout mice were protected from olfactory toxicity of NP. In contrast, CYP1A1- and CYP1A2-null mice pretreated with 5-phenyl-1-pentyne exhibited no NP olfactory toxicity. These results suggest that AHR-mediated enzymes do not contribute significantly to NP bioactivation in the intact animal.

Keywords: Naphthalene; Aryl hydrocarbon receptor; 2,3,7,8-Tetrachlorodibenzo[*p*]dioxin (TCDD); Bioactivation; CYP1A1; CYP1A2

Naphthalene (NP) is a widespread environmental contaminant because of its generation as a combustion by-product, use as a pesticide and pesticide synthesis intermediate, and as component of fuels such as JP-8 [1–3]. Toxicity associated with NP exposure was, until recently, associated primarily with cataract formation, hemolytic anemia, and respiratory tract irritation [4–6]. However, bioassays conducted by the National Toxicology Program have opened the debate as to whether NP poses a cancer risk to exposed humans [7,8]. Specifically, chronic bioassays conducted in B6C3F$_1$ mice and F344/N rats, in which NP was administered via inhalation for 2 yr (0–30 ppm for mice; 0–60 ppm for rats), showed "clear evidence" of carcinogenicity. An excess of nasal respiratory epithelial adenomas was observed in male rats, whereas female rats developed predominantly olfactory mucosal neuroblastomas. Mice exhibited nasal irritation, but not an excess of nasal tumors, and female mice developed an excess of pulmonary alveolar/bronchiolar adenomas. NP is currently the subject of widespread public and scientific scrutiny. NP was listed for the first time in the 11th edition of the NTP Report on Carcinogens as a 'reasonably anticipated human carcinogen' [9]; and NP is classified as a group 2B, or possible human carcinogen, by the International Agency for Research on Cancer [10].

The mechanism of NP's carcinogenic activity is not understood. NP is widely regarded as non-mutagenic, based on the results of *in vitro* mutagenicity assays showing that NP is non-mutagenic ± liver S9 activation in multiple *Salmonella typhimurium* strains. However, cytogenetic tests with cultured Chinese hamster ovary cells showed an increase in sister chromatid exchanges ± metabolic activation and an increase in chromosomal aberrations with liver S9 activation [7,8]. Further, the 1,2- and 1,4-naphthoquinone metabolites of NP are mutagenic *in vitro* [11,12].

Multiple metabolic enzymes, including mouse, human, rat, and goat cytochrome P450s (CYPs), metabolize NP

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(Table 1), but the relative contributions of the various CYPs *in vivo* have not been systematically evaluated. Mouse CYP2F2 has a low K_m and high V_{max} for NP, whereas human CYP2F1 and rat CYP2F4 have a considerably lower turnover rate (less than 0.1% of mouse CYP2F2) [13,14]. Both CYP1A1 and CYP1A2 have been reported to metabolize NP *in vitro* [15,16], and both of these enzymes are under the transcriptional control of the aryl hydrocarbon receptor (AHR). In contrast, AHR-regulated CYP1B1 appears not to have catalytic activity in NP metabolism [17].

Table 1
Cytochrome P450 enzymes shown to catalyze initial epoxidation reaction of NP

Enzyme	Citation
CYP1A1	[15]
CYP1A2	[16]
CYP2A5/6	[18]
CYP2B isoforms	[19,20]
CYP2E1	[21]
CYP2F isoforms	[13,14,22–24]

The goals of this study were to follow up on the *in vitro* observations of other laboratories as to the relative contributions of enzymes under transcriptional control of the AHR to NP bioactivation and to determine whether NP itself can activate gene transcription via the AHR.

Methods

Chemicals. Naphthalene and dimethyl sulfoxide were obtained from Fisher Scientific; 5-phenyl-1-pentyne was purchased from GFS (Columbus OH); TCDD was purchased from Accustandard, Inc. (New Haven, CT).

Mice. *Ahr*−/−, *Cyp1a1*−/−, and *Cyp1a2*−/− knockout mice were generated from in-house breeding colonies, and wild-type littermates were used as controls. Generation of the respective knockout mouse lines has been previously described [25–27], and these mice are maintained on the C57BL/6J background. Female mice were 6–8 weeks of age when treated. Mice were maintained on 12 h light/dark cycles and received rodent chow (Teklad/Harlan LM-485; Indianapolis, IN, USA) and tap water *ad libitum*. Mice received one of the following treatments by intraperitoneal injection: [a] 200 mg/kg of NP in corn oil [Mazola] (all genotypes; $n = 4$/genotype); [b] corn oil only (all genotypes; $n = 4$/genotype); [c] 200 mg/kg 5-phenyl-1-pentyne, diluted in corn oil, followed 2 h later by 200 mg/kg NP (*Cyp1a1*−/−, *Cyp1a2*−/−, and wild type mice; $n = 4$/genotype); [d] 5-phenyl-1-pentyne in corn oil only (*Cyp1a1*−/−, *Cyp1a2*−/−, and wild

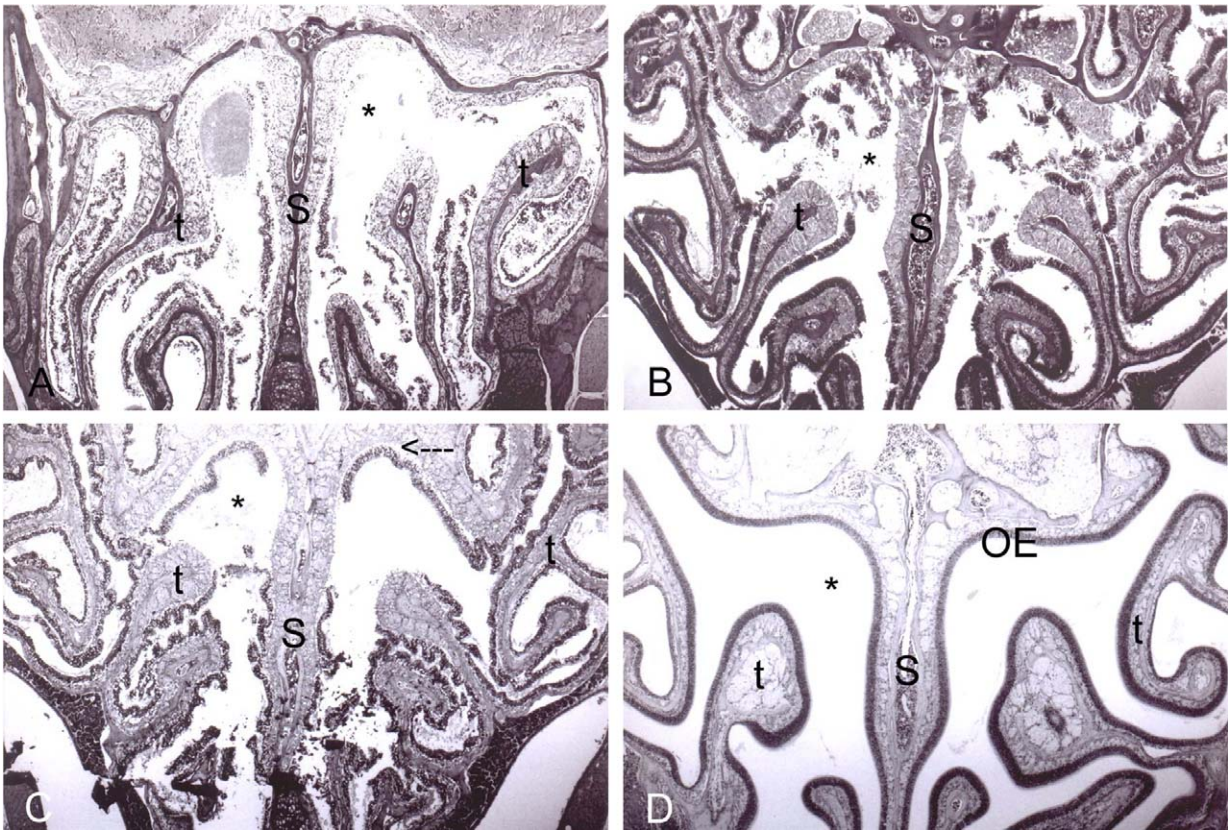

Fig. 1. (A) Frontal section through the ethmoid turbinates of a C57BL/6J (wild type) mouse treated with 200 mg/kg NP and sacrificed 18 h later. Note extensive sloughing of the olfactory epithelium from the dorsal nasal septum (S) and nasal turbinates (t) into the nasal airways (*). (B) Similar damage in the nasal cavity of a *Cyp1a1*−/− mouse following the identical NP treatment. The lesions noted in the nasal cavities of *Cyp1a2*−/− mice are identical (not shown). (C) *Ahr*−/− mice are similarly not protected from NP-induced olfactory epithelial sloughing. Note complete separation of the epithelium from the basement membrane at the top of the panel (arrow). (D) *Cyp1a1*−/− pretreated with 200 mg/kg 5-phenyl-1-pentyne, followed 2 h later by 200 mg/kg NP. Mice were sacrificed 18 h after NP treatment. Note smooth olfactory epithelium (OE) covering the nasal septum (S) and turbinates (t), as well as absence of debris from nasal airways (*). The histological appearance of this nasal cavity is indistinguishable from vehicle treated controls of all genotypes (not shown). Paraffin sections were stained with hematoxylin and eosin; micrographs were photographed at 4× magnification.

type mice; $n = 4$/genotype). Mice were sacrificed by carbon dioxide asphyxiation 18 h after NP administration. Lungs were examined grossly for evidence of hemorrhagic lesions; nasal cavities were flushed with neutral buffered formalin, fixed overnight in formalin, and then decalcified in 10% formic acid containing Rexyn ion exchange resin. After paraffin embedding, sectioning at 5-μm thickness, and staining with hematoxylin and eosin, sections corresponding to approximately Level 3 and Level 4 of the nasal cavity were examined by light microscopy [28]. Olfactory mucosal toxicity has been reported to be a more sensitive endpoint for NP toxicity in mice than lung toxicity [29], and hence our decision to examine this tissue histologically.

AHR-mediated gene transcription assay. A wild-type Hepa-1 cell line with a stably integrated pAhRDtkLuc3 was grown to confluence as previously described [30] and then seeded into 24-well culture plates. After allowing 24 h for cells to adhere to wells, the cultures were treated with DMSO; TCDD (10 nM in DMSO); or NP at concentrations of 0.5, 1, 10, or 50 μM ($n = 4$ wells per treatment). Cells were harvested 24 h later in Reporter Lysis buffer (Promega) and luminescence was determined at 495 nm. Protein concentrations were measured using the Bradford acid assay (Bio-Rad), and results were expressed as means and standard errors of the mean for each treatment condition.

Results

Knockout mouse studies

Wild-type, *Cyp1a1*−/−, and *Cyp1a2*−/− mice treated with 5-phenyl-1-pentyne + NP showed no overt sign of toxicity 18 h after treatment and were completely protected from the olfactory toxicity of NP. In contrast, the non-pretreated NP-treated wild-type, *Cyp1a1*−/−, and *Cyp1a2*−/− mice showed labored breathing and hemorrhagic lungs upon sacrifice, as well as nearly 100% sloughing of the olfactory mucosa (Fig. 1). *Ahr*−/− mice were similarly not protected from NP-induced olfactory mucosal degeneration and pulmonary hemorrhage.

Gene transcription assay

Fig. 2 shows the results of studies designed to determine whether NP activates gene transcription via the AHR. Clearly, whereas TCDD was effective in this assay, NP had no activity over a 100-fold range of concentrations (0.5–50 μM).

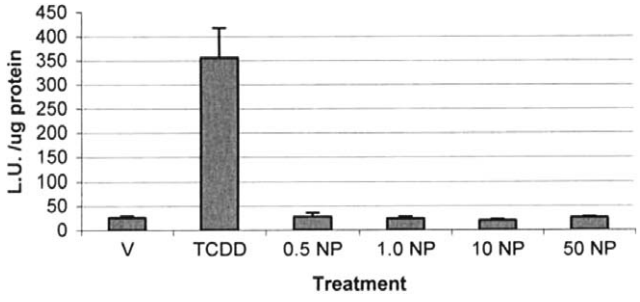

Fig. 2. NP is negative over a 100-fold range of concentrations (0.5–50 μM NP; V = DMSO vehicle treatment) for activity in induction of transcription through the AHR. The AHR ligand 2,3,7,8-tetrachlorodibenzo-[*p*]-dioxin (TCDD; 10 nM) gave a strong positive response, but all NP concentrations tested were indistinguishable from the vehicle control (means ± SEM).

Discussion

The results of our *in vivo* NP treatment studies revealed that mice in which the *Ahr* gene is ablated are not protected from NP cytotoxicity. We also investigated the impact of the absence of the *Cyp1a1* and *Cyp1a2* genes on susceptibility to NP-induced respiratory tract damage, given that both genes are inducible in lung, and the expressed proteins have been shown to convert NP to NP-1,2-oxide *in vitro* [15,16,31]. NP did not stimulate gene transcription via the AHR in our study, suggesting that neither CYP1A1 nor 1A2 would be present in the lung given that they are undetectable in the absence of administration of an inducing agent such as 3-methylcholanthrene [31]. Therefore, our observation that *Cyp1a1*−/− and *Cyp1a2*−/− mice are not different from wild-type mice in their susceptibility to the respiratory tract toxicity of NP is not surprising.

In addition, this study shows that inhibition of CYP2F2 by 5-phenyl-1-pentyne treatment decreases the respiratory tract toxicity of NP in both knockout strains of mice. This effect is presumably due to inhibition of bioactivation of NP to the pneumotoxicant NP-1,2-oxide by CYP2F2 [32–34]. These results suggest that, in the absence of CYP2F2 (or in the presence of a CYP2F isozyme that has less activity toward NP than murine CYP2F2, such as human CYP2F1 or rat CYP2F4), the respiratory tract toxicity and carcinogenicity of NP would be a less significant risk. In the presence of CYP1A1 and 1A2 inducers, however, we cannot rule out the possibility that these enzymes might contribute to the bioactivation or detoxication of NP. These studies confirm previous results that the respiratory tract toxicity of NP in the mouse results from CYP2F2 bioactivation of NP to one or more species that deplete cellular glutathione and thereby cause cytotoxicity [29].

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References

[1] H. Witschi, I. Espiritu, R. Maronpot, K.E. Pinkerton, A.D. Jones, The carcinogenic potential of the gas phase of environmental tobacco smoke, Carcinogenesis 18 (1997) 2035–2042.
[2] J.N. McDougal, D.L. Pollard, W. Weisman, C.M. Garrett, T.E. Miller, Assessment of skin absorption and penetration of JP-8 jet fuel and its components, Toxicol. Sci. 55 (2000) 247–255.
[3] United States Environmental Protection Agency (2000). Health and Environmental Effects Profile for Naphthalene. U.S. Environmental Protection Agency, Washington, DC, EPA/600/X-86/241 (NTIS PB88242383).
[4] ATSDR, ToxFAQs™ for naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene, http://www.atsdr.cdc.gov/tfacts67.html (2005).
[5] E.J. Molloy, B.A. Doctor, M.D. Reed, M.C. Walsh, Perinatal toxicity of domestic naphthalene exposure, J. Perinatol. 24 (2004) 792–793.

[6] M. Kaplan, C. Hammerman, Severe hemolysis and hyperbilirubinemia due to perinatal naphthalene exposure, J. Perinatol. 25 (2005) 359.
[7] National Toxicology Program (1992). Toxicology and carcinogenesis studies of naphthalene (CAS No. 91-20-3) in B6C3F$_1$ mice (inhalation studies). U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH publication 92-3141; http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr410.pdf.
[8] National Toxicology Program (2000). Toxicology and carcinogenesis studies of naphthalene (CAS No. 91-20-3) in F344/N rats (inhalation studies). U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH publication 01-4434; http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr500.pdf.
[9] National Toxicology Program http://ntp.niehs.nih.gov 11th Report on Carcinogens (2005) US Department of Health and Human Services.
[10] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, IARC Monogr. Eval. Carcinog. Risks Hum. 82 (2002) 1–556.
[11] L. Flowers-Geary, W. Bleczinki, R.G. Harvey, T.M. Penning, Cytotoxicity and mutagenicity of polycyclic aromatic hydrocarbon ortho-quinones produced by dihydrodiol dehydrogenase, Chem. Biol. Interact. 99 (1996) 55–72.
[12] A.S. Wilson, M.D. Tingle, M.D. Kelly, B.K. Park, Evaluation of the generation of genotoxic and cytotoxic metabolites of benzo[*a*]pyrene, aflatoxin B1, naphthalene and tamoxifen using human liver microsomes and human lymphocytes, Hum. Exp. Toxicol. 14 (1995) 507–515.
[13] M.A. Shultz, P.V. Choudary, A.R. Buckpitt, Role of murine cytochrome P-450 2F2 in metabolic activation of naphthalene and metabolism of other xenobiotics, J. Pharmacol. Exp. Ther. 290 (1999) 281–288.
[14] R.M. Baldwin, M.A. Shultz, A.R. Buckpitt, Bioactivation of the pulmonary toxicants naphthalene and 1-nitronaphthalene by rat CYP2F4, J. Pharmacol. Exp. Ther. 312 (2005) 857–865.
[15] J.F. Greene, J. Zheng, D.F. Grant, B.D. Hammock, Cytotoxicity of 1,2-epoxynaphthalene is correlated with protein binding and in situ glutathione depletion in cytochrome P4501A1 expressing Sf-21 cells, Toxicol. Sci. 53 (2000) 352–360.
[16] T.M. Cho, R.L. Rose, E. Hodgson, In vitro metabolism of naphthalene by human liver microsomal cytochrome P450 enzymes, Drug Metab. Dispos. 34 (2006) 176–183.
[17] U. Savas, C.P. Carstens, C.R. Jefcoate, Biological oxidations and P450 reactions. Recombinant mouse CYP1B1 expressed in *Escherichia coli* exhibits selective binding by polycyclic hydrocarbons and metabolism which parallels C3H10T1/2 cell microsomes, but differs from human recombinant CYP1B1, Arch. Biochem. Biophys. 347 (1997) 181–192.
[18] A. Asikainen, J. Tarhanen, A. Poso, M. Pasanen, E. Alhava, R.O. Juvonen, Predictive value of comparative molecular field analysis modelling of naphthalene inhibition of human CYP2A6 and mouse CYP2A5 enzymes, Toxicol. In Vitro 17 (2003) 449–455.
[19] K. Nagata, B.M. Martin, J.R. Gillette, H.A. Sasame, Isozymes of cytochrome P-450 that metabolize naphthalene in liver and lung of untreated mice, Drug Metab. Disp. 18 (1990) 557–564.
[20] P.J. van Bladeren, J.M. Sayer, D.E. Ryan, P.E. Thomas, W. Levin, D.M. Jerina, Differential stereoselectivity of cytochromes P-450b and P-450c in the formation of naphthalene and anthracene 1,2-oxides. The role of epoxide hydrolase in determining the enantiomer composition of the 1,2-dihydrodiols formed, J. Biol. Chem. 260 (1985) 10226–10235.
[21] A.S. Wilson, C.D. Davis, D.P. Williams, A.R. Buckpitt, M. Pirmohamed, B.K. Park, Characterisation of the toxic metabolite(s) of naphthalene, Toxicology 114 (1996) 233–242 (Erratum in: Toxicology 1997 120 (1997) 75).
[22] J.K. Ritter, I.S. Owens, M. Negishi, K. Nagata, Y.Y. Sheen, J.R. Gillette, H.A. Sasame, Mouse pulmonary cytochrome P-450 naphthalene hydroxylase: cDNA cloning, sequence, and expression in *Saccharomyces cerevisiae*, Biochemistry 30 (1991) 11430–11437.
[23] H. Wang, D.L. Lanza, G.S. Yost, Cloning and expression of CYP2F3, a cytochrome P450 that bioactivates the selective pneumotoxins 3-methylindole and naphthalene, Arch. Biochem. Biophys. 349 (1998) 329–340.
[24] D.L. Lanza, E. Code, C.L. Crespi, F.J. Gonzalez, G.S. Yost, Specific dehydrogenation of 3-methylindole and epoxidation of naphthalene by recombinant human CYP2F1 expressed in lymphoblastoid cells, Drug Metab. Dispos. 27 (1999) 798–803.
[25] P. Fernandez-Salguero, T. Pineau, D.M. Hilbert, T. McPhail, S.S. Lee, S. Kimura, D.W. Nebert, S. Rudikoff, J.M. Ward, F.J. Gonzalez, Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor, Science 268 (1995) 722–726.
[26] H.C. Liang, H. Li, R.A. McKinnon, J.J. Duffy, S.S. Potter, A. Puga, D.W. Nebert, Cyp1a2(−/−) null mutant mice develop normally but show deficient drug metabolism, Proc. Natl. Acad. Sci. USA 93 (1996) 1671–1676.
[27] T.P. Dalton, M.Z. Dieter, R.S. Matlib, N.L. Childs, H.G. Shertzer, M.B. Genter, D.W. Nebert, Targeted knockout of Cyp1a1 gene does not alter hepatic constitutive expression of other genes in the mouse [Ah] battery, Biochem. Biophys. Res. Commun. 267 (2000) 184–189.
[28] J.T. Young, Histopathologic examination of the rat nasal cavity, Fundam. Appl. Toxicol. 1 (1981) 309–312.
[29] A.J. Phimister, M.G. Lee, D. Morin, A.R. Buckpitt, C.G. Plopper, Glutathione depletion is a major determinant of inhaled naphthalene respiratory toxicity and naphthalene metabolism in mice, Toxicol. Sci. 82 (2004) 268–278.
[30] C.Y. Chang, A. Puga, Constitutive activation of the aromatic hydrocarbon receptor, Mol. Cell. Biol. 18 (1998) 525–535.
[31] A. Dey, J.E. Jones, D.W. Nebert, Tissue- and cell type-specific expression of cytochrome P450 1A1 and cytochrome P450 1A2 mRNA in the mouse localized in situ hybridization, Biochem. Pharmacol. 58 (1999) 525–537.
[32] A. Chang, A. Buckpitt, C. Plopper, W. Alworth, Suicide inhibition of CYP2F2, the enzyme responsible for naphthalene (NA) metabolism to a Clara cell toxicant, Toxicologist 30 (1996) 72.
[33] E.S. Roberts, W.L. Alworth, P.F. Hollenberg, Mechanism-based inactivation of cytochromes P450 2E1 and 2B1 by 5-phenyl-1-pentyne, Arch. Biochem. Biophys. 354 (1998) 295–302.
[34] P.L. Sheets, G.S. Yost, G.P. Carlson, Benzene metabolism in human lung cell lines BEAS-2B and A549 and cells overexpressing CYP2F1, J. Biochem. Mol. Toxicol. 18 (2004) 92–99.