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Impact of hepatic P450-mediated biotransformation on the disposition and respiratory tract toxicity of inhaled naphthalene

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ABSTRACT

We determined whether a decrease in hepatic microsomal cytochrome P450 activity would impact lung toxicity induced by inhalation exposure to naphthalene (NA), a ubiquitous environmental pollutant. The liver-Cpr-null (LCN) mouse showed decreases in microsomal metabolism of NA in liver, but not lung, compared to wild-type (WT) mouse. Plasma levels of NA and NA-glutathione conjugates (NA-GSH) were both higher in LCN than in WT mice after a 4-h nose-only NA inhalation exposure at 10 ppm. Levels of NA were also higher in lung and liver of LCN, compared to WT, mice, following exposure to NA at 5 or 10 ppm. Despite the large increase in circulating and lung tissue NA levels, the level of NA-GSH, a biomarker of NA bioactivation, was either not different, or only slightly higher, in lung and liver tissues of LCN mice, relative to that in WT mice. Furthermore, the extent of NA-induced acute airway injury, judging from high-resolution lung histopathology and morphometry at 20 h following NA exposure, was not higher, but lower, in LCN than in WT mice. These results, while confirming the ability of extrahepatic organ to bioactivate inhaled NA and mediate NA's lung toxicity, suggest that liver P450-generated NA metabolites also have a significant, although relatively small, contribution to airway toxicity of inhaled NA. This hepatic contribution to the airway toxicity of inhaled NA may be an important risk factor for individuals with diminished bioactivation activity in the lung.

1. Introduction

Naphthalene (NA) is a ubiquitous contaminant of the environment (USEPA, 1986; Witschi et al., 1997; Kakareka and Kukharchyk, 2003). NA is classified as a Possible Human Carcinogen (group 2B) (IARC, 2002), in part due to its ability to induce nasal tumors in rats and lung tumors in mice in chronic rodent bioassays conducted by the National Toxicology Program (Abdo et al., 1992, 2001). The current OSHA standard for NA exposure in the workplace is 10 ppm. NA administration injures nonciliated bronchiolar epithelial cells (Club cells) in conductive airways independent of the route of administration in rodents (Plopper et al., 1992a,b; West et al., 2001).

The mechanism of NA carcinogenicity is not fully understood; but it is clear that cytochrome P450 (P450)-mediated NA bioactivation is essential for NA toxicity, and repeated cycles of NA-induced acute cytotoxicity with subsequent tissue repair are believed to be important initiating events for NA carcinogenicity (Buckpitt et al., 2002; Brusick, 2008). Bioactivation of NA to its reactive metabolite NA-oxide (NAO) by P450 enzymes is the key step in NA-induced cellular damage in airways (Warren et al., 1982; Buckpitt and Warren, 1983). The reaction of NAO with reduced glutathione (GSH), to produce NA-glutathione conjugates (NA-GSH), is one of major detoxification pathways for the toxicant, and allows NA-GSH to serve as a marker of NA bioactivation in vitro and in vivo (Buckpitt et al., 1984; Richieri and Buckpitt, 1988; Buckpitt et al., 1992; Tingle et al., 1993; Wilson et al., 1996). Recent studies have provided further details on the involvement of P450 enzymes in NA bioactivation, including the respective roles of mouse CYP2A5 and CYP2F2 in mediating NA-induced nasal and lung toxicity (Li et al., 2011; Hu et al., 2014). The ability of human CYP2A13/CYP2F1 to bioactivate NA in vivo and mediate NA-induced acute nasal and lung toxicity at occupationally relevant inhalation exposure levels has

Abbreviations: NA, naphthalene; NAO, naphthalene oxide; Cpr, cytochrome P450 reductase; LCN, liver-Cpr-null; WT, wild-type; GSH, glutathione; NADPH, β-nicotinamide adenine dinucleotide phosphate; AP-GSH, acetaminophen-glutathione conjugate; NA-GSH, naphthalene-glutathione conjugate; PBS, phosphate-buffered saline; F, bioavailability; CL, clearance; $t_{1/2}$, elimination half-life; FA, filtered air.

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also been demonstrated in a CYP2A13/CYP2F1-humanized mouse model (Li et al., 2017). The latter study provides strong supporting evidence for the potential of NA to cause respiratory toxicity in humans.

The aim of this study was to determine whether a decrease in hepatic microsomal P450 activity would impact lung toxicity induced by inhalation exposure to NA; the answer to this question may impact human risk assessment for NA. We hypothesized that a decrease of P450 activity in liver, as would occur in people with liver diseases, will increase the amount of NA available for bioactivation by lung P450s, resulting in the formation of greater amounts of reactive metabolites and more severe damage to the pulmonary airways. The impact of the loss of hepatic NA metabolic activity on systemic NA clearance has been demonstrated previously in mice exposed to intraperitoneally injected NA at relatively high doses (Li et al., 2011). However, the impact of hepatic metabolic disposition on the pharmacokinetics of NA may differ by exposure route; during an inhalation exposure, the respiratory tract would be exposed to NA delivered directly from the air and NA delivered through the blood circulation following absorption.

In the present study, we exposed mice to occupationally relevant doses (5 and 10 ppm) of NA through inhalation, and compared the pharmacokinetics of NA and NA-GSH, and the extent of NA-induced airway cytotoxicity, between wild-type (WT) and liver-Cpr-null (LCN) mice. The LCN mice undergo tissue-specific deletion of the *Cpr* gene in all hepatocytes, which results in tissue-specific abolishment of microsomal P450 activities in the liver (Gu et al., 2003a). Thus, we can determine whether a decrease in hepatic microsomal P450 activity would impact lung toxicity induced by inhalation exposure to NA. The LCN mouse model has been previously utilized to demonstrate the impact of hepatic P450-mediated NA metabolism on the pharmacokinetics of systemically administered NA (Li et al., 2011).

2. Materials and methods

2.1. Chemicals and reagents

NA (CAS# 91-20-3, purity 99%), NA-d_8 (CAS# 1146-65-2, purity 99%), GSH(CAS# 70-18-8, purity ≥ 98.0%), β-nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexyl ammonium) salt (NADPH) (CAS#, 100929-71-3, purity ≥ 95.0%), and corn oil (highly refined, low acidity) were purchased from Sigma Aldrich (St. Louis, MO). Acetaminophen-glutathione (AP-GSH) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). NA-GSH standard as a mixture of all four stereoisomers was a generous gift from Drs. Alan R. Buckpitt and Dexter Morin (University of California at Davis, Davis, CA) and was prepared as previously described (Richieri and Buckpitt, 1987a). All solvents (dichloromethane, formic acid, methanol and water) were of analytical grade (Fisher Scientific, Houston, TX). Ingredients for Karnovsky's fixative were from Tousimis (Rockville, MD).

2.2. In vitro assay of NA metabolism

Lung and liver microsomes were prepared from three different batches (each prepared from pooled tissue of 3 mice) of 2-month old, male, LCN and WT mice, as described (Gu et al., 1998). In vitro assay of NA bioactivation was performed as described previously (Shultz et al., 1999); reaction mixtures contained 50 mM phosphate buffer (pH 7.4), NA at a wide range of concentrations added in 2 μL of methanol (0; 0.5; 1.0; 2.0; 5.0; 10.0; 20.0; 50.0; 100.0; 200.0 and 400.0 μM), 10 mM GSH, 0.2 mg/mL of liver or lung microsomal protein, and 0 or 1.0 mM NADPH, in a final volume of 0.2 mL. The reaction was carried out at 37 °C in sealed tubes for 5 min and terminated by the addition of 2 volumes of ice-cold methanol containing 2.5 ng of AP-GSH (internal standard). The resultant mixtures were centrifuged to remove precipitated proteins, and NA-GSH was quantified in aliquots of the supernatant using LC-MS/MS (see below).

2.3. Animal experiments

All procedures involving animals were approved by the Wadsworth Center Institutional Animal Care and Use Committee. WT B6 and LCN (Gu et al., 2003b) mice from colonies maintained at Wadsworth Center were housed in an acclimatized environment on a 12-h light:dark cycle, and had access to standard rodent chow and drinking water ad libitum. Two-month-old male mice were used for experiments.

Nose-only inhalation exposure to HEPA-filtered air (sham-exposure control) or NA vapor was conducted in an Oral Nasal Aerosol Respiratory Exposure System (equipped with a 24-port Jaeger rodent inhalation exposure chamber) (CH Technologies, Westwood, NJ). Mice were acclimatized to the holding tube and exposure chamber (once a day for three days) prior to NA exposure. To generate NA vapor, air was passed through a glass column containing crystalline NA, heated to 52 °C; the vapor was mixed with fresh filtered air to achieve desired average NA concentration in the inhalation chamber. All experiments were started in the morning and consisted of two 2-h exposure periods with a 1-h break in between (added to reduce stress to mice). NA vapor at two different doses was studied, 5 and 10 ppm; the latter dose is an OSHA (http://www.osha.gov/dts/chemicalsampling/data/CH_255800.html) permissible exposure limit for human workers. The 4-h total exposure time was selected to mimic daily occupational exposure.

Concentrations of NA, carbon dioxide (CO_2), carbon monoxide (CO), and oxygen (O_2); relative humidity; and air temperature in the exposure chamber were monitored in real time throughout the exposure using a model IQ-604 Total Volatile Organic Compound (TVOC) Monitor (Graywolf Sensing Solutions, Trumbull, CT), which was pre-calibrated for NA as recently described (Li et al., 2017). Air flow through each nose port was maintained at approximately 0.3 L/min.

For toxicokinetics studies, blood samples (~20 μL each) from individual mice were collected from the tail vein using heparinized capillary tubes at various time points (0–360 min) after termination of NA exposure. Plasma was prepared by centrifugation of blood samples at 10,000 rpm for 5 min at 4 °C, and was stored in sealed tubes at −80 °C until use. For detection of tissue levels of NA and NA-GSH, mice were placed in fresh air (immediately after termination of NA exposure) for 0, 2, 4 and 20 h and then euthanized by CO_2 overdose. Lung (lavaged with 1 mL of 1× phosphate-buffered saline (PBS)) and liver were harvested, quick-frozen, and stored in sealed tubes at −80 °C until use.

2.4. NA and NA-GSH detection

For NA detection, plasma (10 μL) or tissue (lung, liver) homogenates (50 μL) were spiked with NA-d_8 (18 pg for plasma and 12 pg for tissue, in 10 μL of methanol), extracted with dichloromethane (100 μL for plasma and 110 μL for tissue). The organic phase (1 μL injection volume) was analyzed for NA using gas-chromatography mass spectrometry in a splitless injection mode, as previously described (Li et al., 2011), using a Restek Rxi-5 ms (30 m × 0.25 mm; 0.25 μm) column (Restek, Bellefonte, PA). The limit for NA detection was 0.8 pmol (on column).

For NA-GSH detection, plasma (10 μL) and tissue homogenate (50 μL) were spiked with an internal standard AP-GSH (2 ng in 10 μL of methanol), and then mixed with methanol (80 and 90 μL, respectively) for protein precipitation. An aliquot of the supernatant (1 μL) was analyzed for NA-GSH using liquid-chromatography mass spectrometry, with a SCIEX 4000 Q-Trap mass spectrometer, as previously described (Li et al., 2011), or with a SCIEX 6500 Q-Trap mass spectrometer (AB-SCIEX, Framingham, MA), as described below.

The 6500 Q-Trap mass spectrometer was coupled to an Agilent 1290 Infinity Series ultra-performance liquid chromatography system (Agilent, Santa Clara, CA) and an Agilent Elipse Plus C18 (2.1 × 50 mm; 1.8 μm) column. Analytes were eluted at room temperature, at a flow rate of 0.2 mL/min, with mobile phases as previously described (Li et al., 2011), using the following program: linear increase from 10%B to 90%B from 0 to 4 min, return to 10%B from 4 to 8 min, and re-