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Kinetics of naphthalene metabolism in target and non-target tissues of rodents and in nasal and airway microsomes from the Rhesus monkey

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ABSTRACT

Naphthalene produces species and cell selective injury to respiratory tract epithelial cells of rodents. In these studies we determined the apparent K_m, V_{max}, and catalytic efficiency (V_{max}/K_m) for naphthalene metabolism in microsomal preparations from subcompartments of the respiratory tract of rodents and non-human primates. In tissues with high substrate turnover, major metabolites were derived directly from naphthalene oxide with smaller amounts from conjugates of diol epoxide, diepoxide, and 1,2- and 1,4-naphthoquinones. In some tissues, different enzymes with dissimilar K_m and V_{max} appeared to metabolize naphthalene. The rank order of V_{max} (rat olfactory epithelium > mouse olfactory epithelium > murine airways ≫ rat airways) correlated well with tissue susceptibility to naphthalene. The V_{max} in monkey alveolar subcompartment was 2% that in rat nasal olfactory epithelium. Rates of metabolism in nasal compartments of the monkey were low. The catalytic efficiencies of microsomes from known susceptible tissues/subcompartments are 10 and 250 fold higher than in rat airway and monkey alveolar subcompartments, respectively. Although the strong correlations between catalytic efficiencies and tissue susceptibility suggest that non-human primate tissues are unlikely to generate metabolites at a rate sufficient to produce cellular injury, other studies showing high levels of formation of protein adducts support the need for additional studies.

Introduction

Naphthalene is a volatile, polycyclic aromatic hydrocarbon that is used as a synthetic starting material in the production of dyes, lubricants and a variety of plasticizers. Naphthalene is generated by combustion of fossil fuels and is present in mainstream and side stream tobacco smoke (Preuss et al., 2003). It is a component of jet fuel and significant exposures occur via both dermal and inhalation routes in fuel cell maintenance workers (Chao et al., 2006). There is widespread exposure of the general public to naphthalene as demonstrated in recent NHANES (National Health and Nutrition Exposure Study) studies where the glucuronide conjugate of 1-naphthol, a primary metabolite of naphthalene, was present in all of the urine samples tested (Li et al., 2008, 2010).

Administration of naphthalene to rodents via either inhalation exposure or by injection into the peritoneal cavity results in dose dependent cytotoxicity to cells of the respiratory tract. In mice, injury is most prominent in the nasal olfactory epithelium and to Clara cells lining the conducting airway epithelium after either exposure route (Plopper et al., 1992; Van Winkle et al., 1995). In rats, injury occurs to both respiratory and olfactory nasal epithelia but importantly, and in contrast to mice, there is no detectable injury to conducting airway epithelial Clara cells (Dodd et al., 2010; Lee et al., 2005; Plopper et al., 1992; West et al., 2001). The toxicity of naphthalene is related to cytochrome P450 dependent metabolism to reactive intermediates which are detoxified by glutathione (Warren et al., 1982). The abundance and high catalytic turnover of naphthalene by cytochrome P450 2F2 in mouse lung appear to be an important determinant in the susceptibility of murine Clara cells to naphthalene, a view supported by recent studies in Cyp2f2 knockout mice (Li et al., 2011). The human ortholog, CYP2F1, has 80% sequence identity with Cyp2f2 yet appears to have strikingly lower catalytic activity (Lanza et al., 1999). The rates of metabolism of naphthalene in dissected airways of mice at saturating substrate concentrations are much greater than in rats or hamsters and this correlates well with differences in the susceptibility to cytotoxic injury (Buckpitt et al., 1995). As a corollary to this, microsomes prepared from either whole lung of Rhesus macaques (Buckpitt et al., 1992) or humans (Buckpitt and Bahnson, 1986) metabolize naphthalene at less than 1% of the rate observed in susceptible rodent tissues. This suggests that primates may not be susceptible to cytotoxic injury associated with naphthalene exposure. However, studies with microsomes prepared from whole lung do not account for differences in the localization of cytochrome P450 monooxygenases within the respiratory tract and results obtained from microsomes prepared from the entire

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lung could seriously under- or over-estimate rates of metabolism. In addition, while the studies with dissected airways focus on target areas within the respiratory tract, this work was previously conducted with saturating substrate concentrations and it is likely that the reactions were not linear for the entire incubation period (Buckpitt et al., 1995).

The overall goal of the current study was to measure the kinetics of naphthalene metabolism in microsomes prepared from known target and non-target respiratory subcompartments of the mouse, rat and rhesus macaque. This work differed from that published previously because the current study uses defined respiratory tract subcompartments whereas the previous studies used microsomes prepared from the entire tissue (Buckpitt et al., 1992).

Materials and methods

Animals. All animals were obtained from Harlan Laboratories and were housed in the AAALAC approved vivarium facilities at UC Davis for at least 5 days following receipt from the supplier. Animals were provided free access to food and water. All animals were males. Mice were Swiss Webster 25–30 g and rats were Sprague Dawley 250–350 g. All animal use was approved by the Animal Care and Use Committee. Tissues from Rhesus macaques were obtained from culled animals at the California National Primate Research Center, UC Davis. The majority of the non-human primates obtained for these studies were euthanized because of chronic diarrhea; for each animal the cause is listed in Supplemental Table 1 along with the age and sex of the animal.

Chemicals and radiochemical. 1,2- and 1,4-naphthoquinones, 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol were purchased from Aldrich Chemical Co, Milwaukee, WI. HPLC solvents were from Fisher Scientific. Components of the NADPH generating system were purchased from Sigma-Aldrich, St. Louis, MO. All other chemicals were of reagent grade or better.

Synthesis and characterization of metabolite standards. Naphthalene oxide was prepared using the method of Yagi and Jerina (1975). The epoxide was recrystallized from 99.5% ethanol/0.5% triethylamine and kept at −80 °C under Ar gas. The concentration was assayed spectrophotometrically (UV) using the published extinction coefficient of 8850 cm^{-1} M^{-1} (Vogel and Klarner, 1968). Caution: Naphthalene oxide is toxic and should be used with care.

1,2-Dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene (diol epoxide) was made with m-chloroperoxybenzoic acid according to the procedure of Tsang et al. (1982). The final product was recrystallized from ethanol and stored in ethanol at −80 °C under Ar gas. NMR of naphthalene diol epoxide in CD_3CN yielded signals identical to those reported previously (Yagi et al., 1975). Caution: Naphthalene diol epoxide is toxic and should be used with appropriate precautions. Additionally, naphthalene diepoxide was prepared from naphthalene by the method of Ishikawa and Griffin (1977).

The general method for synthesis of glutathione conjugates of naphthalene oxide, naphthalene diol epoxide, naphthalene diepoxide and the naphthoquinones was as follows: glutathione (free acid, 0.2 mmol) was dissolved in 100 ml of helium-sparged 0.1 M Na_2HPO_4 in a flask under argon. The pH was adjusted to either 8.5 for the epoxides or 7.0 for the quinones with 10 M NaOH. With rapid stirring, an approximate 5 fold excess of reactive metabolite was added drop-wise. After complete addition, the reaction was continued under argon for 2 h with the epoxides and 30 min for the quinones. The mixture was then extracted with ether. The ether layer was discarded and the water layer partially evaporated under nitrogen to remove residual ether. The solution was acidified with acetic acid to pH 3, desalted on a solid phase extraction column (Phenomenex Strata X) and lyophilized to dryness. The mixture was then purified by preparative HPLC (Phenomenex, Sphericlone C18, 25 × 2 cm, 5 μ packing material) using a 0.5% formic acid/water/acetonitrile gradient system. Major peaks were collected and analyzed by LC/MS using an LTQ orbitrap HRMS in negative ionization mode to verify molecular masses. Sufficient amounts of the naphthalene epoxide glutathione adducts, 1-naphthol and dihydrodiol were available for use as quantitative standards. Glutathione conjugates of the naphthoquinones and naphthalene diol epoxide were prepared in sufficient quantities for structural characterization and to act as retention time standards but were produced in very small amounts in most incubations making exact measurements of their rates of formation unreliable.

^{14}C-[1,4,5,8]-naphthalene was purchased from Moravek Radiochemicals, Brea, CA at a specific activity of 58 mCi/mmol. The sample was >99% pure as measured by reversed phase HPLC.

Tissue specimens, preparation of microsomes and incubations. Rodents were euthanized with an overdose of pentobarbital and airways and nasal olfactory/respiratory epithelium were removed using procedures described in detail earlier (Lee et al., 2005). Intrapulmonary airways were blunt dissected from mice and rats by techniques originally described by Plopper (Duan et al., 1993; Plopper et al., 1991). Briefly, following deep anesthesia, the trachea was cannulated and 1% low melting temperature agarose in Waymouth's medium was used to inflate the lung. The lungs were allowed to cool on ice for at least 30 min to harden the agarose. The intrapulmonary airways of rodents were dissected free of parenchymal tissue and blood vessels under a dissecting microscope. A total of 4 rats and 7 to 8 mice were dissected for an experiment. Dissections were limited to 2 h duration. Since the tissue pieces are very small, no attempt was made to obtain wet weights as the weight of surrounding medium would likely make the measurements unreliable. Likewise, dissection of distal airways from the monkey was limited to 2 h duration to prevent loss of tissue viability.

Washed microsomal fractions were prepared by differential centrifugation as described previously (Buckpitt et al., 1992). The microsomal pellet was resuspended in 0.1 M phosphate buffer at pH 7.4 and protein concentrations were measured using the Bradford procedure (Bradford, 1976). Microsomal yields varied from 500 to 1000 μg from rodents and 50–200 μg from Rhesus macaques.

Incubations were prepared on ice and contained: substrate (added in 2 μl acetonitrile, concentrations of 0.49 to 250 μM as specified in legends to the table or figures), microsomes (25–200 μg, depending upon the tissue), NADPH (25 mM), NADPH generating system (0.14 mM NADP, 3.8 mM glucose 6-phosphate, 0.1 U glucose 6 phosphate dehydrogenase, 10 mM $MgCl_2$), 1 unit glutathione transferase (purified by affinity chromatography) and 5 mM glutathione in a total volume of 200 μl. We note that the addition of 1% acetonitrile does not affect the metabolism of any of the substrates tested in liver microsomal incubations with the exception of tolbutamide (CYP2C8/2C9 substrate) where a <20% decrease was measured. Acetonitrile had the least effect on activity of any of the solvents tested (Chauret et al., 1998). We also note that the volatility of naphthalene precluded its addition to the incubation vial with subsequent evaporation of solvent. Substrate was added last and the reaction vials were capped. Samples were transferred to a shaking incubator at 37 °C. At the appropriate time vials were placed on dry ice to rapidly quench the reaction. Acetonitrile was added to all samples to precipitate protein and samples were transferred to microcentrifuge tubes. Samples were centrifuged at 15,000 ×*g* for 20 min to pellet the protein. The supernatant was removed and solvent was evaporated to dryness on a centrifugal concentrator. Samples were reconstituted in 75 μl of 10% acetonitrile/water for HPLC analysis.

HPLC analysis. Metabolites were separated and measured quantitatively by using UV detection at 215 and 260 nm using a Waters 2487