Metabolism of [D₁₀]Phenanthrene to Tetraols in Smokers for Potential Lung Cancer Susceptibility Assessment: Comparison of Oral and Inhalation Routes of Administration

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are believed to be among the causative agents for lung cancer in smokers. PAHs require metabolic activation for carcinogenicity. One pathway produces diol epoxides that react with DNA, causing mutations. Because diol epoxides are converted to tetraols, quantitation of tetraols can potentially be used to identify smokers who may be at higher risk for lung cancer. Our approach uses [D₁₀]phenanthrene, a labeled version of phenanthrene, a noncarcinogenic PAH structurally analogous to carcinogenic PAH. Although smokers are exposed to PAH by inhalation, oral dosing would be more practical for phenotyping studies. Therefore, we investigated [D₁₀]phenanthrene metabolism in smokers after administration by inhalation in cigarette smoke or orally. Sixteen smokers received 10 µg of [D₁₀]phenanthrene in a cigarette or orally. Plasma and

urine samples were analyzed for [D₁₀]r-1,t-2,3,c-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene ([D₁₀]PheT), the major end product of the diol epoxide pathway, by gas chromatography-negative ion chemical ionization-tandem mass spectrometry. The ratios of [D₁₀]PheT (oral dosing/inhalation) in 15 smokers were 1.03 ± 0.32 and 1.02 ± 0.35 , based on plasma area under the concentration-time curve (0–∞) and total 48-h urinary excretion, respectively. Overall, there was no significant difference in the extent of [D₁₀]PheT formation after the two different routes of exposure in smokers. A large interindividual variation in [D₁₀]PheT formation was observed. These results demonstrate that the level of [D₁₀]PheT in urine after oral dosing of [D₁₀]phenanthrene can be used to assess individual capacity of PAH metabolism by the diol epoxide pathway.

Introduction

Lung cancer kills more than 3000 people in the world each day (International Agency for Research on Cancer, 2004). Approximately 90% of lung cancer mortality is caused by cigarette smoking, entailing exposure to more than 70 established carcinogens (Hecht, 2003; International Agency for Research on Cancer, 2004; Secretan et al., 2009). Polycyclic aromatic hydrocarbons (PAHs) are among the strongest carcinogens in cigarette smoke and are believed to be major

causes of lung cancer in smokers (Hecht, 2003). Benzo[a]pyrene (BaP), a representative PAH, is “carcinogenic to humans” according to the International Agency for Research on Cancer (2010).

PAH require metabolic activation for carcinogenicity (Dipple et al., 1984; Luch and Baird, 2005). One important pathway proceeds through diol epoxides, which react with DNA producing adducts, causing permanent mutations and initiating carcinogenesis (Dipple et al., 1984; Luch and Baird, 2005). BaP diol epoxide-DNA adducts are present in smokers’ lung tissue (Phillips, 2002). This pathway includes initial epoxide formation catalyzed by cytochromes P450, hydration catalyzed by epoxide hydrolase, and further oxidation by cytochromes P450. The resulting diol epoxides react with DNA, but even more readily with H₂O, producing tetraols (Luch and Baird, 2005). These tetraols may be excellent

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; BaP, benzo[a]pyrene; Phe, phenanthrene; PheT, r-1,t-2,3,c-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene; HOPhe, hydroxyphenanthrene; HOPhe-TMS, HOPhe-tetratrimethylsilyl ether; *anti*-PheDE-1-NAC, *N*-acetyl-S-(r-4,t-2,3-trihydroxy-1,2,3,4-tetrahydro-c-1-phenanthryl)-L-cysteine; GC, gas chromatography; MS/MS, tandem mass spectrometry; EI, electron impact; AUC, area under the concentration-time curve; CI, confidence interval.

biomarkers for PAH metabolic activation (Thakker et al., 1985). Competing with this metabolic activation pathway, oxidation to phenols is a representative detoxification pathway (Carmella et al., 2004; Luch and Baird, 2005). Detoxification by glutathione conjugation, glucuronidation, and sulfation of PAH metabolites is also observed (Cooper et al., 1983; Luch and Baird, 2005).

Figure 1 illustrates the diol epoxide pathways of phenanthrene (Phe), the simplest PAH with a bay region, a feature closely associated with carcinogenicity. Phe is generally considered noncarcinogenic, but its metabolism to diol epoxides closely parallels that of BaP (Nordquist et al., 1981; Thakker et al., 1985; Shou et al., 1994). Our group has developed methods for quantitation of total *r*-1,*t*-2,3,*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene [PheT; the sum of Phe-(1*R*,2*S*,3*R*,4*S*)-tetraol (3) and Phe-(1*S*,2*R*,3*S*,4*R*)-tetraol (6) in Fig. 1] in human urine and plasma (Hecht et al., 2003; Carmella et al., 2006). We have demonstrated correlations of urinary PheT with its bay region diol epoxide-derived enantiomer 3 and with the corresponding tetraol derived from BaP (Hochalter et al., 2011). Because PheT is found in all human urine samples in much greater quantities than tetraols derived from carcinogenic PAH (Hecht et al., 2003; Zhong et al., 2011a), we have proposed that PheT could be an indicator of PAH exposure plus metabolic activation by the bay region diol epoxide pathway.

Approximately 11 to 24% of lifelong smokers develop lung cancer over their lifetimes (International Agency for Research on Cancer, 2004). The identification of smokers with higher cancer susceptibility is a challenging and worthwhile goal, because they could be targeted for lung cancer prevention activities and early detection. Our hypothesis is that individuals who metabolically activate tobacco smoke carcinogens more extensively will be at higher risk for cancer (Perera, 1997). Induction of cytochromes P450 1A1, 1A2, and 1B1 is apparently related to higher lung cancer risk in smokers (McLemore et al., 1990; Nebert, 2000). Polymorphisms in genes involved in PAH metabolism may also influence lung cancer risk, but the results of these studies are inconsistent (Bartsch et al., 2000; Nebert, 2000; Hung et al., 2003; Carlsten et al., 2008). We propose that carcinogen metabolite phenotyping has the potential to specifically identify those individuals at high risk. The approach described in this study is unique, involving administration of the stable isotope-

labeled compound [D₁₀]Phe. The use of [D₁₀]Phe allows us to quantify individual differences in metabolism, without complication by environmental exposure to Phe, which is ubiquitous. In the study reported here, we compared levels of [D₁₀]PheT in the plasma and urine of smokers who were given [D₁₀]Phe either by inhalation in cigarette smoke or oral administration. This study was crucial for developing a practical, yet accurate, route of administration for use in potentially large phenotyping studies. A primary objective was to determine and compare the pharmacokinetics of [D₁₀]PheT after the two routes of exposure. A preliminary report of plasma levels of [D₁₀]PheT in smokers given [D₁₀]Phe by inhalation has been published (Zhong et al., 2011b).

The results of the study reported here reveal, for the first time, the comparative metabolic activation profiles of a bay-region PAH administered by different routes to humans and provide direct evidence for significant interindividual variation in PAH diol epoxide metabolism in smokers.

Materials and Methods

Chemicals, Enzymes, and Chromatography Supplies. [D₁₀]Phe (98%) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). [D₁₀]Phe for oral dosing was repurified as described previously in the University of Minnesota Molecular and Cellular Therapeutics GMP facility by normal-phase high-performance liquid chromatography followed by recrystallization from ethanol (Zhong et al., 2011b). It had >99% chemical purity and 98% isotopic purity (Zhong et al., 2011b). All PheT used in this study was racemic. PheT was kindly supplied by Drs. Donald M. Jerina and Haruhiko Yagi, National Institutes of Health (Bethesda, MD). [¹³C₆]PheT was prepared by hydrolysis in 50:50 tetrahydrofuran/H₂O of [¹³C₆]Phe(1*R*,2*S*)diol-(3*S*,4*R*)epoxide (Hecht et al., 2008). [D₁₀]PheT was prepared by in vitro metabolism of [D₁₀]Phe, obtained from Cambridge Isotope Laboratories, Inc., as described previously (Carmella et al., 2006). 1-Hydroxyphenanthrene (1-HOPhe; synthesized), 2-HOPhe (synthesized), 3-HOPhe (Chiron AS, Trondheim, Norway), 4-HOPhe (Chiron), and 9-HOPhe (Sigma-Aldrich, St. Louis, MO) were either synthesized by Drs. Jerina and Yagi or purchased. [¹³C₆]3-HOPhe was procured from Cambridge Isotope Laboratories, Inc.. 2,7-Dihydroxynaphthalene was obtained from Sigma-Aldrich. All stock solutions were prepared in acetonitrile.

β-Glucuronidase and arylsulfatase (from *Helix pomatia*) were obtained from Roche Diagnostics (Indianapolis, IN). *Bis*-trimethylsilyl-trifluoroacetamide containing 1% trimethylchlorosilane was purchased from Regis Technologies, Inc. (Morton Grove, IL). Strata-X polymeric SPE cartridges (200 mg/6 ml) were obtained from Phe-

Fig. 1. Formation of PheT through diol epoxide pathways in the metabolism of Phe. Three steps are required for diol epoxide formation, catalyzed sequentially by cytochromes P450, epoxide hydrolase (EH), and cytochromes P450.

nomenex (Torrance, CA). ChemElut diatomaceous earth partitioning cartridges (5 ml) were procured from Varian, Inc. (Palo Alto, CA). Blue rayon was purchased from MP Biomedicals (Solon, OH), and 100 mg was placed in an empty 6-ml Bond Elute solid-phase extraction cartridge (Varian, Inc.) between two polyethylene frits. Oasis MCX mixed mode cation exchange solid-phase extraction cartridges (60 mg) were purchased from Waters (Milford, MA). Envi-Chrom P SPE columns (styrene-divinylbenzene copolymer resin, Supelclean Envi Chrom P: 0.1 g) were procured from Sigma-Aldrich.

Oral Doses and Cigarettes Containing [D₁₀]Phe. [D₁₀]Phe oral dose solutions were prepared by dissolving 4 mg of purified [D₁₀]Phe in 400 ml of 100% ethanol and delivering 1-ml aliquots of the solution into amber dosing bottles in the Fairview Compounding Pharmacy (Minneapolis, MN), such that each bottle contained 10 µg of [D₁₀]Phe. All oral doses were stored at 4°C before administration. On the day of dosing, 4 ml of drinking water was added to the amber dosing bottle, which was swirled to achieve a composition of 20% ethanol (this was necessary because storage in 20% ethanolic H₂O resulted in decreased concentrations of [D₁₀]Phe). Cigarettes containing [D₁₀]Phe were prepared to deliver approximately 10 µg of [D₁₀]Phe in the mainstream smoke as described previously (Zhong et al., 2011b). Before giving it to a smoker, the spiked cigarette was conditioned in a humidity chamber at 60% relative humidity for 1 to 2 days.

Subject Recruiting and Screening. This study was approved by the U.S. Food and Drug Administration and the University of Minnesota Institutional Review Board. Cigarette smokers were recruited and screened as described previously (Zhong et al., 2011b). Participants had to have been smoking at least 10 cigarettes daily for the past year and be in good physical and mental health. Female subjects who were pregnant or nursing were excluded.

Eligible subjects were invited into the clinic for an orientation visit and to sign an informed consent. A questionnaire including information on age, gender, medical history, medication use, and smoking history including number of years of smoking, age at onset of smoking, number of cigarettes per day, and brand of cigarettes used was completed. Blood pressure, heart rate, height, weight, and carbon monoxide (CO) level were measured. Pregnancy tests were done. Selected subjects were then asked to complete a brief physical exam. Laboratory tests including blood counts, and liver and kidney function were performed to verify health status. Sixteen eligible subjects were each scheduled to attend two sessions.

Study Design. This study was a randomized, open-label, single-dose, nonblinded, within-subject crossover study. Sixteen subjects received 10 µg of [D₁₀]Phe orally or by smoking a cigarette containing [D₁₀]Phe. The order of administration was randomized, and each dose was separated by a washout period of at least 1 week. For the oral dose, each subject was given 10 µg of [D₁₀]Phe in 5 ml of 20% ethanol in water, as described above. The dosing bottle was rinsed twice with water, and the rinsings were administered to ensure that the subjects consumed the entire dose. Administration via cigarette smoking was carried out as described previously (Zhong et al., 2011b). In brief, subjects were asked to smoke the cigarette containing [D₁₀]Phe through a CReSS desktop smoking topography device (Plowshare Technologies, Baltimore, MD), which recorded the number of puffs, puff duration, and puff volume, as used in the Health Canada intense smoking conditions, resulting in delivery of approximately 10 µg of [D₁₀]Phe in mainstream smoke.

The clinical visit procedure was essentially identical to that described previously (Zhong et al., 2011b). Blood samples of 10 ml each were taken before dosing and 15, 30, 45, 60, 90, 120, 150, 240, 360, 540, 720, and 1440 min after the completion of administration. Blood samples were collected into EDTA-containing vacuum collection tubes and centrifuged to obtain plasma, which was frozen at -20°C until analysis. Urine collections were obtained predosing and at the following intervals postdosing: 0 to 30, 30 to 60, 60 to 120, 120 to 360, 360 to 720, 720 to 1440, and 1440 to 2880 min. The volume of each urine collection was measured, and an aliquot of 50 ml was frozen at

-20°C until analysis. Adverse-effect reports, blood pressure, and heart rate were taken several times during the inpatient stay. Subjects were asked not to smoke during the stay and remain abstinent until after the 24-h blood draw the next morning.

Analysis of [D₁₀]PheT in Urine and Plasma. [D₁₀]PheT in urine and plasma were analyzed by gas chromatography (GC)-negative ion chemical ionization-tandem mass spectrometry (MS/MS) as described previously, without the resolution of enantiomers (Carmella et al., 2006; Zhong et al., 2011b).

Analysis of Total [D₉]HOPhe in Urine. The procedure was modified from a previous study (Carmella et al., 2004). A deuterium is lost in the metabolism of [D₁₀]Phe to phenols; therefore the analyte was [D₉]HOPhe. A 3-ml aliquot of urine was placed in a 10-ml centrifuge tube containing 970 µl of NaOAc buffer (0.5 M, pH 5), β-glucuronidase (2000 units), and arylsulfatase (16,000 units). [¹³C₆]3-HOPhe (1 ng) in 10 µl of CH₃CN was added as internal standard. The mixture was incubated in a water bath overnight with shaking at 37°C. The sample was loaded directly on a ChemElut cartridge and allowed to stand for 2 min. The HOPhe were eluted with three 8-ml portions of toluene into a 50-ml centrifuge tube, to which was added 5 ml of H₂O. The toluene was removed on a Speedvac for approximately 3.5 h. Methanol (0.5 ml) was added to the aqueous solution. A blue rayon solid-phase extraction cartridge (100 mg) was preconditioned with 5 ml of CH₃OH, then with 5 ml of H₂O. The sample was applied dropwise to the cartridge, along with 2 ml of H₂O washings of the urine sample tubes. The cartridge was washed with 7 ml of CH₃OH/H₂O (25:75), which was discarded. Phenanthrols were then eluted with 8 ml of CH₃OH. This fraction contained the analyte and internal standard. The solution was concentrated to dryness on a Speedvac overnight in the dark. The residue was dissolved in 200 µl of CH₃OH with sonication and vortexing, transferred to a 0.1-ml insert vial, and concentrated to dryness. To this residue, 9 µl of *bis*-trimethylsilyltrifluoroacetamide and 0.6 ng of 1-hydroxybenz[*a*]anthracene (external standard) in 3 µl of CH₃CN were added. The samples were heated at 60°C for 60 min with periodic mixing, and 1 µl was injected into the GC-electron impact (EI)-MS/MS system.

GC-EI-MS/MS was carried out with a TSQ Quantum instrument (Thermo Scientific, San Jose, CA). The GC was fitted with a 0.25-mm (inside diameter) × 30 m, 0.15-µm film thickness, DB-17MS column (Agilent Technologies, Santa Clara, CA), and a 0.53-mm (inside diameter) × 2-m deactivated fused silica precolumn. The oven temperature program was as follows: 80°C for 2 min, then 80 to 225°C at 25°C/min, then hold for 5 min, then 225 to 310°C at 25°C/min, then hold for 2 min. The injector was operated in the splitless mode at 250°C, and the MS transfer line temperature was 310°C. The flow rate was 1 ml/min He. The EI-MS/MS conditions were as follows: source temperature, 200°C; emission current, 110 µA. Selected reaction monitoring with a collision energy of 14 eV, electron energy of -55 eV, and Ar collision gas pressure of 1.0 mTorr was used to detect HOPhe-trimethylsilyl ethers (HOPhe-TMS), [¹³C₆]3-HOPhe-TMS, and [D₉]HOPhe-TMS at *m/z* 266.1 → *m/z* 251.1, *m/z* 272.1 → *m/z* 257.1, and *m/z* 275.1 → *m/z* 260.1, respectively. External standard 1-hydroxybenz[*a*]anthracene was detected at *m/z* 316.1 → *m/z* 301.1.

Analysis of Unchanged [D₁₀]Phe in Plasma and Urine. [D₁₀]Phe analysis was performed essentially as described, with modifications (Grova et al., 2005). A 5-ml aliquot of urine or 1-ml aliquot of plasma was placed in a 10-ml centrifuge tube containing 50:50 (v/v) of cyclohexane/ethyl acetate and [¹³C₆]Phe (1 ng) as internal standard. After 30 min of gentle shaking followed by 20-min centrifugation (2000g at 15°C), the supernatant was collected and concentrated to dryness, and 600 µl of cyclohexane was added. An Envi-Chrom P solid-phase extraction (styrene-divinylbenzene copolymer resin, Envi Chrom P: 0.1 g) column was conditioned with 1 ml of H₂O, 1 ml of methanol, and twice with 1 ml of cyclohexane. The sample was applied to the column, which was then washed with 600 µl of cyclohexane, then with 1.8 ml of cyclohexane/ethyl acetate (50:50; v/v) to elute [D₁₀]Phe. The solution was evaporated to dryness

(at 37°C using a stream of N₂), and 100 µl of cyclohexane was added and mixed by vortexing at ambient temperature. After centrifugation, the sample was transferred into a 0.1-ml insert vial and concentrated to dryness on a Speedvac. Twenty microliters of cyclohexane was added to the residue before GC-EI-MS/MS analysis.

GC-EI-MS/MS quantitation for [D₁₀]Phe was performed on a TSQ Quantum instrument (Thermo Scientific) with a 0.25-mm (inside diameter) × 30 m, 0.15-µm film thickness, DB-17MS column (Agilent Technologies). The splitless injector was maintained at 250°C, and the MS transfer line temperature was 320°C. The EI-MS/MS conditions were as follows: source temperature, 220°C; emission current, 500 µA. Selected reaction monitoring with a collision energy of 18 eV, electron energy of -70 eV, and Ar collision gas pressure of 1.2 mTorr were used to detect Phe, [¹³C₆]Phe, and [D₁₀]Phe at *m/z* 178.1 → *m/z* 152.1, *m/z* 184.1 → *m/z* 158.1, and *m/z* 188.1 → *m/z* 160.1, respectively.

The recovery of internal standard was 55% in plasma and 80% in urine. The limit of quantification was 5 pg/ml urine or 25 pg/ml plasma.

Analysis of [D₁₀]N-acetyl-S-(*r*-4,*t*-2,3-trihydroxy-1,2,3,4-tetrahydro-*c*-1-phenanthryl)-L-cysteine in Urine This was carried out as described previously (Hecht et al., 2008).

Determination of Urinary Creatinine. The creatinine concentration in each urine aliquot was determined at the University of Minnesota Medical Center, Fairview, Diagnostic Laboratories, using Vitros CREA slides (Ortho Clinical Diagnostics, Rochester, NY).

Pharmacokinetic Analysis. Pharmacokinetics parameters were calculated using noncompartmental methods with Phoenix WinNonlin v6.1 (PharSight, Mountain View, CA). The trapezoidal rule was used to calculate the area under the concentration-time curve (AUC_{0-t}). The remainder of the AUC (AUC_{t-∞}) was calculated by dividing the last measured time point by the rate constant associated with the terminal monoexponential phase, λ. AUC_{0-∞} was estimated by combining the two above AUC portions. The half-life associated with the terminal phase was determined by dividing 0.693 by λ.

Statistical Analysis. Ninety percent confidence intervals (CIs) for the geometric means of ratios of pharmacokinetic parameters (plasma AUC_{0-∞} and total urinary excretion) were calculated and evaluated using the paired *t* test and Pearson correlation coefficients. Grubb's test was used to test for outliers. Two-way analysis of variance was used to determine the effects of route of administration and type of sampling on the elimination half-life of [D₁₀]PheT.

Results

Sixteen subjects (six males) were enrolled in the single-dose (10 µg of [D₁₀]Phe) randomized crossover study. The mean age (±S.D.) was 37.9 ± 10.1 years, with a mean weight (±S.D.) of 85.0 ± 17.0 kg. Nine were white (one male), six were African-American (four male), and one was of mixed race (male). All subjects received the dose of 10 µg of [D₁₀]Phe orally or through cigarette smoking in two separate visits with at least a 1-week washout period in between. Smoking conditions were monitored by a topography apparatus so that the inhaled dose of [D₁₀]Phe was approximately 10 µg (Zhong et al., 2011b). Blood samples were drawn at baseline before dosing and at a series of time points from 15 min until 24 h after dosing. Urine samples were collected at baseline and at intervals during a 48-h postdose period. All subjects completed the entire protocol and did not experience any significant adverse effects.

Our major endpoint was [D₁₀]PheT, a biomarker of the diol epoxide metabolism pathway of [D₁₀]Phe. Quantitation of plasma and urinary [D₁₀]PheT were accomplished by GC-negative ion chemical ionization-MS/MS with selected reaction monitoring using [¹³C₆]PheT as the internal standard as

reported previously (Carmella et al., 2006; Zhong et al., 2011b). The transitions monitored for PheT, [D₁₀]PheT, and [¹³C₆]PheT were *m/z* 372 → *m/z* 210, *m/z* 382 → *m/z* 220, and *m/z* 378 → *m/z* 216, respectively. The analysis of baseline samples showed peaks for natural PheT and the internal standard [¹³C₆]PheT, but no detectable peak for [D₁₀]PheT. Natural PheT arises from exposure to Phe in cigarette smoke, the diet, and the environment. Clean, readily quantifiable peaks for [D₁₀]PheT were observed as early as the first time points after both oral and smoking doses in all subjects: at 15 min postdose for plasma samples and 0- to 30-min intervals for urine samples. Positive and negative control samples produced the expected values.

Typical plasma concentration-time profiles after a single dose of 10 µg of [D₁₀]Phe administered orally or through cigarette smoking are illustrated in Fig. 2. To determine whether the route of administration affected the extent of [D₁₀]PheT formation, ratios ([D₁₀]PheT from oral dose divided by [D₁₀]PheT from smoking dose for each subject) of plasma AUC_{0-∞} and amount excreted in 48 h urine were calculated. The results are summarized in Table 1. Subject 7, with ratios of 10.06 and 3.05, was determined to be an outlier (*p* < 0.001). The ratios in the other 15 subjects were 1.03 ± 0.32 (90% CI 0.84–1.14) and 1.02 ± 0.35 (0.83–1.13) for

Fig. 2. A, plasma concentration-time plots for [D₁₀]PheT in subject 3 after smoking a cigarette containing [D₁₀]Phe (▲) or receiving an oral dose of [D₁₀]Phe (○). B, urinary excretion rate-time plots for [D₁₀]PheT in subject 3 after smoking dose (▲) or the oral dose (○). Plasma data after the smoking dose have been previously published for 12 of the subjects (Zhong et al., 2011); one is included in A for comparison to the oral dose.

TABLE 1

Ratios (oral dose/smoking dose) of geometric means of two pharmacokinetic parameters for [D₁₀]PheT after oral and smoking administration of 10 µg of [D₁₀]Phe to 16 healthy smokers

Subject	Ratios of Plasma AUC _{0-∞}	Ratio of Total Urinary Excretion
1	0.73	0.7
2	1.08	0.66
3	0.98	0.98
4	0.96	0.65
5	0.49	0.66
6	0.72	0.91
7	10.06	3.05
8	0.9	0.77
9	1.27	1.41
10	1.13	1.03
11	1.18	1.9
12	0.83	1.09
13	0.75	0.83
14	1.51	1.33
15	1.75	1.36
16	1.11	1.02
Mean ± S.D.	1.59 ± 2.28	1.15 ± 0.61
90% CI	(0.85, 1.52)	(0.86, 1.26)
Mean ± S.D. ^a	1.03 ± 0.32	1.02 ± 0.35
90% CI ^a	(0.84, 1.14)	(0.83, 1.13)

^a Without subject 7.

plasma AUC_{0-∞} and urinary excretion, respectively, showing that excretion of [D₁₀]PheT after administration of [D₁₀]Phe either orally or in a cigarette was the same. Furthermore, a two-sided paired *t* test of all subjects comparing oral and smoking doses indicated that there was no statistically significant difference in either plasma AUC_{0-∞} or the total urinary excretion of [D₁₀]PheT between the two routes of administration (*p* = 0.42 and 0.51, respectively). Figure 3 shows plasma AUC_{0-∞} of [D₁₀]PheT and the total urinary excretion of [D₁₀]PheT over 48 h following the two routes of administration for 16 subjects, ranking from low to high by the level of [D₁₀]PheT after smoking administration.

Plasma levels of [D₁₀]PheT after smoking doses were maximum at early time points examined in all subjects, 15 to 30 min after smoking the cigarette containing [D₁₀]Phe, and decreased thereafter, as we have published previously for 12 of these subjects (Zhong et al., 2011b). Oral administration produced delayed maximum blood concentrations of [D₁₀]PheT from 30 min to 6 h postdose (Fig. 2). However, there was no significant difference in the elimination half-life of [D₁₀]PheT, determined by the slope of the terminal phase of the plasma concentration-time profile and the urinary excretion rate-time profile, between the two routes of administration (*p* = 0.43). The mean elimination half-lives (±S.D.) of [D₁₀]PheT in 16 subjects determined by the plasma concentration-time profiles were 8.93 ± 5.40 h after the oral dose and 7.69 ± 1.99 h after the smoking dose. The mean elimination half-lives (±S.D.) determined by urinary excretion rate-time profiles were 7.78 ± 1.85 h after the oral dose and 7.95 ± 1.04 h after the smoking dose.

The plasma concentration-time profile provides a direct measurement of systemic exposure to [D₁₀]PheT. However, a measurement of the urinary level of [D₁₀]PheT will be preferred for future phenotyping clinical studies because it is noninvasive and simpler. We thus examined whether total urinary excretion can substitute for plasma AUC_{0-∞} to predict individual metabolic activation. The results demonstrated that total 48-h urinary excretion of [D₁₀]PheT as

Fig. 3. A, plasma AUC_{0-∞} of [D₁₀]PheT after oral dose (open bars) and smoking administration (closed bars) in 16 subjects, ranking from low to high by AUC_{0-∞} of [D₁₀]PheT after smoking dose (*p* = 0.42). B, total 48-h urinary excretion of [D₁₀]PheT after oral dose (open bars) and smoking administration (closed bars) for 16 subjects, ranking from low to high by the level of [D₁₀]PheT after smoking administration (*p* = 0.51). Plasma data for 12 subjects after the smoking dose have been previously published and are included here for comparison (Zhong et al., 2011).

percentage of dose administered was highly correlated with plasma AUC_{0-∞} of [D₁₀]PheT, either after smoking or oral dose (*r* = 0.95 and 0.81, respectively), as illustrated in Fig. 4. Furthermore, 2- and 6-h postdose amounts of [D₁₀]PheT excreted in urine were significantly correlated with the 48-h urinary excretion (*r* = 0.83 and 0.95, respectively; *p* < 0.001), as shown in Fig. 5.

We observed a large interindividual variation among the 16 subjects in their capacity to metabolize [D₁₀]Phe to [D₁₀]PheT. Amounts of urinary [D₁₀]PheT excreted in 48 h ranged from 0.54 to 12.0% of the dose after oral dosing and from 0.74 to 15.1% after smoking dosing (Fig. 3B). These results show that the extent of metabolism of [D₁₀]Phe by the diol epoxide pathways varied by as much as 20-fold among the 16 smokers after both routes of administration. There was a large interindividual variation in natural urinary PheT in these 16 smokers as well, ranging from 0.17 to 5.03 pmol/mg creatinine. However, there was a weak correlation between the urinary excretion of [D₁₀]PheT (percentage of dose) and urinary level of natural PheT (*r* = 0.42; *p* < 0.05), probably because the PheT level was also affected by Phe exposure.

The PheT/HOPhe ratio in urine has been used as a measurement of an individual's capacity to metabolize Phe, because this ratio presumably would not be influenced by variations in Phe exposure (Hecht et al., 2005). We further analyzed the level of urinary total HOPhe and total [D₉]HOPhe in the three subjects who had the highest levels of urinary excretion of [D₁₀]PheT and three with the lowest levels. PheT and HOPhe were detected in all samples and

Fig. 4. Relationship of total 48-h urinary excretion of [D₁₀]PheT as percentage of dose to plasma AUC_{0-∞} of [D₁₀]PheT after smoking (A) or oral dose (B) ($n = 16$).

remained relatively constant throughout the 48-h period. Table 2 summarizes the levels of total 48-h urinary excretion of [D₁₀]PheT, total [D₉]HOPhe, and their ratios after oral and smoking dosing, compared with the PheT/HOPhe ratios in these six subjects' urine samples. Levels of [D₁₀]PheT were not correlated with the ratios of [D₁₀]PheT/[D₉]HOPhe ($r = 0.52$; $n = 12$; $p = 0.08$). However, there was a statistically significant correlation of [D₁₀]PheT with PheT/HOPhe ratios ($r = 0.71$; $n = 12$; $p < 0.01$). The ratios [D₁₀]PheT/[D₉]HOPhe and PheT/HOPhe were strongly correlated ($r = 0.91$; $n = 12$; $p < 0.001$), as illustrated in Fig. 6. The ratio [D₁₀]PheT/[D₉]HO-Phe among individuals showed a 50- to 100-fold spread, much greater than the variation in [D₁₀]PheT and PheT/HOPhe ratios.

Table 2 shows that the group including subjects 1, 6, and 14, who formed the largest amounts of [D₁₀]PheT, excreted similar amounts of [D₉]HOPhe as did subjects 12, 7, and 2, who excreted the lowest levels of [D₁₀]PheT. To further investigate the reason for the interindividual variation in [D₁₀]PheT formation between these two groups, we analyzed plasma and urinary levels of [D₁₀]Phe and urinary levels of [D₁₀]N-acetyl-S-(*r*-4,*t*-2,3-trihydroxy-1,2,3,4-tetrahydro-*c*-1-phenanthryl)-L-cysteine (*anti*-[D₁₀]PheDE-1-NAC) in these six subjects. Levels of [D₁₀]Phe in both plasma and urine samples were below our limit of quantitation (5 pg/ml), which suggests that unmetabolized [D₁₀]Phe comprised less than 0.2% of the dose in all six subjects. There was no significant difference in the 24-h urinary excretion of *anti*-[D₁₀]PheDE-1-NAC between the two groups: $0.018 \pm 0.021\%$ of the dose for the group forming higher

Fig. 5. Relationship of 48-h urinary excretion of [D₁₀]PheT as percentage of dose to 2-h (A) and 6-h (B) postdose urinary excretion of [D₁₀]PheT ($n = 32$). ▲, smoking dose; ○, oral dose.

amounts of [D₁₀]PheT (subjects 1, 6, and 14) and $0.020 \pm 0.021\%$ of the dose for the lower [D₁₀]PheT forming group (subjects 12, 7, and 2). Collectively, these results demonstrate that the difference in [D₁₀]PheT excretion between the high excretion group (subjects 1, 6, and 14) versus the low excretion group (subjects 12, 7, and 2) was not caused by differences in the extent of formation of other metabolites (e.g., [D₉]HOPhe or *anti*-[D₁₀]PheDE-1-NAC) or differences in unmetabolized [D₁₀]Phe.

Discussion

This study used a unique strategy for phenotyping of PAH metabolic activation in humans. Urinary biomarkers of exposure plus metabolism of PAHs such as PheT, BaP-tetraol, and 1-hydroxypyrene have been described previously (Hecht et al., 2003; Hansen et al., 2008; Zhong et al., 2011b). However, the levels of these biomarkers reflect individual differences in both exposure to and metabolic activation of PAHs. All humans, smokers and nonsmokers, are exposed to PAHs through the diet and the general environment. The use of deuterium-labeled [D₁₀]Phe makes it possible to exclude the contribution of environmental or dietary exposure. Thus we were able to specifically track diol epoxide formation after a single PAH dose. This approach allowed us to directly assess in vivo interindividual variation in PAH metabolism in smokers.

Only a fraction of lifelong smokers develop lung cancer,

TABLE 2

Levels of urinary [D₁₀]PheT, total [D₉]HOPhe, and the ratios of [D₁₀]PheT/[D₉]HOPhe and natural PheT/HOPhe in subjects 1, 6, and 14 (with high [D₁₀]PheT formation) and 12, 7, and 2 (with low [D₁₀]PheT formation)

Subject	Gender	Age	CPD	Percentage of Administrated Dose				Ratios			
				[D ₁₀]PheT		[D ₉]HOPhe ^a		[D ₁₀]PheT/[D ₉]HOPhe		PheT/HOPhe	
				Smoking	Oral	Smoking	Oral	Smoking	Oral	Smoking	Oral
1	F	40	15	15	11	5.6	1.8	2.7	5.8	0.67	1.5
6	M	54	25	13	12	1.9	5.4	6.9	2.2	1.8	1.0
14	F	25	12	8.2	11	0.6	1.7	13	6.4	2.0	1.6
12	F	28	25	3.0	3.2	2.6	2.8	1.2	1.1	0.27	0.37
7	F	31	13	2.0	5.9	1.4	4.0	1.5	1.5	0.47	0.94
2	M	47	23	0.8	0.5	7.2	4.0	0.11	0.13	0.063	0.056

CPD, cigarette per day; M, male; F, female.

^a Total [D₉]HOPhe = [D₉]1-HOPhe + [D₉]2-HOPhe + [D₉]3-HOPhe + [D₉]4-HOPhe.

Fig. 6. Relationship of the ratios of [D₁₀]PheT/[D₉]HOPhe to natural PheT/HOPhe ($n = 12$). ▲, smoking dose; ○, oral dose.

whereas others with comparable smoking histories remain free of lung cancer. This large interindividual variation is believed to be influenced not only by the extent of tobacco carcinogen exposure but also by differences in carcinogen metabolic activation or detoxification, among other factors. Although the extent of carcinogen exposure in a smoker is clearly important, this can be estimated by the measurement of urinary nicotine metabolites and other tobacco-specific compounds. The approach described here potentially will allow us to determine interindividual differences in PAH metabolic activation in smokers, unconfounded by dietary or environmental exposures that may not be relevant to lung cancer. We hypothesize that differences in PAH metabolic activation will be related to lung cancer risk. Our ultimate goal is to identify those smokers who are at highest risk for lung cancer, so they can be targeted for preventive measures. This clinical study was designed to establish the basics for using [D₁₀]PheT as a biomarker for PAH metabolic activation in future large-scale risk assessment studies in smokers.

A main aim of this study was to determine and compare the pharmacokinetic profile of [D₁₀]PheT after two routes of administration of [D₁₀]Phe: oral ingestion and inhalation through cigarette smoke. This information is essential for developing a reliable phenotyping method. If the two routes of administration produce markedly different results, smoking administration will be preferred because it mimics the major PAH exposure route in smokers. If there are no significant differences between the two routes of administration, an oral dose will be used, because it is simpler and more

practical. Several previous studies have examined the bioavailability and urinary excretion of BaP and pyrene metabolites after oral, intravenous, cutaneous, intraperitoneal, or intrapulmonary administration to rats (Jacob et al., 1989; Van de Wiel et al., 1993; Bouchard and Viau, 1997). They found that the formation of metabolites varied greatly after different administration routes. In contrast to these results in animal studies, we found comparable plasma AUC, half-life, and total urinary excretion of [D₁₀]PheT after oral and cigarette smoking administration. There was no significant difference in the rate or extent of metabolism of [D₁₀]Phe to diol epoxides between the two routes of exposure. This supports the use of oral dosing in future phenotyping studies. [D₁₀]PheT was formed rapidly after the smoking dose and was already present at its highest levels in plasma at 15 to 30 min postdose in all subjects, as reported previously (Zhong et al., 2011b). We found here in the oral dosing arm that the [D₁₀]PheT level peak time varied from 30 min to 6 h. These results suggest that there was a significant “first-pass” biotransformation of [D₁₀]Phe in the lung as well as in the liver. Absorption in the gastrointestinal tract probably is related to our observation that the [D₁₀]PheT peak was reached more slowly after the oral dose than after the smoking dose in some subjects, but this requires further study.

Our results indicate that 6-h urinary excretion of [D₁₀]PheT was sufficient to reflect the extent of metabolism to [D₁₀]PheT in smokers, because it was highly correlated with 48-h excretion. After both routes of administration, excretion of [D₁₀]PheT was almost complete over the 48-h period, which is consistent with the 24- to 48-h period reported in animal studies (Bouchard and Viau, 1997). By 6 h after administration, approximately half of [D₁₀]PheT transformed from the parent dose had already been excreted in urine. The 6-h time period will be practical for phenotyping studies in smokers.

Many previous studies clearly demonstrated large interindividual differences in PAH metabolism in humans (Harris et al., 1976; Nebert, 2000), leading to the logical hypothesis that individuals who extensively metabolically activate these carcinogens are at a higher risk for cancer. Beginning with the work of Conney and coworkers in the 1960s (Welch et al., 1968) and continuing to the present, these studies have used a variety of in vitro approaches, most commonly using mitogen-activated human lymphocytes to investigate the inducibility of PAH metabolism via the aryl hydrocarbon receptor, which regulates the *CYP1A1*, *CYP1A2*, and *CYP1B1* genes, coding for cytochromes P450 1A1, 1A2, and 1B1, which are

involved in PAH metabolism (Nebert et al., 2004; Tang et al., 2005). Approximately 10% of the white population has a high inducibility phenotype. It is well established that cigarette smoking induces CYP1A and CYP1B enzymes, most likely through the binding of PAH and other smoke constituents to the aryl hydrocarbon receptor (International Agency for Research on Cancer, 1986, 2004; Kim et al., 2004; Nebert et al., 2004; Port et al., 2004; Tang et al., 2005). The relationship between aryl hydrocarbon hydroxylase inducibility and lung cancer risk in smokers has been examined with conflicting results (International Agency for Research on Cancer, 1986, 2004; Kiyohara et al., 1998; Nebert et al., 2004), possibly because aryl hydrocarbon hydroxylase inducibility affects both the metabolic activation and detoxification of PAH. In contrast to these studies, our approach is apparently the first to use a PAH for *in vivo* phenotyping of smokers.

Although our approach was different, our results are consistent with these previous studies. A 20-fold interindividual variation in the formation of [D₁₀]PheT was observed after both oral and smoking dosing. This is a striking finding considering that only 16 smokers were included in this study. We investigated other pathways of [D₁₀]Phe metabolism in the subjects with the three highest and three lowest levels of urinary [D₁₀]PheT. The differences in [D₁₀]PheT excretion were not explained by differences in levels of [D₁₀]HOPhe or *anti*-[D₁₀]PheDE-1-NAC, nor could we detect [D₁₀]Phe in the urine or plasma of these subjects. Apparently, there are other pathways of Phe metabolism that differ in the subjects with high versus low amounts of excreted [D₁₀]PheT. One possibility is Phe-diols, which have previously been identified in human urine (Jacob et al., 1999), but we did not investigate their levels in this study. There may be uncharacterized routes of Phe metabolism as well.

One limitation of this study is that the dose of [D₁₀]Phe (10 µg) used was approximately two to three times as much as the amount of natural Phe a regular smoker (20 cigarettes/day) receives per day from smoking cigarettes, based on machine measurements (Ding et al., 2005). The 10-µg dose was used to facilitate analysis of [D₁₀]PheT in plasma and urine. Our plasma data suggest that Phe-metabolizing enzymes were not saturated by this dose. Unchanged [D₁₀]Phe was not detected in urine after either route of administration, which suggests a complete and rapid metabolism of Phe in our subjects. The metabolites quantified here in six of our subjects, [D₁₀]PheT, [D₉]HOPhe, and *anti*-[D₁₀]PheDE-1-NAC, together accounted for approximately 3 to 21% of the dose of [D₁₀]Phe (Table 2). Previous studies have identified two other types of Phe metabolites in humans—dihydrodiols, formed in amounts similar to HOPhe, and a mercapturic acid resulting from conjugation of Phe-9,10-epoxide with glutathione, formed in amounts similar to *anti*-[D₁₀]PheDE-NAC (Jacob et al., 1999; Upadhyaya et al., 2006). Including these metabolites, the percentage of dose in these six subjects might increase by a few percent, but there is still a considerable amount of unknown metabolism. We speculate that this might result from further oxidation of Phe-9,10-diol to Phe-9,10-quinone and subsequent ring cleavage, but further studies are required to more fully characterize Phe metabolism in smokers. The study reported here focused mainly on the diol epoxide pathway believed to be critical in the induction of cancer by PAH.

A second limitation of our approach is related to [D₁₀]Phe

itself. As it is a relatively low molecular weight PAH, its pharmacokinetics and metabolism may be quite different from those of carcinogenic PAH such as BaP, but it would be unethical to give a carcinogenic PAH to smokers. Phe is generally considered noncarcinogenic, and we have reported a correlation between levels of PheT and BaP-tetraols in human urine (Hochalter et al., 2011). In addition, the approach described here using [D₁₀]Phe as a surrogate for carcinogenic PAH does not reveal any information about differences in uptake or metabolism of other carcinogens in tobacco smoke such as tobacco-specific nitrosamines or volatile organic compounds. Alternate techniques would be required to assess the contributions of these compounds to individual susceptibility.

In summary, we demonstrate that the metabolic activation of Phe, as represented by the biomarker [D₁₀]PheT, is comparable after ingestion and inhalation in smokers. Our approach is apparently the first to directly phenotype PAH metabolism *in vivo* in humans. This noninvasive phenotyping method potentially can become part of an individual-based, predictive model for lung cancer risk assessment in smokers in the future.

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Authorship Contributions

Participated in research design: Zhong, Rauch, Oliver, Jensen, Hatsukami, and Hecht.

Conducted experiments: Zhong and Hochalter.

Contributed new reagents or analytic tools: Carmella and Upadhyaya.

Performed data analysis: Zhong, Wang, and Zimmerman.

Wrote or contributed to the writing of the manuscript: Zhong and Hecht.

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