Nicotine metabolism in African Americans and European Americans: variation in glucuronidation by ethnicity and UGT2B10 haplotype

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ABSTRACT

Nicotine is the major addictive agent in tobacco smoke, and it is metabolized extensively by oxidation and glucuronide conjugation. The contributions of ethnicity and UGT2B10 haplotype on variation in nicotine metabolism were investigated. Nicotine metabolism was evaluated in two populations of smokers. In one population of African American and European American smokers (n=93) nicotine and its metabolites were analyzed in plasma and 24-hour urine over 3 days while participants were abstinent and at steady state on the nicotine patch. In a second study of smokers (n=84) the relationship of a UGT2B10 haplotype linked with Asp67Tyr to nicotine and cotinine glucuronidation levels was determined. We observed that both African American ethnicity and the UGT2B10 Asp67Tyr allele were associated with a low glucuronidation phenotype. African Americans excreted less nicotine and cotinine as their glucuronide conjugates compared to European Americans; percent nicotine glucuronidation, 18.1 versus 29.3 (p < 0.002); percent cotinine glucuronidation, 41.4 versus 61.7 (p < 0.0001). In smokers with a UGT2B10 67Tyr allele, glucuronide conjugation of nicotine and cotinine was decreased by 20% compared to smokers without this allele. Two key outcomes are reported here. First, the observation that African Americans have lower nicotine and cotinine glucuronidation was confirmed in a population of abstinent smokers on the nicotine patch. In addition, we provide the first convincing evidence that UGT2B10 is a key catalyst of these glucuronidation pathways in vivo.
INTRODUCTION

Lung cancer is the second most common cancer and the leading cause of cancer death among both African Americans and European Americans (Jemal A., 2009). African Americans have higher rates of lung cancer than European Americans despite consistently reporting smoking fewer cigarettes per day (cpd) (Haiman et al., 2006). Yet higher levels of the nicotine metabolite cotinine have been documented in African Americans at all levels of smoking (Caraballo et al., 1998; Kandel et al., 2007; Perez-Stable et al., 1998; Pirkle et al., 1996; Wagenknecht et al., 1990). In the National Health and Nutrition Examination Study (NHANES) III, cotinine was also higher in African American nonsmokers than European American nonsmokers who reported similar environmental tobacco smoke exposure (Caraballo et al., 1998). Higher cotinine among African American compared to European American smokers could reflect higher exposure per cigarette, differences in nicotine metabolism, or a combination of both. Benowitz et al. conducted a nicotine infusion study in smokers and reported that African Americans had decreased urinary levels of nicotine and cotinine glucuronide conjugates, slower conversion of nicotine to cotinine, and increased nicotine exposure per cigarette compared to European Americans (Benowitz et al., 1999; Perez-Stable et al., 1998). Ethnic differences in nicotine metabolism may affect cancer risk through an effect on smoking behavior or because the enzymes that metabolize nicotine also metabolize carcinogens in tobacco smoke.

In most smokers, nicotine is metabolized primarily by P450 2A6 catalyzed 5′-oxidation (Hukkanen et al., 2005). The product of this reaction is further metabolized to cotinine either by P450 2A6 or aldehyde oxidase (von Weymarn et al., 2006). Cotinine, in turn is metabolized to trans-3′-hydroxycotinine. Nicotine may also be metabolized by N-oxidation or N-glucuronidation (figure 1); each of these pathways on average accounts for less than 10% of the
total nicotine dose excreted. However, in some smokers, nicotine glucuronide may account for up to 50% of the nicotine metabolites excreted (Murphy et al., 2004).

In the urine of a smoker, or an individual on the nicotine patch, >80% of the nicotine dose is accounted for by nicotine, cotinine, \textit{trans}-3'\text{\-}hydroxycotinine and their glucuronide conjugates, (Hukkanen et al., 2005; Scherer et al., 2007). The sum of these six compounds, referred to as total nicotine equivalents, has been used by us and others as an estimate of tobacco exposure (Derby et al., 2008; Scherer et al., 2007). Overall, glucuronide metabolites of nicotine and cotinine account for about 25% of the nicotine metabolites excreted in urine, and from 0.8 to 84% of the nicotine or cotinine in a smoker's urine is present as its glucuronide conjugate (Benowitz et al., 1994; Murphy et al., 2004).

Variation in nicotine metabolism has been associated with differences in smoking behavior (Benowitz, 1999; Malaiyandi et al., 2006; Schoedel et al., 2004; Strasser et al., 2007). Metabolism largely determines the rate of decline in nicotine levels and to avoid onset of nicotine withdrawal, smokers adjust their smoking. (Benowitz, 1999). The P450 2A6 (CYP2A6) gene is highly polymorphic, and several variants are associated with altered (increased or decreased) nicotine metabolism, including variants found in African Americans (Ho et al., 2009; Mwenifumbo et al., 2008; Mwenifumbo and Tyndale, 2007). Yet even among African Americans who are genotypically considered CYP2A6 wild-type, there is significant variation in phenotype, assessed by the ratio of \textit{trans}-3'\text{\-}hydroxycotinine to cotinine (Ho et al., 2009).

The role of glucuronidation on variation in nicotine metabolism and smoking behavior is not well characterized. \textit{N}-Glucuronide conjugation of nicotine and cotinine is catalyzed by UDP-
glucuronosyl transferases (UGT) (Kaivosaari et al., 2007; Kuehl and Murphy, 2003) and in vitro the most efficient catalyst of both reactions is UGT2B10, followed by UGT1A4 (Kaivosaari et al., 2007). Both enzymes are polymorphic but few variants have been evaluated in nicotine metabolism (Chen et al., 2007; Ehmer et al., 2004; Saeki et al., 2005). An Asp67Tyr substitution in UGT2B10 is associated with a 20-30% decrease in nicotine and cotinine glucuronidation in vitro (Chen et al., 2007). In European Americans the Asp67Tyr allele is linked with a haplotype with an allelic prevalence of 11%; UGT2B10 haplotype analysis or genotyping of Asp67Tyr has not been reported for African Americans (Chen et al., 2007).

We report here the results of a controlled dose nicotine metabolism study of African Americans and European Americans. Nicotine and its major metabolites were analyzed in urine from abstinent smokers on the nicotine patch, as well as while participants were smoking ad libitum. In addition, we evaluated the effect of UGT2B10 haplotype on nicotine and cotinine glucuronidation.
METHODS

Patch Study Recruitment

The study was approved by the University of Minnesota’s Institutional Review Board. Smokers were recruited from the Minneapolis-St. Paul area through newspaper advertisements, flyers, and word of mouth. Potential participants were scheduled by telephone for a screening visit. Inclusion criteria were (1) age 18-74, (2) grandparents who were all either African American or European American, (3) daily smoking > 10 cpd, and (4) in good health. A medical history questionnaire and vital signs were used to assess health. Individuals who used illicit drugs, were taking barbiturates or anticonvulsants and women who were pregnant were ineligible. A total of 105 individuals enrolled.

Patch Study protocol

The 8-day study included baseline smoking assessment, followed by administration of the nicotine patch (Nicoderm, GlaxoSmithKline). Participants completed questionnaires about demographics and smoking behavior. Current smoking was verified by exhaled carbon monoxide (CO) and a urine spot check for nicotine (NicCheck). Participants collected 24-hour urine while smoking as usual. Subsequently, participants were instructed to abstain from smoking and to use 21 mg nicotine patches. On days 5-7 of using the patch, after reaching steady state, participants visited the study center for CO testing, blood pressure and heart rate assessment, and 24-hour urine drop-off. A subset of participants provided plasma (n= 70, 75%) during days 5-7. Blood for genotyping was obtained from 32 participants. Compliance was determined as having a CO < 8 ppm and turning in used nicotine patches. Participants were offered referrals to smoking cessation programs and received compensation ($200).
TRIP Study

UGT2B10 genotype-phenotype analyses involved samples from a prior study of nicotine metabolism among European American smokers. Tobacco Reduction Intervention Program (TRIP) participants were smokers aged 18 – 70 who smoked > 14 cpd (Hecht et al., 2004; Murphy et al., 2004). While smoking as usual, participants provided first morning urine; 2 – 4 samples were collected with each at least one week apart. Urinary nicotine and nicotine metabolite concentrations were previously reported (Murphy et al., 2004).

Chemicals and reagents

(S)-[5-3H]-Nicotine was purified as previously described (Murphy et al., 2005). Nicotine N-βD-glucuronide was purchased from Toronto Research Chemicals (North York, ON, Canada). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Nicotine and Metabolite analysis

All urine samples were stored at -20 °C prior to analysis. Four 24-hour urine samples were analyzed (days 1 and 5-7) per participant. Base treatment was used to cleave glucuronide conjugates of nicotine and cotinine. Free nicotine, total nicotine (free nicotine + N-glucuronide), free cotinine, and total cotinine (free cotinine + N-glucuronide) were quantified by gas chromatography/mass spectrometry (GC/MS) (Hecht et al., 1999b; Hecht et al., 1999a; Murphy et al., 2004). Total cotinine and total nicotine values for urine samples, stored at -20 °C and analyzed 8 months apart did not differ significantly. The mean difference in the pair of values was 7.3% for cotinine and 12% for nicotine (n= 62). Free cotinine concentrations in urine stored
at 5 °C for 30 days are no different then when analyzed immediately (Hagan et al., 1997). Total trans-3'-hydroxycotinine (trans-3'-hydroxycotinine + its glucuronides) was analyzed after treatment with β-glucuronidase by GC/MS (Hecht et al., 1999b). From whole blood, plasma was separated and stored at -20 °C. Nicotine and cotinine were then quantified by GC/MS as performed for urinary metabolites except for an initial solid phase extraction using an Oasis MCX column (Waters Corporation, Milford, MA) (Hecht et al., 1999a). Analyses were performed in duplicate and repeated if values differed by > 10%. The mean difference in duplicates was 3%.

**UGT2B10 Haplotyping**

DNA was isolated from blood (Ethnic study, n=32; TRIP study, n=84) with the GFX DNA purification kit (Amersham Biosciences, Piscataway, NJ) or from frozen human liver tissue (n=28) using the DNeasy kit (Qiagen, Valencia, CA). PCR-restriction fragment length polymorphism (RFLP) analysis was performed as previously described on the UGT2B10 SNP rs7657958 that is linked with Asp67Tyr in Caucasians (haplotype C) (Chen et al., 2008). DNA samples that tested positive for the rs7657958 variant and an equal number of samples that were called wild-type were subjected to a second RFLP targeting the codon 67 position with HinfI-digestion (Chen et al., 2008). DNA from all African Americans and from all European Americans in the ethnic study was genotyped at the codon 67 position. The Asp67Tyr substitution was confirmed by DNA sequencing in 8 individuals (2 African Americans and 6 European Americans) and its absence was confirmed in 2 wild-type individuals.
Nicotine glucuronidation by human liver microsomes (HLMs)

Frozen normal liver tissues from African American (n=14) or European American (n=14) donors were obtained through the Liver Tissue Cell Distribution System from the University of Pittsburgh repository (NIH Contract # N01-DK-7-0004/HHSN26700700004C). Donor characteristics were average age (49 years), gender (50% female), BMI (26.6 kg/m²), and smoker (36%). Microsomes were prepared by the method of Fowler et al. and aliquots were stored at -80 °C (Fowler et al., 1994). Total protein concentrations were determined by the Bradford assay with Coomassie Plus (Pierce, Rockford, IL). Microsome quality was evaluated by p-nitrophenol O-glucuronidation activity. Product formation was quantified by UV-HPLC after incubation with 500 µM p-nitrophenol and 5 mM UDPGA (Kuehl and Murphy, 2003). These HLMs had p-nitrophenol glucuronidation activity ranging from 10-55 nmol/min/mg protein.

HLMs (2mg/ml) were incubated with 0.5 or 5 mM (S)-[5-3H]nicotine (specific activity, 25 Ci/mol and 2.5 Ci/mol, respectively) and 5 mM UDPGA essentially as described by Kuehl et al. (Kuehl and Murphy, 2003). Metabolites were quantified by radioflow HPLC using a Gemini C18 5 µM 250 x 4.60 mm column (Phenomenex, Torrance, CA), SPD-10Avp UV-VIS detector (Shimadzu, Kyoto, Japan), and β-RAM radioflow detector (IN/US Systems, Tampa, FL). The mobile phase was 20 mM ammonium bicarbonate (pH 9.2) in water (A) and acetonitrile (B) and the gradient was 2.5%B for 0-12 min., to 25% B in 15 min., hold at 25% B for 5 min. The eluant flow was 0.8 ml/min and the scintillant flow was 2.4 ml/min using Monoflow 5 (National Diagnostics, Atlanta, GA). This HPLC system provided good separation between nicotine N-glucuronide (11 min.) and nicotine (41 min.). No significant cleavage of nicotine N-glucuronide was observed during HPLC analysis. The tritiated reaction product co-eluted with nicotine N-glucuronide standard, and formed nicotine following β-glucuronidase treatment. Activity assays
were performed with microsomes that underwent a single freeze-thaw since repeated freeze-thaw resulted in significantly decreased p-nitrophenol and nicotine glucuronidation activity; however, this did not appear to vary by donor ethnicity or haplotype.

*Other analyses*

Exhaled CO was determined using a Medical Gas Monitor (Bedfont Scientific Ltd., Kent, United Kingdom). Urine spot checks for nicotine were performed using NicCheck (Mossman Associates, Blackstone, MA).

*Statistics*

Patch participants were excluded from analyses if they were noncompliant. Specifically, participants were excluded if on more than one visit they excreted < 20% or > 120% of the patch dose or had exhaled CO levels > 8 ppm. Single visits that met noncompliance criteria were excluded from analysis (8 of 279, 3%). Overall, 12 participants (7 African American) were excluded due to: ethnicity was American Indian (n=1), not completing the study (n=2), dose recovery was < 20% (n=6), dose recovery was > 120% (n=3, two had plasma cotinine levels greater than 700 ng/ml, suggesting concurrent smoking). Included in analyses were 93 participants. Baseline urine was available for 59 participants, after exclusion of 4 participants who returned < 400 ml of 24-hour urine at baseline.

Statistical analyses were conducted using SAS (SAS Institute, Cary, NC) and Excel (Microsoft, Redmond, WA). Wilcoxon two-sided t-approximation statistics were calculated to compare means of continuous variables that did not have a normal distribution, and a p-value less than 0.05 was considered significant. Chi-square approximation statistics were analyzed for categorical variables. Spearman nonparametric correlation was used to assess univariate
correlations. Multivariate linear regression models were evaluated to assess predictors of
cotinine glucuronidation (square-root transformed ratio of cotinine glucuronide to free cotinine)
and the nicotine metabolite ratio (square-root transformed ratio of total trans-3’-hydroxycotinine
to total cotinine) (Derby et al., 2008).
RESULTS

Patch study population

African American and European American participants were not statistically different in age (mean = 38; range 26-56), gender (51% female), cpd (mean = 21), or quit attempts (mean = 4). The distribution of time to first cigarette was 48%, 0-5 min.; 37%, 6-30 min.; 11.5%, 31-60 min.; 3.5%, > 60 min, and there was no difference by ethnicity. African American women reported more quit attempts than men (6.1 versus 3.2, p =0.012) or European American women (6.1 vs. 3.3, p = 0.02). Mentholated cigarettes were smoked by 86% of African Americans and 24% of European Americans (p < 0.0001). Fewer African Americans than European Americans reported attending at least some college in this study population (43% vs. 64%, p = 0.04).

Nicotine metabolism on the patch

Free nicotine and free cotinine were quantified in plasma to assess nicotine dosing achieved with the patch in 70 of 93 (75%) of participants. At steady-state (day 6), free cotinine levels were comparable to levels observed in smokers. Mean free nicotine and free cotinine were 28.1 ± 30.6 ng/ml and 311 ± 170 ng/ml for African Americans and 18.3 ± 11.8 ng/ml and 245 ± 129 ng/ml for European Americans and were not statistically different by ethnicity. Mean plasma free cotinine and nicotine concentrations also did not differ by gender. Plasma free cotinine was correlated with 24-hour urinary free cotinine; 0.49, p < 0.0001.

Nicotine and nicotine metabolites were quantified in 24-hour urine from days 4,5,6 of patch use and their average urinary concentrations were determined for each individual. The mean intraindividual variation in the concentrations for all analytes measured was much less than the interindividual variation (Table 1). To estimate the nicotine dose each subject obtained during
patch use we calculated the percent of the patch dose excreted in each 24-hour urine collection. Specifically the molar sum of nicotine and its metabolites in 24-hour urine were expressed as a molar percentage of nicotine in a 21 mg (130 µmoles) patch. The mean estimated dose on days 5-7 was 61 ± 20% for African Americans and 70 ± 25% for European Americans (p = 0.09). The intraindividual variation in the estimated dose recovered was 13% for both African Americans and European Americans. In addition, nicotine equivalents, defined as the sum of nicotine, cotinine, trans-3'-hydroxycotinine and their respective glucuronides, expressed as nmol/ml or µmol/24-hour urine, were the same for African Americans and European Americans; 81.2 ± 39.4 nmol/ml and 84.0 ± 42.7 nmol/ml, respectively (Scherer et al., 2007). Therefore, any differences in absorbance from the patch or compliance, in patch use or urine collection, did not vary by ethnicity.

The urinary concentration of free cotinine did differ significantly by ethnicity. The mean free cotinine concentration for African Americans was 33% higher than for European Americans, 12.7 ± 6.2 nmol/ml versus 9.5 ± 4.2 nmol/ml, respectively (p = 0.01). Free nicotine was also higher among African Americans than European Americans, 12.9 ± 9.4 nmol/ml versus 9.9 ± 6.5 nmol/ml, but the means were not statistically different (p = 0.17). There was no significant difference by ethnicity in mean total nicotine, total cotinine or total trans-3'-hydroxycotinine, the product of cotinine oxidation (Table 1). Therefore, the observation that African Americans had higher free cotinine but similar total cotinine levels reflects a difference in glucuronidation.

Glucuronide conjugation of both nicotine and cotinine was significantly lower among African Americans compared with European Americans (Table 1). The percent of nicotine excreted as a glucuronide conjugate in 24-hour urine was 18.1 ± 12.7% for African Americans.
versus 29.3 ± 16.9% for European Americans (p = 0.002). The percent of cotinine excreted as a glucuronide conjugate was 41.4 ± 20.7% for African Americans versus 61.7 ± 14.2% for European Americans (p < 0.0001). Nicotine and cotinine glucuronidation were correlated in African Americans and European Americans, 0.73 and 0.53 respectively (p = 0.11 for difference in correlation by ethnicity).

The distribution of percent cotinine excreted as a glucuronide was distinct for African Americans (Figure 2). Few European Americans excreted less than 50% of cotinine as its glucuronide while this was common among African Americans. We observed large interindividual variation in percent glucuronidation regardless of ethnicity. The ranges of percent glucuronidation for nicotine and cotinine were 0.4 – 67.5% and 8.6 – 84.4% respectively. Intraindividual variation in glucuronidation on days 5 – 7 was low with a mean standard deviation of 7.3% for nicotine (range, 0.1-53%) and 4.9% for cotinine (range, 0.1-21.8%). There was no difference in intraindividual variation by ethnicity.

In contrast, we observed no difference in nicotine 5'-oxidation by ethnicity. We evaluated nicotine 5'-oxidation as the urinary ratio of total trans-3'-hydroxycotinine to total cotinine (Benowitz et al., 2003; Derby et al., 2008). The value of this ratio ranged from 0.10 – 7 across all subjects with one outlier of 15.8. The mean ratio was 2.28 ± 2.42 for African Americans and 1.62 ± 1.12 for European Americans (p = 0.27). The ratio of total trans-3'-hydroxycotinine to total cotinine (total:total) was highly correlated with the ratio of total trans-3'-hydroxycotinine to free cotinine (total:free) in both ethnic groups, 0.79 (p < 0.0001). Neither ratio varied by ethnicity. There was also no influence of gender on either ratio.
Predictors of glucuronidation, assessed as the ratio of cotinine glucuronide to cotinine, were evaluated by univariate correlations and multivariate linear regression models (Table 2). In univariate comparisons, a lower glucuronidation ratio was significantly correlated with African American ethnicity, male gender, older age, lower nicotine equivalents, higher \textit{trans}-3'-hydroxycotinine: cotinine ratio, lower percent of dose excreted as nicotine, and lower percent dose recovered. The glucuronidation ratio was not correlated with baseline cpd or urine volume. The multivariate model to evaluate predictors of low glucuronidation ratio (square-root of cotinine glucuronide: cotinine) using stepwise selection included African American ethnicity, low nicotine equivalents, and high \textit{trans}-3'-hydroxycotinine: cotinine ratio, and not gender. The adjusted $R^2$ value for the model was 0.41, compared with 0.35 if the \textit{trans}-3'-hydroxycotinine:free cotinine ratio was not included.

\textit{Baseline nicotine metabolism}

Nicotine and nicotine metabolites were also quantified in 24-hour urine at baseline while participants were smoking \textit{ad libitum} (Table 1). As was observed when these subjects were using the nicotine patch, free nicotine and free cotinine were higher in African Americans compared to European Americans, 8.8 versus 6.0 nmol/ml ($p = 0.02$) and 6.1 versus 4.4 nmol/ml ($p = 0.01$), respectively. Between ethnic groups there were no statistically significant differences in total nicotine ($p = 0.07$), total cotinine ($p = 0.54$), or total \textit{trans}-3'-hydroxycotinine ($p = 0.1$). The correlation between total nicotine and total cotinine was 0.70 ($p < 0.0001$). Mean nicotine equivalents were 77.4 nmol/ml and 61.7 nmol/ml among African Americans and European Americans respectively ($p = 0.09$). Notably, mean nicotine equivalents per cigarette were higher
for African Americans compared to European Americans, 4.7 nmol/ml versus 3.0 nmol/ml (p = 0.02).

At baseline, the percent nicotine excreted as its glucuronide was 51.4% and 57.4% among African Americans and European Americans respectively (p = 0.05). The percent cotinine excreted as its glucuronide was lower among African Americans compared to European Americans, 71.2% versus 79.8% (p = 0.01). The correlation between percent nicotine and cotinine glucuronidation was 0.63 (p < 0.0001).

There was no difference in the mean trans-3'-hydroxycotinine: cotinine ratio (total: total) by ethnicity at baseline. The ratio had an overall range of 0.1 – 3.7. The mean ratios were 1.5 ± 1.0 and 1.1 ± 0.6 among African Americans and European Americans respectively (p = 0.17). There was also no ethnic difference if the ratio was calculated as total trans-3'-hydroxycotinine to free cotinine (p = 0.92). However, the correlation between total trans-3'-hydroxycotinine and total cotinine concentration was lower among African Americans than European Americans; 0.36 versus 0.72 with 95% confidence intervals, 0.002-0.637 and 0.456-0.864, respectively (p = 0.06).

We analyzed predictors of the glucuronidation ratio during smoking (Table 2). In univariate comparisons, the strongest correlation was between the glucuronidation ratio and trans-3'-hydroxycotinine: cotinine ratio; - 0.56, p < 0.0001). In addition, a low glucuronidation ratio was correlated with African American ethnicity, male gender, and lower nicotine equivalents. In a multivariate model, African American ethnicity, low nicotine equivalents, and a high trans-3'-hydroxycotinine: cotinine ratio were significant predictors of low cotinine glucuronidation with an adjusted R^2 value of 0.50 (Table 3).
In vitro nicotine glucuronidation by human liver microsomes

Nicotine glucuronidation was characterized in vitro for 28 liver samples (14 from African American donors) that were genotyped for UGT2B10 Asp67Tyr. Correlation between PNP-O-glucuronidation and nicotine-N-glucuronidation was \( r = 0.4 \). A 50-fold variation was observed in glucuronidation activity when liver microsomes were incubated with either 0.5 mM or 5 mM nicotine; range, 1.1 to 51 (mean 14.7) and not detectable (< 15) to 112 (mean 46.4) pmol/min/mg protein, respectively. Mean nicotine glucuronidation activity was not different between samples obtained from African American and European American donors at both nicotine concentrations, 15.1 ± 12 versus 14.3 ± 13 pmol/min/mg protein (p = 0.90) respectively at 0.5 mM nicotine. Unlike ethnicity, UGT2B10 genotype did appear to predict nicotine glucuronidation (figure 3). The UGT2B10 Asp67Tyr variant was present in 2 African American and 6 European American samples. The mean rate of nicotine glucuronidation was 46-50% lower for Asp67Tyr samples at both nicotine concentrations compared to wild-type, 8.1 pmol/min/mg protein compared to 17.5 pmol/min/mg protein (p = 0.07) at 0.5 mM nicotine. Due to the small sample numbers the power to detect a difference in the means was limited.

Relationship of Nicotine and cotinine glucuronidation in vivo to UGT2B10 haplotype

DNA was available from only 32 subjects in the patch study (18 African Americans and 14 European Americans). These subjects were genotyped for the UGT2B10 Asp67Tyr allele (Chen et al., 2008). In this small number of subjects there was no evidence that the Asp67Tyr allele was more common in African American individuals. Five participants, 3 African Americans and 2 European Americans, were heterozygous and one African American was homozygous for the Asp67Tyr allele. No statistical differences were observed in nicotine metabolites by genotype.
Interestingly, the percent nicotine and cotinine glucuronidation in the single homozygous Asp67Tyr variant, was low, 7.5 and 11.9%, respectively.

In a second population of smokers, for which both urinary nicotine metabolites and DNA were available, we further investigated the relationship of the UGT2B10 Asp67Tyr variant to nicotine and cotinine metabolism in vivo. Nicotine metabolite data on this population had been previously gathered (Hecht et al., 2004; Murphy et al., 2004). For the current study, participants were genotyped for the UGT2B10 haplotype that is tagged by the rs7657968 SNP and linked to Asp67Tyr (haplotype C) (Chen et al., 2008). Genotyping results were concordant in the subset of samples, which included all heterozygous individuals, that were analyzed by two RFLP analyses and DNA sequencing. Asp67Tyr was detected as a G>T substitution at nucleotide +199, and this co-occurred with a synonymous SNP at nucleotide 111 (T>C) as reported previously (Chen et al., 2008). The allele frequency was 0.11 and was in Hardy-Weinberg equilibrium. The mean percent glucuronidation and glucuronidation ratio of urinary metabolites in these smokers was determined for each haplotype (Table 3). Using either measure the extent of glucuronide conjugation for both cotinine and nicotine was significantly lower among smokers who were heterozygous for UGT2B10 Asp67Tyr relative to smokers with no Asp67Tyr alleles. Moreover, nicotine equivalents were significantly lower among individuals with a UGT2B10 Asp67Tyr allele (Table 3); the mean was 35% lower than wild-type. The distribution of percent cotinine excreted as a glucuronide was related to UGT2B10 genotype (Figure 4). However, there were wild-type individuals who excreted less than 10% of cotinine as its glucuronide conjugate and heterozygotes excreting as much as 80% of cotinine as a glucuronide conjugate.
DISCUSSION

Higher free cotinine levels have been consistently reported among African Americans compared to European Americans (Caraballo et al., 1998; Kandel et al., 2007; Wagenknecht et al., 1990). To the extent that higher cotinine levels reflect higher nicotine exposure and correspondingly exposure to tobacco carcinogens, higher cotinine could explain a portion of the increased cancer risk observed among African Americans. In our study, as previously observed (Benowitz et al., 1999; Perez-Stable et al., 1998), African Americans received a higher dose of nicotine per cigarette than European Americans, presumably due to a difference in smoking behavior. However, differences in nicotine metabolism independent of dose per cigarette also contribute to ethnic differences in free cotinine levels. Here we report that among abstinent smokers on the nicotine patch, African Americans excreted less nicotine and cotinine as their glucuronide conjugates than European Americans. Importantly, the observed difference in glucuronidation occurs in the absence of exposure to other constituents in tobacco smoke. Higher free cotinine among African Americans was accounted for by lower glucuronide conjugation in the controlled dose setting.

In an elegant pharmacokinetic study of nicotine and cotinine Benowitz et al. (1999) reported that nicotine and cotinine glucuronidation, as well as cotinine clearance, cotinine half-life, and the conversion of nicotine to cotinine were lower in African American compared to European American smokers. They concluded from their study that cotinine and nicotine metabolism is slower in African Americans, due in part to decreased \textit{N}-glucuronidation and also to decreased oxidation. However, the observed ethnic difference in cotinine's pharmacokinetic parameters could all be explained by decreased cotinine glucuronidation. The metabolic clearance of nicotine to cotinine, as defined by the authors is significantly influenced by the clearance of
cotinine, which was 25% lower in African Americans (Benowitz et al., 1999). Therefore,
decreased cotinine glucuronidation not decreased nicotine oxidation may be driving the reported
decrease in the metabolic clearance of nicotine to cotinine. In the present study there was no
evidence that nicotine C-oxidation differed between African Americans and European
Americans. Neither the urinary trans-3'-hydroxycotinine concentration nor the ratio of trans-3'-
hydroxycotinine:cotinine was significantly different in these two ethnic groups.

The urinary trans-3'-hydroxycotinine:cotinine ratio is highly correlated with the plasma
trans-3'-hydroxycotinine:cotinine ratio and is used as a biomarker for P450 2A6 activity
(Benowitz et al., 2003). Yet, Swan et al. reported that variation in the urine ratio is greater than
the plasma ratio and it is likely influenced by the contribution of other genes (Swan et al., 2009).
We observed that glucuronidation is associated with the urinary trans 3'-hydroxycotinine:
cotinine ratio (in both studies reported here), and contributes to variation in this ratio.
Consequently, caution is warranted in comparing the urinary nicotine metabolite ratio as a
marker of P450 2A6 activity between populations with different phenotypic variation of cotinine
glucuronidation.

Since the majority of African Americans smoke menthol cigarettes compared to a minority of
European Americans, this has been proposed as an explanation for ethnic differences in nicotine
metabolism. However in a recent study of both African American and European American
smokers of menthol cigarettes no difference in urinary nicotine metabolites was observed
between menthol and non-menthol cigarette smokers (Heck, 2009). In addition, in a large study
(n=1424) of young adult smokers, use of menthol cigarettes did not explain higher cotinine
levels among African Americans compared to European Americans, and African Americans who
smoked non-mentholated cigarettes (n=89) still had higher cotinine (Wagenknecht et al., 1990).
In our study, menthol was not a factor since the half-life of menthol is about an hour, and all participants had not smoked mentholated cigarettes for a period of 4 days before sample collection (Gelal et al., 1999).

Differences in glucuronidation that result from differences at the enzyme level, due to variation in enzyme amount or activity, could be confirmed in vitro. We compared the in vitro glucuronidation of nicotine by human liver microsomes prepared from African American and European American donor tissue. Regretfully, the availability of liver tissue from African American donors was limited and in the small in vitro study no ethnic difference in nicotine glucuronidation was observed. However, UGT2B10 haplotype appeared to predict low versus high rates of nicotine glucuronidation independent of donor ethnicity. The UGT2B10 Asp67Tyr variant was present in samples from both African Americans and European Americans. We expect that this polymorphism affects nicotine metabolism in both populations, and that it is unlikely to explain the observed decreased levels of glucuronidation in African Americans compared to European Americans. However, an uncharacterized variant in UGT2B10 may well contribute.

UGT2B10 is the most efficient catalyst of nicotine and cotinine glucuronidation in vitro and it is predicted to be the major enzyme responsible for nicotine and cotinine glucuronidation in smokers (Chen et al., 2007; Kaivosaari et al., 2007). This is the first report of in vivo nicotine and cotinine glucuronidation by UGT2B10 haplotype, and our finding that smokers with a variant Asp67Tyr allele had decreased excretion of glucuronide conjugates provides convincing evidence that UGT2B10 significantly contributes to nicotine metabolism in vivo. However, as illustrated in Figure 4, the Asp67Tyr UGT2B10 variant is clearly only one contributor to nicotine and cotinine glucuronidation. The relatively high percentage of cotinine glucuronidation in some
individuals heterozygous for this variant could be due to either variation in UGT2B10 expression or the contribution of UGT1A4, as a second catalyst of cotinine glucuronidation.

Interestingly, individuals with the UGT2B10 Asp67Tyr variant had lower nicotine equivalents than individuals without this allele, and in both studies reported here, individuals with a low glucuronidation phenotype (irrespective of genotype) had lower nicotine equivalents while smoking \textit{ad libitum}. The potential role of UGT2B10 on smoking behavior merits further investigation based on these intriguing and novel findings.

We expect that differences in UGT2B10 activity, due to genetic or environmental factors, will affect the disposition of drugs and xenobiotics, beyond nicotine. For instance, UGT2B10 was reported to catalyze the metabolism of the analgesic, medetomidine, and its role in the metabolism of other drugs that undergo N-glucuronidation is being investigated (Kaivosaari et al., 2008). UGT2B10 has been shown to catalyze the \textit{N}-glucuronidation of the tobacco carcinogen 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanol (NNAL) \textit{in vitro} and is predicted to contribute to its detoxification \textit{in vivo} (Chen et al., 2008; Lazarus et al., 2005). UGT2B10 activity may modulate cancer risk through an effect on smoking behavior or carcinogen metabolism. In brief, low UGT2B10 activity, independent of ethnicity may have important health consequences.
ACKNOWLEDGEMENTS

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REFERENCES


FOOTNOTES

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**LEGENDS FOR FIGURES**

Figure 1. Glucuronide conjugation pathways of nicotine metabolism

Figure 2. Percent cotinine excreted as its glucuronide in 24-hour urine by ethnicity

Figure 3. In vitro nicotine glucuronide conjugation by UGT2B10 haplotype. Human liver microsomes were incubated with 0.5 mM $^3$H-nicotine (S.A. = 25 nCi/nmol) and 5 mM UDPGA.

Figure 4. Percent cotinine excreted as its glucuronide in 24-hour urine by UGT2B10 genotype
**TABLE 1.** Urinary nicotine and nicotine metabolite levels

<table>
<thead>
<tr>
<th>Metabolite Mean (nmol/ml)</th>
<th>Nicotine Patch a</th>
<th>Baseline Smoking b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A.A. (N=51)</td>
<td>White (N=42)</td>
</tr>
<tr>
<td>Free nicotine</td>
<td>12.9 (9.4; 4.8)</td>
<td>9.9 (6.5; 4.0)</td>
</tr>
<tr>
<td>Total nicotine c</td>
<td>15.6 (11.2; 5.4)</td>
<td>13.7 (8.1; 4.4)</td>
</tr>
<tr>
<td>Free cotinine</td>
<td>12.7 * (6.2; 2.2)</td>
<td>9.5 * (4.2; 1.9)</td>
</tr>
<tr>
<td>Total cotinine c</td>
<td>24.4 (14.0; 7.7)</td>
<td>28.2 (16.1; 6.8)</td>
</tr>
<tr>
<td>Total trans-3'-hydroxycotinine c</td>
<td>41.2 (27.7; 11)</td>
<td>42.0 (25.5; 12.7)</td>
</tr>
<tr>
<td>Nicotine equivalents d</td>
<td>81.2 (39.4; 16.8)</td>
<td>84.0 (42.7, 21.0)</td>
</tr>
<tr>
<td>Nicotine equivalents (µmoles/24 hr)</td>
<td>78.7 (25.6)</td>
<td>90.2 (32.8)</td>
</tr>
<tr>
<td>Percent nicotine glucuronide e</td>
<td>18.1 * (12.7)</td>
<td>29.3 * (16.9)</td>
</tr>
<tr>
<td>Percent cotinine glucuronide e</td>
<td>41.4 ** (20.7)</td>
<td>61.7 ** (14.2)</td>
</tr>
</tbody>
</table>
a Nicotine and metabolites were quantified in 24-hour urine while participants were on the nicotine patch; mean values from 3 days per participant

b Nicotine and metabolites were quantified in a 24-hour urine sample while participants were smoking *ad libitum*

c Total analyte represents the sum of the analyte and its glucuronide conjugate

d Sum of urinary total nicotine, total cotinine, and total *trans*-3'-hydroxycotinine

e Percent glucuronide = \[\frac{(\text{total analyte} - \text{free analyte})}{\text{total analyte}} \times 100\]

Note: Difference in means, * p-value < 0.05, ** p-value < 0.005
### TABLE 2. Predictors of the glucuronidation ratio\(^a\): multivariate linear regression models and univariate spearman correlation coefficients

<table>
<thead>
<tr>
<th></th>
<th>Adj. ( R^2 ) (% )</th>
<th>Independent variables(^b)</th>
<th>( p)-value</th>
<th>Univariate correlation (( p)-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patch:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucuronidation ratio</td>
<td>41</td>
<td>African American vs. European American</td>
<td>&lt;0.0001</td>
<td>- 0.49 (&lt;0.0001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male vs. female</td>
<td>0.536</td>
<td>- 0.21 (0.04)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.138</td>
<td>- 0.22 (0.03)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicotine equivalents(^c)</td>
<td>0.0002</td>
<td>0.30 (0.003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( trans-3')-Hydroxycotinine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cotinine ratio(^d)</td>
<td>0.0031</td>
<td>- 0.37 (0.0003)</td>
</tr>
<tr>
<td><strong>Baseline:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucuronidation ratio</td>
<td>50</td>
<td>African American vs. European American</td>
<td>0.032</td>
<td>- 0.35 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Male vs. female</td>
<td>0.103</td>
<td>- 0.36 (0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Age</td>
<td>0.180</td>
<td>- 0.20 (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nicotine equivalents(^c)</td>
<td>0.001</td>
<td>0.27 (0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( trans-3')-Hydroxycotinine:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>cotinine ratio(^d)</td>
<td>&lt;0.0001</td>
<td>- 0.56 (&lt;0.0001)</td>
</tr>
</tbody>
</table>

\(^a\) Glucuronidation ratio = cotinine-\(N\)-glucuronide / free cotinine; square-root transformed

\(^b\) Stepwise selection was used to select variables included in the models presented, though demographic variables (ethnicity, age, gender) were forced into the model if rejected during selection. Nicotine and its metabolites were measured in 24-hour urine.
Nicotine equivalents = sum of urinary total nicotine, total cotinine, and total trans-3'-hydroxycotinine

trans-3'-Hydroxycotinine:cotinine ratio = total trans-3'-hydroxycotinine / total cotinine; square-root transformed
**TABLE 3.** Urinary nicotine metabolites in smokers by UGT2B10 haplotype\(^a\)

<table>
<thead>
<tr>
<th>UGT2B10(^b)</th>
<th>N</th>
<th>Nicotine equivalents(^c)</th>
<th>Cotinine glucuronidation</th>
<th>Nicotine glucuronidation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percent(^d)</td>
<td>Ratio(^e)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>66</td>
<td>101.3 (58.1)</td>
<td>65.4 (13.5)</td>
<td>2.42 (1.39)</td>
</tr>
<tr>
<td>Asp67Tyr</td>
<td>18</td>
<td>69.2 (42.0)</td>
<td>52.7 (11.4)</td>
<td>1.34 (0.82)</td>
</tr>
</tbody>
</table>

\(^{a}\text{Metabolites were quantified in morning (1}\text{st void) urine, 2-4 baseline visits per participant and the effect of haplotype was tested by repeated measures analysis of variance. Values are mean (SD).}\)

\(^{b}\text{Asp67Tyr genotype includes only heterozygotes}\)

\(^{c}\text{Sum of total nicotine, total cotinine, and total trans-3'-hydroxycotinine}\)

\(^{d}\text{Percent of analyte excreted as a glucuronide}\)

\(^{e}\text{Ratio of conjugated analyte to free analyte; square-root transformed}\)
Figure 1

Nicotine $\rightarrow$ Nicotine-glucuronide

P450s $\rightarrow$ Cotinine $\rightarrow$ Cotinine-glucuronide

P450s $\rightarrow$ Trans-3'-hydroxycotinine $\rightarrow$ Trans-3'-hydroxycotinine glucuronide
Figure 2

The bar chart shows the frequency of individuals (No. of individuals) excreting cotinine as a glucuronide at different percentages. The categories are as follows:

- European American
- African American

The percentages are divided as follows:

- 10%
- 20%
- 30%
- 40%
- 50%
- 60%
- 70%
- 80%

The frequencies range from 0 to 18, with notable peaks at 60% for both European American and African American individuals, and another peak at 70% for African American individuals.
Figure 4

- Wild type
- Asp67Tyr (heterozygous)

Number of individuals

Percent cotinine excreted as a glucuronide