Exposure Levels and Cytochrome P450 1A2 Activity, but not N-Acetyltransferase, Glutathione S-Transferase (GST) M1 and T1, Influence Urinary Mutagen Excretion in Smokers

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Abstract

We investigated the polymorphic enzymes cytochrome P450 1A2 (CYP1A2), N-acetyltransferase (NAT2), glutathione S-transferase (GST) M1 (GSTM1), and T1 (GSTTI) in relation to cigarette smoking-associated urinary mutagenicity detected on YG1024 Salmonella typhimurium strain with 59 mix in 97 smokers. In each subject, cigarette smoke intake was checked by analysis of urinary nicotine plus its metabolites. NAT2 and CYP1A2 phenotypes were determined by the molar ratio of urinary caffeine metabolites detected by high-performance liquid chromatography, and GSTT1 and GSTM1 genotypes were determined by PCR. An increase in urinary mutagenicity was significantly related to levels of exposure to cigarette smoke and CYP1A2 N-hydroxylation activity (linear multiple regression analysis t = 4.51 and P < 0.001 and t = 3.09 and P = 0.003; F = 6.31, P < 0.001). Urinary mutagenicity was significantly higher in CYP1A2 extensive metabolizer smokers (n = 49) than in CYP1A2 poor metabolizer ones (n = 48; 2176 ± 1525 versus 1384 ± 1206 revertants/mmol creatinine, Mann-Whitney U-test, z = 2.65, P < 0.001). The highest mutagenic activity was seen in subjects CYP1A2 extensive metabolizer/NAT2 slow acetylators (n = 29) with respect to the other phenotype combinations (n = 68; 2392 ± 1660 versus 1525 ± 1238 revertants/mmol creatinine, Mann-Whitney U-test, z = 2.37, P = 0.017). NAT2 acetylation activity was slightly but inversely related to urinary mutagenicity, and the association was not significant. No effect of GSTM1 and GSTT1 genotypes in lowering (detoxifying) urinary mutagens was found. The significant enhancement of urinary mutagenicity associated with increased CYP1A2 activity, as already seen for diet-caused urinary mutagenicity, allows for many analogies between the process of mutagen formation derived from cooked meat and that from cigarette smoke condensate. In conclusion, the intensity of tobacco smoke exposure, modulated by CYP1A2 activity, is the major determinant of mutagenic urine among smokers, whereas GSTM1 and GSTT1 genotypes have no influence on this biomarker. This study suggests that CYP1A2 should definitely be determined in future studies involving urinary mutagenicity in cases in which smoking is a factor.

Introduction

Mainstream tobacco smoke is a complex mixture containing at least 4000 compounds, both volatile and particulate, including several carcinogenic/mutagenic agents. CSC,3 the particulate matter of mainstream smoke, is active in several short-term tests for genotoxicity (1) including the Salmonella/microsome assay (2). Smokers’ CSC intake may range from a few to some hundreds of milligrams per day. After smoking, CSC and/or its metabolites rapidly appear in the urine, in which they have been detected as increased mutagenic activity after urine concentration on adsorbents (3, 4).

Mutagenic compounds or their metabolites from CSC may be responsible for the mutagenicity of smokers’ urinary extracts. These mutagens are relatively not polar aromatic compounds, mainly have two or more aromatic rings, and belong to three major classes of genotoxins [PAHs, HAAs, and AAs (5–7)]. The daily intake of these compounds by a heavy smoker has been estimated at a maximum amount of hundreds of nanograms for AAs 2-naphthylamine and 4-aminobiphenyl and some micrograms for PAHs benzo(a)pyrene and benzo(a)anthracene and HAAs 2-amino-1-methyl-6 phenylimidazo[4,5-b]pyridine and α-aminocarboline (8). The urinary levels of these compounds and their metabolites, considered singly, cannot explain the finding of increased urinary mutagenicity in smokers.

After being inhaled, tobacco smoke condensate undergoes several metabolic transformations of activation/detoxification in various organs (mainly the liver) before its unmetabolized form or its metabolites appear in urine. CYP1A2 and NAT2 enzymes have been identified as being involved in the activation and detoxification of AAs and HAAs (9). Recently, two genetic polymorphisms (C734A and G2964A) of CYP1A2 have been identified (10, 11) as being associated with the high

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3 The abbreviations used are: CSC, cigarette smoke condensate; AA, aromatic amine; AFMU, 5-acetylamin-6-formyl-3-methyluracile; CYP1A2, cytochrome P450 1A2; GST, glutathione S-transferase; HAA, heterocyclic aromatic amine; PAH, polycyclic aromatic hydrocarbon; NAT2, N-acetyltransferase; 17U, 1,7-dimethyluric acid; 1U, 1-methyluric acid; 1X, 1-methylxanthine; EM, extensive metabolizer; PM, poor metabolizer; CV, coefficient of variation.
inducibility of enzyme activity in smokers, whereas a bimodal distribution of CYP1A2 activity in smokers and nonsmokers has been suggested (12–14). NAT2 polymorphism, which divides human populations into slow and fast acetylators, has been well known for a long time (15). PAHs undergo several metabolic transformations, and their reactive intermediates are detoxified by GSTs. The μ (GSTM1) and θ (GSTT1) members of the GST multigene family are involved in the detoxification of several tobacco smoke-derived carcinogens, including intermediate metabolites of PAHs, and deletion variants at both these loci, associated with a lack of detoxifying function, are well known (16). Polymorphisms of these metabolizing enzymes can thereby modulate the presence of tobacco smoke mutagens in urine.

The aim of this study was to investigate the influence of CYP1A2 and NAT2 phenotypes and GSTM1 and GSTT1 genotypes on modulating the presence of mutagens in the urine of cigarette smokers.

Materials and Methods

Subjects. A total of 97 healthy smokers comprised the sample population. Exclusion criteria were pregnancy, liver or kidney diseases, caffeine intolerance, antibiotic therapy and/or use of urinary disinfectants within the past 3 months, and occupational exposure to PAHs or other genotoxicants. For each subject, personal data were collected by means of a questionnaire. Subjects were informed of the study’s purpose and instructed about the protocol. All participants gave their informed consent. All information regarding participants was rendered anonymous after collection of data and blood and urine samples.

Study Design. This study was conducted between October 2000 and February 2001. Subjects were asked to abstain from consumption of charcoaled and pan-fried meat, alcohol-containing beverages, and any foods or beverages containing methylxanthines for 24 h before as well as during the day of the study. Participants were also instructed to keep their urine in a methylxanthines for 24 h before as well as during the day of the study. On the day of the experiment, after emptying their bladders in about 250 ml of water. Urine was collected for 6 h after the caffeine dose in 500-ml bottles preloaded with 6 ml of 6N HCl. Acidification of urine (pH 2) was further checked before urine storage at −20°C until analysis. In addition, subjects were asked for a blood sample for genotype analysis.

On the day of the experiment, after emptying their bladders in the morning, at 7 a.m. the subjects ingested an instant coffee beverage containing 140 mg of caffeine (2 packets of Nescafé) in about 250 ml of water. Urine was collected for 6 h after the caffeine dose in 500-ml bottles preloaded with 6 ml of 6N HCl. Acidification of urine (pH 2) was further checked before urine storage at −20°C. Urine samples for the analysis of mutagenicity and nicotine and its metabolites (at least 250 ml) were collected in the late afternoon. The compliance of each volunteer with these instructions had been checked with the questionnaire.

Analysis of Urinary Nicotine plus Metabolites. Urine concentration of nicotine plus metabolites was determined colorimetrically by the diethylthiobarbituric acid extraction method (17), based on the Koenig reaction. Diethylthiobarbituric acid, used as condensing agent, gives a pink product that can easily be extracted in ethyl acetate. Absorbance was measured spectrophotometrically at a wavelength of 532 nm. This simple method is very useful to estimate active tobacco smoke exposure, and, as also seen in our previous study (18), the value of urinary nicotine plus metabolites correlates better with urinary mutagenicity than urinary cotinine levels. In each urine sample, nicotine plus metabolites were adjusted for urinary creatinine, determined according to the Boehringer-Mannheim colorimet-
and slow acetylators, CYP1A2 EM and PM, GSTM1 active and *0/*0, and GSTT1 active and *0/*0, respectively. Analysis was carried out using the BMDP package (27).

Results

Table 1 shows the characteristics of the examined population (number of subjects, age, sex, number of cigarettes/day, frequencies of NAT2 rapid and slow acetylators, CYP1A2 EMs and PMs, and GSTM1 and GSTT1 genotypes). The levels (mean ± SD and range) of nicotine plus its metabolites and of mutagenic activity in urine samples, together with CYP1A2 [(AFMU + 1X + 1U)/17U] and NAT2 [(AFMU/AFMU + 1X + 1U)] ratios (mean ± SD and range), are also reported.

CYP1A2 activity in smokers was distributed over a wide range; metabolite ratios were 1.68–15.32, with a median value of 5.5. In our population, the percentages of slow acetylators and GSTM1- and GSTT1-null subjects were 71%, 57%, and 25%, respectively, with frequencies similar to those already reported for Caucasian populations. Urinary mutagenicity ranged from very low values (no detectable mutagenic activity in urine) to more than 6700 revertants/mmol creatinine, and values of urinary nicotine plus metabolites also ranged from values comparable with those of nonsmokers to 3.56 mg/mmol creatinine. Both the latter parameters were quite well correlated (Spearman correlation coefficient (Rho) = 0.52, P < 0.001).

Table 2 shows urinary mutagenicity levels (range and mean ± SD) of the 97 smokers, according to NAT2 and CYP1A2 phenotypes and GSTM1 and GSTT1 genotypes in 97 cigarette smokers.

Table 1  Characteristics of smoking sample population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No. of subjects</th>
<th>Gender</th>
<th>Age (yrs)</th>
<th>Cigarettes/day</th>
<th>Nicotine and its metabolites (mg/mmol creatinine)</th>
<th>Urinary mutagenicity (revertants/mmol creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>Range</td>
<td>Range</td>
<td>Range</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>38 ± 12</td>
<td>18 ± 8</td>
<td>7–50</td>
<td>1.68–15.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19–49</td>
<td>18 ± 8</td>
<td>184 ± 1425</td>
<td>3.56 mg/mmol creatinine</td>
</tr>
</tbody>
</table>

Table 2  Nicotine plus its metabolites and urinary mutagenicity according to NAT2 and CYP1A2 phenotypes and GSTM1 and GSTT1 genotypes in 97 cigarette smokers

<table>
<thead>
<tr>
<th>Pheno-genotype</th>
<th>Subjects N (%)</th>
<th>Nicotine plus metabolites (mg/mmol creatinine; mean ± SD)</th>
<th>Urinary mutagenicity* (revertants/mmol creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>48 (50%)</td>
<td>0.76 ± 0.64</td>
<td>1384 ± 1206</td>
</tr>
<tr>
<td>EM</td>
<td>49 (50%)</td>
<td>0.76 ± 0.42</td>
<td>2176 ± 1525**</td>
</tr>
<tr>
<td>NAT2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slow</td>
<td>69 (73%)</td>
<td>0.77 ± 0.58</td>
<td>1844 ± 1520</td>
</tr>
<tr>
<td>Rapid</td>
<td>28 (27%)</td>
<td>0.72 ± 0.44</td>
<td>1636 ± 1172</td>
</tr>
<tr>
<td>GSTM1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>42 (43%)</td>
<td>0.64 ± 0.43*</td>
<td>1775 ± 1454</td>
</tr>
<tr>
<td>*0/*0</td>
<td>55 (57%)</td>
<td>0.86 ± 0.43</td>
<td>1817 ± 1418</td>
</tr>
<tr>
<td>GSTT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>72 (75%)</td>
<td>0.79 ± 0.56</td>
<td>1834 ± 1476</td>
</tr>
<tr>
<td>*0/*0</td>
<td>24 (25%)</td>
<td>0.66 ± 0.48</td>
<td>1536 ± 1205</td>
</tr>
<tr>
<td>CYP1A2 EM/NAT2 slow</td>
<td>30 (30%)</td>
<td>0.78 ± 0.41*</td>
<td>2392 ± 1660*</td>
</tr>
<tr>
<td>Other combinationsa</td>
<td>68 (70%)</td>
<td>0.78 ± 0.41</td>
<td>1525 ± 1238</td>
</tr>
</tbody>
</table>

a On YG1024 + 89.

b GSTT1 was not determined in one subject.

c CYP1A2 EM/NAT2 rapid, CYP1A2 PM/NAT2 rapid, and CYP1A2 PM/NAT2.

Statistical comparisons: urinary mutagenicity (revertants/mmol creatinine) CYP1A2 PM versus EM and CYP1A2 EM/NAT2 slow versus other combinations Mann-Whitney U test, z = 2.65, **, P < 0.001 and 2.37; *, P = 0.017; urinary nicotine plus its metabolites GSTM1 *0/*0 versus active Mann-Whitney U test, z = 2.34, *, P = 0.02.
active genotype to the lowering of urinary mutagenicity in smokers was observed.

**Discussion**

In this study, we investigated polymorphic enzymes CYP1A2 and NAT2 (phenotype) and GSTM1 and GSTT1 (genotype), involved in the metabolism of several genotoxic compounds, in relation to cigarette smoking-associated urinary mutagenicity.

An increase in S9-mediated urinary mutagenicity in smokers, related to levels of tobacco smoke exposure, was easily detectable with the YG1024 strain, as observed previously by other authors (28–30).

The CYP1A2 activity of our smoking population had a very wide interindividual variability and was significantly different from that of nonsmokers, as reported by many authors (31–36) and by us as well (37). CYP1A2 is an inducible enzyme. Besides smoking, PAHs, HAAs, and certain dietary components (38) are known to induce enzyme activity.

Our results indicate that an increase in urinary mutagenicity was significantly related to CYP1A2 activity in smokers, also confirming other authors’ reports (39). Although CYP1A2 is expressed mainly in the liver, considering its great relevance in the activation of many environmental carcinogens (i.e., conversion of aromatic or heterocyclic amines to their proximate N-hydroxy derivatives), CYP1A2 activity may be a risk factor for the development of cancers in other tissues, targets for activated carcinogens. In a previous study, the CYP1A2 phenotype was significantly associated with increased risk of nonoccupational urinary bladder cancer (40). In another case-control study, persons with the high inducibility variant C734A polymorphism in intron 1 of CYP1A2 genotype were overrepresented in bladder cancer, but only if they were smokers or had slow NAT2 genotypes (41).

In agreement with other authors (29, 42), we did not find any clear effect of NAT2 phenotype on cigarette smoke-induced urinary mutagenicity. NAT2 slow acetylation alone, in workers professionally exposed to AAs (43), or in combination with GSTM1 in smoking coke-oven workers (44) has been shown to increase mutagenic activity. The slight increase in urinary mutagenicity in the subgroup of slow acetylators (which, in the present work, was significant only if extensive CYP1A2 activity was present) may indicate that aromatic and heterocyclic amine N-hydroxy derivatives from tobacco smoke do not undergo sequestration as stable DNA adducts in organs where NAT2 O-acetyltransferase is active and/or detoxification via NAT2 N-acetylation in the liver (45). Certainly, we cannot exclude the role of other conjugation pathways, e.g., glucuronidation, in the urinary elimination of tobacco-derived mutagenic aroyl/heterocyclic amines.

Neither the GSTM1 nor GSTT1 genotype influenced urinary mutagenicity in smokers. Only one report deals with urinary mutagenicity in smokers with GSTM1-null genotype (29). A significant increase in S9-mediated urinary mutagenicity, detected with YG1024 and TA 98 Salmonella strains, has been reported in smokers with GSTM1-null genotype compared with GSTM1 active ones, but the small number of observations and the poor control of smoke exposure (6 of 7 GSTM1-null subjects were heavy smokers, but only 5 of 10 were GSTM1 active) may explain this discrepancy. The influence of the GSTM1-null genotype on increasing mutagenic activity in humans highly exposed to PAHs alone has been reported by our research group (46). One consequence of the present results is that the contribution to smokers’ urinary mutagenicity of PAHs, the GSTM1-related detoxification pathway of which is well-known, is slight.

Urinary mutagens in smokers are a complex mixture containing indirectly acting mutagens, the identification of which is quite far from being achieved, although some attempts have been made (47). Most of smokers’ urinary mutagens have been found in the relatively nonpolar chemical acid extractable fraction, which contains both PAHs and HAAs (48, 49). PAHs and their metabolites have been detected in small quantities in smokers’ urine (50–53), much lower than the detection limit of many urinary mutagenesis assays (54). Instead, the bacterial mutagenic potency of some HAAs is extraordinarily high (55). Because smokers’ urinary mutagens act by means of a frameshift mechanism and can easily be extracted with “cotton bleu,” and their activity is abolished by nitrite treatment, it has been suggested that they are primary AAs including HAAs (56, 57). Moreover, later studies showed that the mutation spectrum of cigarette smoke more closely resembles that of AAs than that of PAHs (58). Tobacco pyrolysis is one indispensable step in the formation of mutagenic substances in condensate (59–61), which depends on combustion temperature (62) and tobacco protein contents (63). Subjects who use tobacco but do not burn it or burn only a little of it do not show detectable urinary mutagenic activity (64), or their values are greatly reduced (30). The process has many analogies with that of the formation of mutagens in cooked meat (65), and both these types of environmental exposure give rise to frameshift urinary mutagens in man that are evident only after metabolic activation (5, 66). Previously, we showed the significant enhancement of urinary mutagenicity associated with increased CYP1A2 activity in diet-caused urinary mutagenicity (37).

In conclusion, the intensity of tobacco smoke exposure modulated by CYP1A2 activity is the major determinant of mutagenic urine among smokers, whereas GSTM1 and GSTT1 genotypes have no influence on this biomarker. This study suggests that CYP1A2 should definitely be determined in fa-

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Table 3: Influence of nicotine and its metabolites, CYP1A2 and NAT2 phenotypes and GSTM1 and GSTT1 genotypes on urinary mutagenicity in 96 smokers: results of multiple linear regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Nicotine and its metabolites</th>
<th>CYP1A2</th>
<th>NAT2</th>
<th>GSTM1</th>
<th>GSTT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>b (std. error)</td>
<td>1091 (242)</td>
<td>815</td>
<td>−370</td>
<td>196</td>
<td>264</td>
</tr>
<tr>
<td>t</td>
<td>4.51 (3.09)</td>
<td>1.28</td>
<td>0.73</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>≤0.001 (0.003)</td>
<td>0.155</td>
<td>0.047</td>
<td>0.382</td>
<td></td>
</tr>
<tr>
<td>Partial r²</td>
<td>16.7% (7.9%)</td>
<td>1.3%</td>
<td>0.4%</td>
<td>0.3%</td>
<td></td>
</tr>
</tbody>
</table>

*a Coefficients of regression (b), corresponding standard error (SE(b)), t test of partial significance, and partial explained variance (r²) were estimated for each term included in the model. F = 6.31, P < 0.001.
ture studies involving urinary mutagenicity in cases in which smoking is a factor.

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