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ORIGINAL ARTICLE

U. Heudorf · J. Angerer

Urinary monohydroxylated phenanthrenes and hydroxypyrene – the effects of smoking habits and changes induced by smoking on monooxygenase-mediated metabolism

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Abstract ***Objectives***: Internal polycyclic aromatic hydrocarbon (PAH) exposure is usually studied by determining 1-hydroxypyrene in urine. In many studies, increased urinary levels of 1-hydroxypyrene have been found in smokers compared with non-smokers. The disadvantage of this procedure, however, is that it is based on only one substance. Therefore, in our study, urine specimens from smokers and non-smokers were tested for four monohydroxylated phenanthrenes in addition to 1-hydroxypyrene. ***Subjects and methods***: Spot urine samples from 288 non-smokers and 100 smokers were analysed for 1-, 2-, 3- and 4-hydroxyphenanthrene and 1-hydroxypyrene by a very sensitive high performance liquid chromatography (HPLC) method with fluorescence detection. The detection limit of the method is 5 ng metabolite/l urine. The data were calculated on a creatinine basis (ng/g creatinine). ***Results***: Highly significant differences and dose-response relationships with regard to cigarettes smoked per day were found for 2-, 3- and 4-hydroxyphenanthrene and 1-hydroxypyrene, but not for 1-hydroxyphenanthrene. When the ratio of the sum of hydroxyphenanthrenes to 1-hydroxypyrene, and the ratio of 1- and 2-/3- and 4-hydroxyphenanthrene were taken into consideration, significant negative dose-response relationships to the numbers of cigarettes smoked per day, were found. ***Conclusion***: 1-Hydroxypyrene as well as 2-, 3- and 4-monohydroxylated phenanthrenes in urine may be used as parameters to detect PAH exposure from cigarette smoking. Moreover, 3,4-oxidation of phenanthrenes was found to be enhanced in smokers, with a significant dose-response relationship. This phenomenon is thought to be caused by an induction of the CYP 1A2 (or CYP 3A4) monooxygenase system in smokers. Therefore, it may be recommended that monohydroxylated phenanthrenes be analysed in order to assess the balance between the PAH-metabolising cytochrome isoforms, and the activity or induction of cytochrome P450 isoforms, respectively.

Key words Polycyclic aromatic hydrocarbons (PAH) · Cigarette smoking · PAH exposure · Human biomonitoring · Urinary 1-hydroxypyrene · Urinary monohydroxylated phenanthrenes · Cytochrome P450

U. Heudorf
Public Health Department, Braubachstrasse 18-22,
60311 Frankfurt am Main, Germany

J. Angerer
Institute for Occupational, Social, and Environmental Medicine,
University of Erlangen-Nürnberg, Schillerstrasse 27,
91054 Erlangen, Germany

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during the incomplete combustion of wood, oil, coal and gas, and of other organic substances e.g. during the roasting of meat and smoking of tobacco. The predominant source of exposure to PAHs is the diet, with daily ingestion of PAHs estimated to be 1–16 μg and the mean daily ingestion of benzo(a)pyrene (BaP) 0.1–0.2 μg to 1–2 μg (Buckley et al. 1995; Dennis et al. 1983; deVos et al. 1990; Lioy et al. 1988; Lodovici et al. 1995; Menzie et al. 1992; Santodonato et al. 1981; Vaessen et al. 1988). The level of PAH exposure resulting from inhalation is comparatively low, except in smokers, whose level of exposure from smoking may be in the same range as exposure via the diet.

More than ten years ago, Jongeneelen proposed urinary 1-hydroxypyrene as an indicator of PAH exposure (Jongeneelen et al. 1988; Jongeneelen and Anzion 1991). Meanwhile, all over the world, studies of occupational and environmental PAH exposure have been carried out using proposed urinary 1-hydroxypyrene as an appropriate indicator of exposure (Jongeneelen 1998; Levin 1995; Strickland et al. 1996).

Increased levels of urinary 1-hydroxypyrene have been found in smokers relative to non-smokers (Angerer et al. 1992; Gilbert and Viau 1997; Göen et al. 1995;

Gündel et al. 1994 and 1996; Herikstad et al. 1993; Hong et al. 1999; Jacob et al. 1999; Jongeneelen et al. 1990; Levin 1995; Santella et al. 1993; Scherer et al. 2000; Sherson et al. 1992; Sithisarankul et al. 1997; van Rooij et al. 1994; van Schooten et al. 1995), although this effect could not be verified in some studies (Jongeneelen et al. 1988; Martin et al. 1989; Ny et al. 1993; Omland et al. 1994; Zhao et al. 1992).

The disadvantage of the procedure for analysing 1-hydroxypyrene in urine developed by Jongeneelen et al. (1988) and Jongeneelen and Anzion (1991) as a marker of PAH exposure is that it is based on only one substance, a metabolite of pyrene. In 1994, a high performance liquid chromatography (HPLC) procedure was published which allows simultaneous determination of different monohydroxylated phenanthrenes and 1-hydroxypyrene in urine (Lintelmann et al. 1994).

Phenanthrenes, i.e. PAHs with several non-equivalent double bonds, have been used for studying the activity and balance of diverse cytochrome P450 isoforms with different toxifying and detoxifying activities. In metabolism studies using V 79 Chinese hamster cells, genetically engineered for diverse human cytochromes P450, it was found that CYP 1A1 activity predominantly ended up in 1,2-oxidation of phenanthrene, whereas CYP 1A2 activity enhances 3,4-oxidation (Jacob et al. 1996). In smokers, an increase of 3,4-oxidation had been demonstrated, indicating the induction of the CYP 1A2 system by smoking (Jacob et al. 1999). Similar results were obtained in another study assessing environmental PAH exposure in women in an industrial area in Germany: significant differences were found in urinary concentration of 1-hydroxypyrene and 2-, 3- and 4-hydroxyphenanthrene according to smoking behaviour. This was not the case for 1-hydroxyphenanthrene (Gündel et al. 1996).

Subjects and methods

In 1998, spot urine samples were collected from 495 adults (20–64 years of age, 37.9 ± 7.9 years; 40% male, 60% female) living in former American Forces housing in Frankfurt am Main, an urban area without heavy industrial pollution. Occupational exposure to PAHs was excluded by questionnaire. Of the adults, 288 of them stated they were non-smokers. A total of 100 persons said they were smokers, and stated the number of cigarettes they smoked per day. The urine specimens were frozen and stored at −18 °C until required for analysis for 1-, 2-, 3- and 4-hydroxyphenanthrene, and 1-hydroxypyrene. Analysis was carried out using a very sensitive HPLC method with fluorescence detection, which has been proven for its analytical reliability by the working group "analytical chemistry" of the senatcommission for the investigation of health hazards of chemical compounds in the work area of the Deutsche Forschungsgemeinschaft (Lintelmann and Angerer 1999). The method is, in brief: after enzymatic hydrolysis of the glucuronides and sulfates of the hydroxylated phenanthrenes and pyrene, an aliquot is injected into an HPLC instrument for a system internal sample processing. The PAH metabolites are first enriched on a phthalocyanine-modified silica gel within the HPLC instrument, and the analytes are separated from the urine matrix. Then the analytes are transferred on to a reversed phase column by means of an automatic switching valve and quantified with the fluorescence detector. The calibration is carried out with aqueous standards which are processed and analysed in the same manner as the urine samples. The between-day impression for the determination of these PAH metabolites lay between 5 and 12% in a concentration range between 100 and 300 ng/l.

The limit of detection of this method is 5 ng metabolite/l urine.

Creatinine in urine was determined photometrically as picrate according to the Jaffé method (Taussky 1954). The data were calculated on a creatinine basis (ng/g creatinine). The SPSS program, version 8, was used for statistical analysis.

Results

The mean, median, 95th percentile and maximum values for the hydroxylated phenanthrenes and 1-hydroxypyrene in urine of smokers and non-smokers are shown in Table 1. The differences were highly significant for 2-, 3- and 4-hydroxyphenanthrene, the sum of monohydroxyphenanthrenes, and 1-hydroxypyrene, but not for 1-hydroxyphenanthrene (Mann-Whitney test). A highly significant positive dose-response relationship was found (Table 2; Kruskal-Wallis test; Fig. 1).

On the other hand, a highly significant negative dose-response relationship was found between the ratio of the sum of hydroxyphenanthrenes to 1-hydroxypyrene, and the ratio of 1- and 2-hydroxyphenanthrene to 3- and 4-hydroxyphenanthrene and the numbers of cigarettes smoked per day (Table 3; Fig. 2 (box-plots)).

There were significant positive correlations (Spearman rank correlation, two-tailed) between the numbers of cigarettes smoked per day and 2-, 3-, 4- and the sum of hydroxyphenanthrenes and 1-hydroxypyrene, whereas highly significant negative correlations were found with the ratios 1-hydroxyphenanthrene/1-hydroxypyrene, the sum of hydroxyphenanthrenes/1-hydroxypyrene, and 1- and 2-hydroxyphenanthrene/3- and 4-hydroxyphenanthrene (Table 4).

Discussion

In the study presented here, a clear dose-response relationship was found between PAH exposure due to smoking and 2-, 3- and 4-monohydroxyphenanthrene and 1-hydroxypyrene, but not for 1-hydroxyphenanthrene. These data may be compared with the data presented in two other publications assessing the impact of smoking on the levels of urinary pyrene and phenanthrene metabolites (Gündel et al. 1996; Jacob et al. 1999).

In 1996, urine specimens from 124 women in Germany were analysed for 1-hydroxypyrene and 1-, 2-, 3- and 4-hydroxyphenanthrene (Gündel et al. 1996). Women who smoked ($n = 27$) were found to have significantly higher concentrations of 2-, 3- and 4-monohydroxyphenanthrene and 1-hydroxypyrene (median values: 410; 610; 100; 480 ng/g creatinine) than non-smoking women ($n = 97$) (median values: 310; 310; 40; 150 ng/g creatinine), while no differences in urinary 1-hydroxyphenanthrene concentrations could be detected (530 compared with 510 ng/g creatinine). These

Table 1 Urinary hydroxylated phenanthrenes and hydroxypyrene in adult smokers and non-smokers (*LOD* limit of detection, *OHPhen* hydroxyphenanthrene, *OHPyr* hydroxypyrene)

	Non-smokers (n = 288)					Smokers (n = 100)					Mann–Whitney-U test
	< LOD n	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	P 95 ng/g Creatinine	Maximum ng/g Creatinine	< LOD n	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	P 95 ng/g Creatinine	Maximum ng/g Creatinine	
1-OHPhen	2	445 ± 358	350	1,104	3,075	1	437 ± 311	357	878	2,290	0.522
2-OHPhen	2	266 ± 231	206	625	2,395	1	314 ± 216	240	745	1,209	0.005
3-OHPhen	2	305 ± 209	244	682	1,371	2	473 ± 302	389	946	1,850	0.001
4-OHPhen	79	58 ± 170	30	147	2,600	9	65 ± 140	39	176	1,328	0.040
1-OHPyr	16	100 ± 101	77	263	1,172	2	195 ± 142	152	537	716	0.001

Table 2 Urinary hydroxylated phenanthrenes and hydroxypyrene in adult smokers and non-smokers according to the numbers of cigarettes smoked per day (*OHPhen* hydroxyphenanthrene, *OHPyr* hydroxypyrene)

	Non-smokers (n = 288)		Smokers < 10 cigarettes/day (n = 27)		Smokers 10–< 20 cigarettes/day (n = 42)		Smokers ≥ 20 cigarettes/day (n = 31)		Kruskal-Wallis test
	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	Mean ± SD ng/g Creatinine	Median ng/g Creatinine	
1-OHPhen	445 ± 358	350	400 ± 235	320	370 ± 165	325	559 ± 460	455	0.191
2-OHPhen	266 ± 231	206	254 ± 135	232	290 ± 200	230	397 ± 268	314	0.007
3-OHPhen	305 ± 209	244	335 ± 168	346	448 ± 211	403	626 ± 416	460	0.001
4-OHPhen	58 ± 170	30	80 ± 251	31	55 ± 53	39	66 ± 78	50	0.029
Phen (sum)	1,077 ± 834	869	1,071 ± 580	974	1,164 ± 502	1,079	1,648 ± 1,080	1,280	0.001
1-OHPyr	100 ± 101	77	134 ± 76	139	193 ± 135	147	251 ± 173	230	0.001

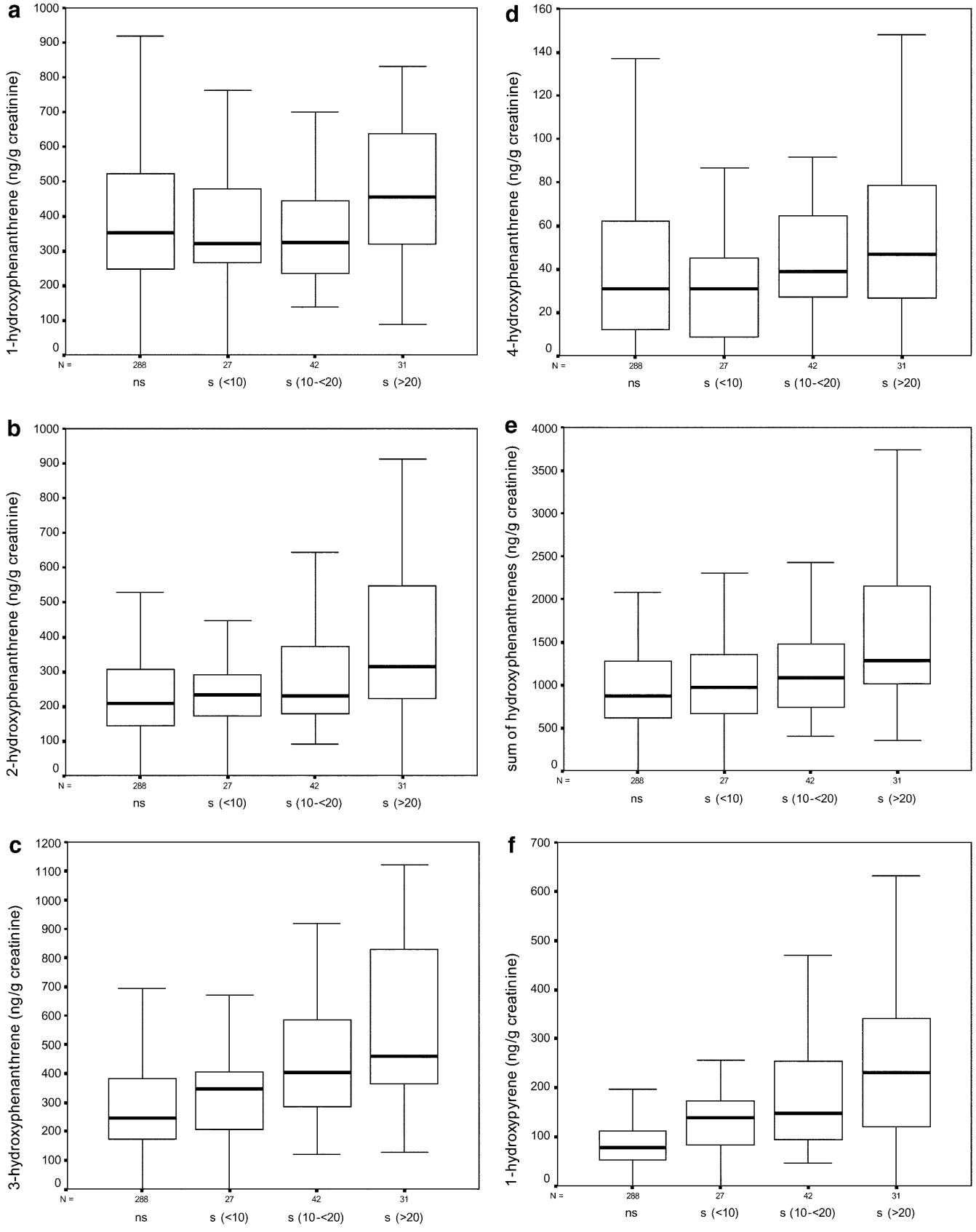

Fig. 1a–f Urinary 1-, 2-, 3-, 4- monohydroxylated phenanthrenes, total hydroxylated phenanthrenes, and 1-hydroxypyrene in relation to the number of cigarettes smoked per day. *Figures in parentheses* are number of cigarettes smoked per day. *Box-plots* indicate P 10, P 25, P 50, P 75, and P 90 (*ns* non-smoker, *s* smoker)

Table 3 Ratios of 1-hydroxyphenanthrene and the sum of hydroxyphenanthrenes to 1-hydroxypyrene, and the ratio of 1- and 2-hydroxyphenanthrene to 3- and 4-hydroxyphenanthrene in urine according to smoking behaviour (*OHPhen* hydroxyphenanthrene, *OHPyr* hydroxypyrene)

	Non-smokers (n = 288)	Smokers < 10 cigarettes/day (n = 27)	Smokers 10–< 20 cigarettes/day (n = 42)	Smokers ≥ 20 cigarettes/day (n = 31)	Kruskal–Wallis test
1-OHPhen/1-OHPyr	5.7 ± 4.9	3.6 ± 2.2	2.4 ± 1.3	2.8 ± 2.2	0.000
Phen (sum)/1-OHPyr	13.9 ± 12.0	10.1 ± 7.3	7.6 ± 4.2	8.5 ± 6.4	0.000
1- and 2-OHPhen/ 3- and 4-OHPhen	2.2 ± 1.0	1.8 ± 0.7	1.4 ± 0.4	1.4 ± 0.5	0.000

results are confirmed by our data. In addition, we could demonstrate a dose-response relationship with the number of cigarettes smoked per day.

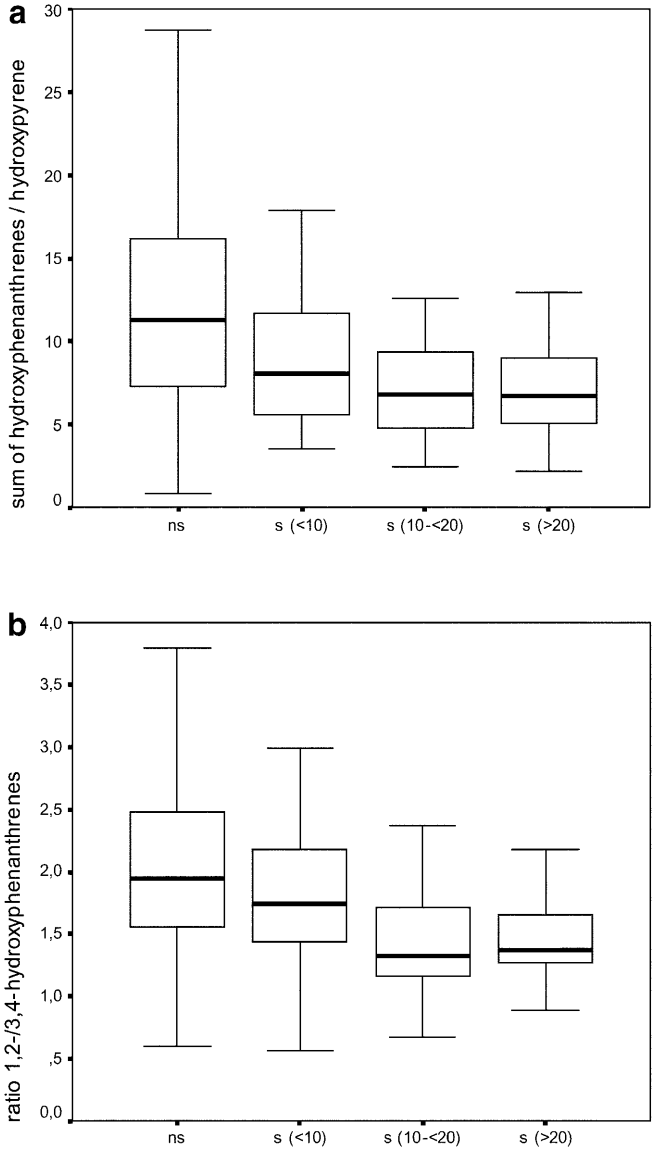

Fig. 2a, b Ratio of total hydroxyphenanthrenes/hydroxypyrene, and 1,2- vs. 3,4-hydroxyphenanthrene in urine specimens from non-smokers and smokers, in relation to the number of cigarettes smoked per day. *Figures in parentheses* are number of cigarettes smoked per day. *Box-plots* indicate P 10, P 25, P 50, P 75, and P 90 (*ns* non-smoker, *s* smoker)

In 1999, the levels of phenanthrene phenols, phenanthrene dihydrodiols and 1-hydroxypyrene in 24-h urine samples from ten smokers and ten non-smokers were published (Jacob et al. 1999). Although the study design was somewhat different – i.e. repeated 24-h urine samples analysed for the total amount of metabolites in ten smokers and ten non-smokers, versus single spot urine samples in 100 smokers and 288 non-smokers in our study – the results are comparable. The authors detected significantly higher 1-hydroxypyrene concentrations in smokers than in non-smokers (mean 603 compared with 218 ng/24 h), which reflects the higher intake of pyrene by smokers. They found a significantly lower ratio of total phenanthrene metabolites (phenanthrene phenols and dihydrodiols) to 1-hydroxypyrene in smokers than in non-smokers (4.2 compared with 10.4). Significant differences were detected between smokers and non-smokers in the ratios for regio-specific oxidation of phenanthrenes, i.e. 1,2-oxidation versus 3,4-oxidation (1.45 compared with 2.45), indicating increased 3,4-oxidation of phenanthrene as a result of the induction of the CYP450 1A2 (or CYP 3A4) monooxygenase system in smokers. Since a ratio of 2.6 was detected in urine specimens from coke plant workers highly exposed to PAHs – a value comparable with that found for non-smokers not occupationally exposed to PAHs – the authors argued that induction of this enzyme is obviously caused by cigarette constituents other than PAHs (Jacob et al. 1999; Grimmer et al. 1993). Moreover, comparison with occupationally exposed workers indicates that saturation of 1,2-oxidation need not be assumed in smokers (Jacob et al. 1999; Grimmer et al. 1993).

Table 4 Correlation of urinary monohydroxylated phenanthrenes, hydroxypyrene and various ratios with the numbers of cigarettes smoked per day (persons = 388) (Spearman correlation, two-tailed)

Parameter	Correlation (r)	P
1-OH-phenanthrene	+0.048	0.342
2-OH-phenanthrene	+0.160	0.002
3-OH-phenanthrene	+0.336	0.000
4-OH-phenanthrene	+0.119	0.01
OH-phenanthrenes (sum)	+0.195	0.000
1-OH-pyrene	+0.399	0.000
Phenanthrenes (sum)/1-OH-pyrene	−0.316	0.000
1- and 2-OH-phenanthrene/3- and 4-OH-phenanthrene	−0.379	0.000

Our study confirms these results. Additionally, we found a significant dose-response relationship for the different ratios indicating regio-specific oxidation of phenanthrenes according to the number of cigarettes smoked per day. We therefore confirm that induction of the CYP 450 1A2 (or CYP 3A4) monooxygenase system by PAHs (including phenanthrenes) in smokers may be readily assessed using monohydroxylated phenanthrenes.

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