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Substances in human urine that strongly inhibit bacterial mutagenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and related heterocyclic amines

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Extracts of human urine were shown to contain substances that strongly inhibited the liver S9-mediated mutagenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in *Salmonella typhimurium* TA98 strain in a liquid incubation assay. The inhibitory effect was unrelated to cytotoxicity and was similar with urine extracts from smokers and non-smokers. Under similar assay conditions, the mutagenicity of the related amino-imidazoazaarenes, 2-amino-3-methylimidazo[4,5-*f*]quinoline, 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoline and 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline was also found to be strongly inhibited by urine extracts. Decreased or enhanced mutagenicity was seen with 2-acetylaminofluorene and 2-aminoanthracene depending on the type of assay, and the time of incubation in liquid medium. A weak inhibition of the mutagenicity of 2-nitrofluorene, a direct-acting mutagen, was observed only after a short incubation time. Mutagenicity of 4-nitroquinoline *N*-oxide was not altered by the presence of urine extracts at concentrations shown to be inhibitory for the mutagenicity of heterocyclic aromatic amines. Our data suggest that the inhibitory substances in urine act through their capacity to non covalently bind the parent heterocyclic and aromatic amines, thus affecting their availability in aqueous medium for diffusion into liver microsomes where metabolic activation takes place.

Introduction

^{32}P-Postlabelling analysis of DNA adducts derived from mutagens in smokers' urine has implicated 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) as a major DNA-damaging agent present in the urine of smokers of black tobacco (1). When the mutagenic activity of PhIP was measured in *Salmonella typhimurium* TA98 strain under conditions (liquid incubation assay) used for assaying urinary extracts (2), substances that strongly inhibited PhIP mutagenicity were detected in urine from both non-smokers and smokers. We report herein on the capacity of such extracts to inhibit the liver S9-mediated mutagenicity of PhIP and related amino-imidazoazaarenes. Similar inhibition experiments were carried out to obtain mechanistic data, with 2-aminoanthracene (2-AA), 2-nitrofluorene (2-NF), 2-acetylaminofluorene (2-AAF) and 4-nitroquinoline *N*-oxide (4-NQO). Preliminary results of attempts to characterize the anti-mutagenic substances in urine are also presented.

Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 2-AAF, 2-acetylaminofluorene; 4-ABP, 4-aminobiphenyl; 4-NQO, 4-nitroquinoline *N*-oxide; DMSO, dimethylsulphoxide; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; B[*a*]P, benzo[*a*]pyrene; AP, alkaline phosphatase.

Materials and methods

Preparation of urine extracts

Samples of 24 h urine from randomly selected non-smokers and cigarette smokers were collected in polyethylene bottles, stored at +4°C during the collection period and then frozen at −20°C. Extracts were prepared within 3 months according to a procedure described previously (2), involving passage of a 220 ml urine aliquot through a column of XAD-2 resin, and elution of the mutagens with dichloromethane, followed by acetone. The combined organic phases were evaporated to dryness under reduced pressure at 40°C, dissolved in 500 μl of dimethylsulphoxide (DMSO), stored at −70°C and analysed within 4 months.

Salmonella mutagenicity assay

S.typhimurium TA98 and YG1024 strains were supplied by B.N.Ames (Berkeley, CA, USA) and T.Nohmi (Tokyo, Japan) respectively. Using a modified liquid incubation assay described by Malaveille *et al.* (2), the mutagenic activities of the urine extracts, a PhIP standard and a PhIP standard to which urine extract had been added, were measured in *S.typhimurium* TA98 in the presence of a 2.5 μl Aroclor 1254-treated σ BDVI rat liver S9 (final volume 210 μl). Incubation was carried out for 90 min at 37°C with shaking. The inhibitory effect of each urine extract on PhIP mutagenicity was measured in assays as above, containing 10 ng of PhIP (added in 10 μl 25% DMSO aqueous solution) and up to 10 μl DMSO solution of urine extract, equivalent to 4.4 ml of urine (5.7% final DMSO concentration in each assay of 220 μl). Surviving bacteria were determined by plating in duplicate 100 μl of 10^6 and 10^7-fold diluted incubation medium mixed with 2 ml selective soft agar medium onto minimal agar as described previously (3). The modifying effect of urine extract on the mutagenicity in *S.typhimurium* YG1024 of 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQX), 2AAF, 2-AA, 2-NF and 4-NQO was measured using liquid incubation assay conditions as indicated above for PhIP and in Table I. YG1024 strain is an *O*-acetyltransferase over-producing derivative of TA98 and thus is extremely sensitive to the mutagenic action of aromatic amines as shown by Watanabe *et al.* (4). The test compounds were each added in 10 μl 25% DMSO aqueous solution.

The plate incorporation assay of PhIP, benzo[*a*]pyrene (B[*a*]P) and 2-aminofluorene mutagenicity in *S.typhimurium* TA98 was carried out according to Maron and Ames (5) with minor modifications (6) in the presence of 50 μl Aroclor 1254-treated rat liver S9 per plate.

Assay for SOS-function-inducing activity in Escherichia coli PQ37

E.coli PQ37 strain was supplied by P.Quillardet (Paris, France). The SOS chromotest (7) was carried out and enzyme activities, SOS-inducing potency were calculated as described previously (2).

Analysis of methylxanthines by HPLC

Eight randomly selected urine extracts were analysed using a method adapted from Berthou *et al.* (8) and Campbell *et al.* (9). Urine extracts were diluted 100-fold with demineralized water; 200 μl aliquots were saturated by ammonium sulphate, then mixed with 100 μl solution of an internal standard, 7-(β-hydroxypropyl)theophylline. Methylxanthines were extracted by 6 ml (5 min) and 4 ml (3 min) of a mixture of chloroform and isopropanol (85:15 v/v) using a mechanical shaker. The dry residues obtained after evaporating the organic phase under a stream of nitrogen at ~40°C, were dissolved in HPLC mobile phase A. Methylxanthines were separated on a column (25 cm × 4.6 mm ID) containing Ultrapack C-18 (5 μm particle size). Solvent A was tetrahydrofuran, acetic acid and acetonitrile in water (3.3:2.7:10:984 v/v/v/v), solvent B was tetrahydrofuran, acetic acid and acetonitrile in water (7.5:4.0:75:913.5 v/v/v/v). The solvents were programmed from an initial concentration of 10% B for 5 min followed by a linear increase to 50% B in 20 min, then to 80% B in 10 min. The flow rate was 1 ml/min. The separation was monitored by UV at 254 nm.

Results and discussion

The mutagenicity of PhIP in the TA98 strain in the presence of rat liver S9 showed a linear dose–response relationship up to

Table I. Inhibition by urinary extracts of the mutagenicity of heterocyclic/aromatic amines and related direct-acting mutagens in *S.typhimurium* YG1024 using liquid incubation assay

Test compounds	Assay conditions		Range of the mutagenicity scored (rev./assay)	% inhibition produced by extract (2.2 ml urine equiv.)	
	Range (ng/assay)	Time of liquid incubation (min)		Range[a]	Mean
IQ	0.025–0.25	20	90–1460	79–100	94
MeIQ	0.05–0.10	10–20	465–1850	96–100	98
MeIQx	0.05–0.20	20	310–1080	97–100	99
2-AAF	25	5–20	300–1510	46–83	71
2-AA	20–60	5	90–380	24–44	34
2-NF	20	5–20	70–350	20–34	27
4-NQO	20	10–50	90–490	0	0

[a]Mean values from two or three series of experiments done in duplicate except for results on 2-AA and MeIQx that were calculated from one duplicate experiment. Urine from two non-smokers was used for the preparation of urinary extracts; 2.2 ml urine equivalent corresponds to 10 ml urine equivalent/ml liquid incubation medium.

25 ng per assay, with a sp. act. of 70 000 revertants/μg (calculated from two series of duplicate experiments with PhIP concentrations ranging from 1 to 100 ng/assay). The 17- to 35-fold higher specific mutagenicity of PhIP as compared to previously published values (reviewed in 10), is due to the high sensitivity of our conditions for the liquid incubation assay as discussed (2) and not due to a mutagenic contaminant of PhIP. This assay revealed a 12-fold higher mutagenicity of PhIP than the plate incorporation assay (Table II) using the same rat liver S9 concentration (per ml soft agar layer) as used per ml liquid incubation medium. The mutagenicity of PhIP was strongly reduced by addition of urine extracts at amounts that did not affect bacterial survival (Figure 1). The absence of a toxic effect of urine extracts was indicated by the unchanged number of viable *Salmonella* after incubation with up 10 μl pooled extracts prepared from the urine of five non-smokers (see Materials and methods). Similarly, the SOS chromotest, which is also a sensitive test for cytotoxicity, when carried out with an identical metabolic activation system as used for the *Salmonella* liquid incubation assay, showed that urine extract at concentrations close to that used in the *Salmonella* assay did not decrease the survival of bacteria, expressed in terms of the alkaline phosphatase (AP) activity (Figure 2). None of the extracts from non-smokers were mutagenic, while those of 10 smokers produced 40–190 revertants per 2.2 ml urine equivalent (Table III). For a better comparison of the quenching effect exerted by the urine extracts on PhIP mutagenicity, activity was expressed as revertants induced by 10 ng PhIP per assay (Figure 1). In the presence of 10 μl urine extract (the highest amount tested) from either smokers or non-smokers, the mutagenicity of PhIP was decreased 50-fold. The percentage inhibition for 5 μl extract per assay was similar to urine extracts from 10 smokers (mean ± SD: 91.9 ± 4.8) and 10 non-smokers (mean ± SD: 87.6 ± 5.1) (Table III). The mutagenicity of related amino-imidazoazaarenes, IQ, MeIQ and MeIQx, which are present in urine of smokers and fried meat consumers (11–13), was also strongly inhibited by urine extracts (Table I). Assay conditions identical to those for the PhIP experiments were used, except that *S.typhimurium* YG1024 (to permit measurements at very low test compound concentrations as compared to TA98) and shorter incubation times (located within an almost linear part of the time–response curves) were employed. Somewhat surprising results were obtained with 2-AAF and 2-AA, an aromatic amine and amide that are metabolized by cytochrome P450IA2, like the other amino-imidazoazaarenes assayed (14,15). For incubation times of ≤20 min and of ≤5 min in the case of 2-AAF and 2-AA respectively, the urine extracts inhibited their mutagenicity, although less strongly than with PhIP (Table I). For longer

Table II. Effect of urinary extract on the mutagenicity of PhIP, B[*a*]P and 2-AA in *S.typhimurium* TA98 strain using plate incorporation assay

Urinary extract equivalent to ml urine[a]	Mutagenicity as rev./assay[b] (% change)		
	100 ng PhIP[c]	2 μg B[*a*]P[c]	0.1 μg 2-AA[c]
Vehicle (DMSO)	595	490	90
11	500	460	300
22	430 (−28%)[d]	450	450 (+400%)
44	240 (−60%)	405 (−17%)	680 (+655%)

[a]Urine from two non-smokers was used for the preparation of urinary extracts; 11–44 ml urine equivalent correspond to 4.1–16.2 ml urine eqivalent per ml soft agar.
[b]Mean value of two plates. The number of revertants occurring spontaneously has been subtracted.
[c]The amounts assayed were located within the range of concentrations giving linear dose–response curves.
[d]% inhibition (−) or % enhancement (+) relative to a control value obtained in the absence of urinary extract.

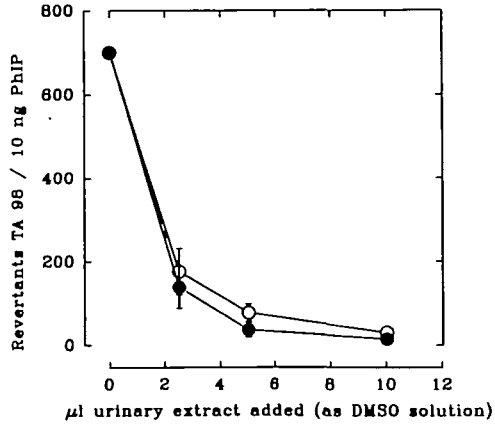

Fig. 1. Inhibition of PhIP mutagenicity in *S.typhimurium* TA98 in the presence of rat liver S9 and increasing amounts of urine extract. After XAD-2 chromatography, extracts were tested as DMSO solution: (○) means ± SD for three non-smokers, (●) means ± SD for five smokers. Mutagenicity is expressed as number of revertants per 10 ng PhIP per assay.

Table III. Inhibitory effect of urinary extracts on the mutagenicity of PhIP in *S.typhimurium* TA98

Subject no.	Cigarettes per day[a]	Urine volume (ml/24 h)	Urinary nicotine[b] plus cotinine (μmol/24 h)	Urinary mutagenicity[c] (rev/2.2 ml)	Inhibition of PhIP mutagenicity[d] (%)
Non-smokers					
1	0	980	4.2	0	92
2	0	1000	0.0	0	86
3	0	800	0.5	0	89
4	0	1150	0.0	0	92
5	0	500	0.0	0	94
6	0	900	0.0	0	87
7	0	750	0.0	0	80
8	0	1150	0.0	0	91
9	0	1200	0.0	0	86
10	0	800	0.0	0	79
					Mean ± SD: 87.6 ± 5.1
Smokers					
11	25	800	71.9	75	94
12	20	1500	6.6	65	94
13	25	850	52.5	55	94
14	20	500	62.5	105	99
15	10	820	18.4	55	93
16	10	1400	36.2	190	88
17	15	900	20.2	40	91
18	60	850	65.3	120	81
19	40	700	25.8	140	94
20	20	1400	163.6	190	91
					Mean ± SD: 91.9 ± 4.8

[a]Based on questionnaire.
[b]Determined by gas chromatography as described by Malaveille *et al.* (2).
[c]Measured as 5 μl DMSO solution of urinary extract (equivalent to 2.2 ml urine); <30 revertants per plate above the background level indicates lack of mutagenicity; the number of spontaneous revertants per plate ranged from 37 to 48.
[d]Measured for each urinary extract (equivalent to 2.2 ml urine) as described in Materials and methods; results are from two series of duplicate experiments.

incubation times (up to 60 min), the extracts increased their mutagenicity up to 400% (data not shown). We also investigated the modifying effect of urine extracts on the mutagenicity of two direct-acting mutagens, 4-NQO and 2-NF (the latter is structurally related to 2-AAF). Weak inhibition of 2-NF mutagenicity was observed only for incubation times of ≤20 min, while for longer times the mutagenicity was unchanged. 4-NQO mutagenicity was not affected by urine extracts at concentrations shown to be inhibitory for heterocyclic and aromatic amines and 2-NF.

Our findings are somewhat at variance with the reported enhancement of the mutagenic activity in *Salmonella* TA98 of aromatic amines (2-AA and 2-AF) by urine concentrates from smokers (16) and non-smokers (17,18). In previous experiments, urinary extracts were prepared by a method similar to ours, but plate incorporation instead of liquid incubation assays were employed. We have therefore re-investigated the modifying effect of our urine extracts on the mutagenicity of PhIP, 2-AA and B[*a*]P using a plate incorporation assay. The results presented in Table II indicate that a concentration of urine extracts (i.e. extract equivalent to 22 ml of urine, corresponding to 8.1 ml urine per ml soft agar) shown to inhibit PhIP mutagenicity strongly in the liquid incubation assay, reduced the mutagenicity of PhIP by only 28%. The mutagenicity of B[*a*]P was unchanged and that of 2-AA increased by 400% (Table II), in accordance with published data (16,17). A further increase in concentration of urine extracts enhanced the modifying effects on PhIP and 2-AA, and at variance with previous results (17), caused a 17% inhibition of B[*a*]P mutagenicity. The discrepancy in the observed modifying action of urine extracts on amine mutagenicity is reminiscent of the effects of norharman (known to act as a co-

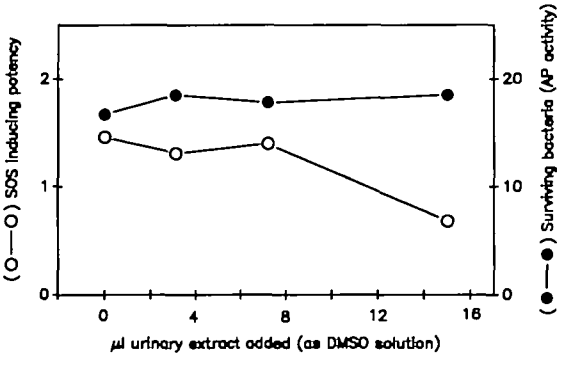

Fig. 2. Inhibition of PhIP SOS-inducing potency (○) in *E.coli* PQ37 and survival (●) in the presence of rat liver S9 and increasing amounts of urine extract after XAD-2 chromatography, which was prepared from one non-smoker's urine and tested as DMSO solution. Means, calculated from two series of experiments each involving two to seven determinations (PhIP at 200 and 400 ng per assay) are plotted.

mutagen), which either inhibited or potentiated the mutagenicity of two amino-azobenzene derivatives when liquid and plate incorporation assays were used respectively (19). Indeed, our experimental conditions used for mutagenicity assays ensure results that accurately reflect the rates of metabolic activation reactions of a compound (6), and thus reveal the presence of substances in human urine that inhibit the mutagenicity of PhIP and related heterocyclic and aromatic amines. Our observations that (i) the mutagenicity of 2-NF, a direct-acting substance, was weakly inhibited by urine extracts at incubation times of ≤20 min, and (ii) the mutagenicity of 2-AAF and 2-AA were

either decreased or enhanced by urinary extracts, depending on incubation time (see above) suggest that the inhibiting substances act through their capacity to bind non-covalently the mutagen and metabolites. The strength of such binding determined by structural features of the amine, could prevent the test substance from diffusing from the aqueous medium into bacterial cells and into microsomal membranes where it is oxidized by cytochrome P450. In the case of 2-AA and 2-AAF, where the urine extract caused an increase in mutagenicity, partial trapping of not only the parent compound (that would account for the observed decrease) but also of metabolites inhibiting microsomal metabolism may occur, thus permitting *N*-hydroxylation of the parent compound (mutagen activation) to proceed at the initial rate.

As described above, the SOS chromotest was used to complement the *Salmonella* assay in order to measure the cytotoxicity of urine extracts. These experiments were performed with a metabolic activation system similar to that used for the *Salmonella* assay. The result not only permitted dissociation of the cytotoxicity from the anti-mutagenicity of urine extracts, but also indicated that at a strong inhibitory concentration in the *Salmonella* assay (7.2 μl DMSO solution of urine extract per assay corresponding to 2.2%, v/v), the extracts did not affect the genotoxicity of PhIP in *E.coli*. A concentration twice as high was found to be necessary to obtain ~50% inhibition of SOS inducing potency (Figure 2). In keeping with the proposed mode of action of the urinary anti-mutagens, the much lower inhibitory effect in this system is probably due to the higher ratio of PhIP:anti-mutagen concentration in the SOS chromotest than in the *Salmonella* assay: PhIP concentrations, required to get measurable SOS DNA-repair induction, being 13- to 27-fold higher than those that mutate *Salmonella*.

As extracts were prepared from urine of subjects who were coffee drinkers, they could contain caffeine and paraxanthine. These are substrates for cytochrome P450IA2, as they are aromatic and heterocyclic amines (5,20,21) and caffeine was found to inhibit the mutagenicity in *Salmonella* of various heterocyclic amines (22). Analysis of eight urine extracts by HPLC (see Materials and methods) showed concentrations of caffeine and paraxanthine at 3.9 ± 1.7 and 9.6 ± 3.6 μmol/ml DMSO solution of extract respectively. These concentrations (corresponding to 45.2 μM caffeine and 109 μM paraxanthine in assay medium with 2.5 μl urine extract) were far too low to account for the inhibitory effect of the urine extracts on PhIP mutagenicity. Under our assay conditions, at least 400 μM caffeine and paraxanthine is required to get 60 and 10% inhibition respectively.

Work is in progress to characterize and trace the origin of these anti-mutagenic substances that are present in the urine of smokers and non-smokers. Studies are being undertaken in smoking volunteers to see whether the anti-mutagens may reduce the levels of DNA adducts in the urinary bladder of smokers arising from exposure to aromatic and heterocyclic amines (23,24).

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