Urinary Porphyrins in Patients with Endemic Chronic Arsenic Poisoning Caused by Burning Coal in China

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Abstract

Objective: To evaluate the effect of arsenic (As) on the porphyrin biosynthetic pathway, urine samples from patients with endemic chronic arsenic poisoning were examined.

Subjects and Methods: The subjects were 16 patients, who had been exposed to As from burning coal for 8 to 25 years, and 16 controls living in the same region in Guizhou Province in southwest China. Concentrations of urinary As, porphyrins and ALA were determined by induced coupled plasma mass spectrometry (ICP-MS), high performance liquid chromatography (HPLC) with a reversed-phase column and fluorescence detector, and colorimetric spectrophotometry, respectively.

Results: Concentrations of As in patients and controls, 184.40 ± 200.04 and $86.82 \pm 64.20 \mu\text{g/g}$ creatinine (mean $\pm$ SD) respectively, were significantly different ($p < 0.05$). The concentrations of various kinds of urinary porphyrins, including isomers I and III of coproporphyrin and pentacarboxylporphyrin, were determined. Positive correlations were observed between As and porphyrins (e.g. total porphyrins, hexacarboxylporphyrin and coproporphyrin III) or between As and ALA in male and female patients. However, porphyrin and ALA concentrations were not significantly different between the patients and the controls. Urinary porphyrin concentrations in females were higher than those in males.

Conclusion: Exposure to As from burning coal may influence porphyrin biosynthesis.

Key words: Endemic arsenic poisoning, porphyrin, urine, coal, China

Introduction

Porphyrins and δ -aminolevulinic acid (ALA) are intermediates in the heme biosynthetic pathway, which is highly susceptible to environmental factors; disorders in this pathway may cause changes in urinary porphyrins and ALA. The effects of arsenic (As) on the heme biosynthetic pathway and urinary excretion of porphyrins have been reported in humans¹⁻⁵ and animals.⁶⁻¹¹⁾

In Guizhou Province in southwest China, about 200,000 local residents are exposed to As from burning coal through air

and food, and at least 3,000 of them suffer from endemic chronic As poisoning, which is characterized by dermal hyperkeratoses.¹²⁻²⁰⁾ Some local residents in the endemic areas burn coal containing high As (about 600 ppm) in indoor stoves without chimneys to cook, dry food and warm themselves in winter; thus the indoor air and food are contaminated by As. The absorption route of As in the patients in Guizhou is different from other places; they are affected by air and food, and those in other places in the world, for example, some regions in north China,²¹⁻²⁵⁾ Taiwan²⁶⁻²⁷⁾ and Mexico,^{1, 2)} are affected by drinking water. The clinical manifestations of this disease in Guizhou are also different from those of the water-caused ones in north China²¹⁻²⁵⁾ or Taiwan.²⁶⁻²⁷⁾ It is conceivable that the biological effects, including the effect on the heme biosynthetic pathway, of As from coal in Guizhou are different from the water borne As in other regions. Though there are some reports on the effect of As on human heme metabolism,¹⁻³⁾ there is no such report concerning As from burning coal.

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Materials and Methods

1. Subjects

The subjects were 16 patients with endemic chronic As poisoning (7 males and 9 females) living in a village, a severe endemic area, in the southwest of Guizhou Province in China. As controls, 16 unexposed healthy subjects (8 males and 8 females) living in the same county were used (Table 1). The patients had been exposed to As for 8 to 25 years and had typical clinical manifestations of dermal injuries due to endemic As poisoning, which were diagnosed using the Diagnostic Criteria of Endemic Chronic Arsenic Poisoning by LF Wang,²⁸⁾ according to which As poisoning was diagnosed mainly on the basis of dermal hyperkeratoses on the palms and soles of the feet. All subjects gave their informed consent to be studied.

Urine samples were collected in dark rooms in the early morning on one day in late March, 2000. One drop of 0.1% HCl was added to about 25 ml of the urine soon after collection. Urine samples were stored in the dark at -20 ~ -30 °C until the analysis was performed.

2. Determination of As concentration

After digestion of 1 ml urine with 0.4 ml concentrated HNO₃ and 0.2 ml H₂O₂ in a microwave oven (MLS-1200 MEGA, Milestone, Italy), the solution was filled up to 10 ml with mil-Q water. The As concentration was determined by an induced coupled plasma mass spectrometer (ICP-MS, Yokogawa Co. Japan).

3. Porphyrin analyses

Urinary porphyrin carboxylic acid isomers were determined by high-performance liquid chromatography (HPLC) with a fluorescence detector according to the method described by Kondo and Kajiwara²⁹⁾ with some modifications. Each urine sample was diluted with an equal volume of concentrated acetic acid containing 0.08% iodine, and all porphyrinogens were converted to porphyrins by oxidation with iodine. Then, 20 µl of the mixture was directly injected onto the C18 reverse-phase column of an HPLC Autoanalyser (Table 2). A standard porphyrin mixture containing only type I isomers (Porphyrin Products, Logan, UT, USA) was used.

4. Determination of ALA concentration

Urinary ALA was determined by the method of Urata and Granick³⁰⁾ with some modifications: 1 ml of urine was applied to a Dowex 50W-X8 column (10 mm × 10 mm, 200-400 mesh, H⁺ form). The column was washed with distilled water and ALA was subsequently eluted with 4 ml of 2 mol/l pyridine, pH 6.4. During this elution, aminoketone in the urine, which is a monoacid-noncarboxylicketone, was retained on the column and was not eluted. After 0.2 ml of ethyl acetoacetate was added to 4 ml of the eluates, the mixture was boiled for 10 minutes to convert ALA to pyrrole. The cooled solution was diluted to 20 ml with distilled water and then applied to a Dowex 1-X8 column (8 mm × 10 mm, 200-400 mesh, CH₃COO⁻ form). The resin was washed with 10 ml of water. Finally, the pyrrole was eluted with 1.5 ml of 12 mol/l acetic acid. Pyrrole formed from ALA in the eluate developed a red color with modified Ehrlich reagent (containing 8 g of *p*-dimethylaminobenzaldehyde, 100 ml of acetic acid, 80 ml of 70% perchloric acid and 20 ml of 0.2 mol/l HgCl₂). After 10 minutes, the absorbance was measured at 553

nm, and the ALA concentration was calculated assuming that the molar absorption coefficient of the ALA pyrrole-Ehrlich complex was 64000 at 553 nm.

Urinary creatinine (Cr.) was determined with a commercially available kit (Eiken Kagaku, Tokyo, Japan), and then the above concentrations of As, porphyrin and ALA were adjusted by Cr. concentration.

5. Statistical analysis

Analyses of significant differences between the groups were performed with the Student's *t* test. The level of significance was taken as *P* < 0.05.

Table 1 Basic information on the 32 subjects for urinary porphyrin analysis

Endemic chronic arsenic poisoning patients					Controls		
No.	Sex	Age (year)	Exposure duration ^c (year)	Dermal Injuries ^d	No.	Sex	Age (year)
D-1	M ^a	30	25	Slight	C-1	M	27
D-2	M	31	25	Moderate	C-2	M	30
D-3	M	32	25	Severe	C-3	M	36
D-4	M	37	25	Moderate	C-4	M	36
D-5	M	38	25	Slight	C-5	M	38
D-6	M	44	20	Slight	C-6	M	44
D-7	M	49	25	Severe	C-7	M	58
D-8	F ^b	28	8	Slight	C-8	M	58
D-9	F	28	8	Slight	C-9	F	25
D-10	F	32	12	Very Slight	C-10	F	30
D-11	F	32	25	Slight	C-11	F	32
D-12	F	35	18	Slight	C-12	F	35
D-13	F	36	19	Slight	C-13	F	39
D-14	F	40	25	Moderate	C-14	F	43
D-15	F	48	25	Slight	C-15	F	57
D-16	F	54	25	Slight	C-16	F	54
Mean		37.1	20.9				40.1
SD		7.9	6.3				11.2

a. M: male

b. F: female

c. Exposure duration: Duration of exposure to arsenic from burning coal

d. Dermal Injuries (as main clinical manifestations, diagnosed according to the Diagnostic Criteria of Endemic Chronic Arsenic Poisoning by LF Wang):²⁸⁾

Slight: only mild keratoses were observed

Moderate: obvious papular keratoses were observed

Severe: dense and larger keratoses were observed

Table 2 HPLC conditions for urinary porphyrin analysis

HPLC system: Jasco. Gulliver Series	
System Controller (802-sc)	
Autosampler (AS-950)	
HPLC Pump (PU-980)	
Degasser (DG-980-50)	
Gradient Unit (LG-980-02)	
Column Oven (CO-965)	
Detector Spectrofluorometer (821-FP)	
Integrator (SIC-21J)	
Analytical conditions:	
Column: Finepak C18-5 (4.6 mm I.D. × 150 mm, Jasco)	
Column Filter: pore size 0.45 µm (Irika Co.)	
Wavelength: Ex 404 nm, Em 620 nm	
Solvents: A acetic acid : acetonitrile : 50 mM ammonium acetate (7 : 80 : 13)	
B acetic acid : acetonitrile : 50 mM ammonium acetate (4 : 10 : 86)	
Gradient: A/B (30/70) → (90/10) 14 min	
linear gradient, A/B (100/0) 14 min hold	
Flow rate: 1.0 ml/min	
Column temperature: 40 °C	

Results

1. As concentration

The As concentrations (mean $\pm$ SD) of the patients and the controls were 184.40 ± 200.04 and $86.82 \pm 64.20 \mu\text{g/g Cr.}$, respectively. The mean value of the patients was significantly higher than that of the controls ($P < 0.05$).

2. Porphyrin concentrations

The chromatogram of the standard porphyrins and typical profiles of the urine samples of male and female controls and patients are shown in Fig. 1. The abbreviated porphyrin names are explained in the Table 3 legend. 8-(URO), 7-, 6-, 5- and 4-(COPRO) carboxylic porphyrins, along with the isomers of COPRO I and III, were clearly separated within 16 minutes. Isomers of PENTA I and III were also separated. However, isomers of URO, HEPTA and HEXA could not be separated, so the peaks of these porphyrins in the chromatogram contained both isomers. In some urine samples, unknown peaks appeared, such as the one with a retention time of 14.4 minutes in Fig. 1. The excellent reproducibility of this method was shown by the small coefficients of variation (CV) for the retention time of porphyrin peaks (0.10 ~ 0.47%). Clear peaks were calculated as porphyrin concentrations ($\mu\text{g/g Cr.}$). There was no significant difference between the patients and the controls in porphyrin concentrations, the COPRO III/COPRO I ratio, or the total COPRO/URO ratio (Table 3).

Results of correlation analysis showed that there was no significant correlation between urinary porphyrins and As in patients or controls when sexual difference was not considered (data are not shown). As described below, the results were different when the sexual difference was considered.

3. ALA concentration

ALA concentrations are shown on the bottom line in Table

3. When sexual difference was not considered, no significant difference was found between the patients and controls. Also, there was no significant correlation between ALA and As concentrations.

4. As, porphyrins, and ALA concentrations in both sexes

After separation into male and female groups, total porphyrins, COPRO III, COPRO/URO ratio and ALA were

Table 3 Urinary porphyrins and ALA of arsenic poisoning patients and controls

	(μ g/g creatinine)			
	Patients (n=16)		Controls (n=16)	
URO (I +Ⅲ)	23.23 ±	11.43	24.82 ±	13.13
HEPTA (I +Ⅲ)	8.01 ±	5.57	7.94 ±	6.34
HEXA (I +Ⅲ)	0.90 ±	1.95	1.61 ±	4.66
U-peak 1 (Rt=9.81)	2.19 ±	2.19	1.67 ±	2.18
U-peak 2 (Rt=11.42)	0.43 ±	0.64	0.60 ±	0.86
PENTA I	0.81 ±	0.64	0.87 ±	1.15
PENTA Ⅲ	0.11 ±	0.46	0.34 ±	0.45
U-peak 3 (Rt=14.37)	2.36 ±	1.98	3.13 ±	1.74
COPRO I	16.25 ±	7.40	19.52 ±	7.26
COPRO Ⅲ	24.68 ±	22.30	20.50 ±	14.74
U-peak 4 (Rt=15.77)	0.20 ±	0.35	0.14 ±	0.31
Total porphyrins	79.18 ±	39.03	81.16 ±	36.14
COPRO Ⅲ/COPRO I ratio	1.68 ±	1.58	1.07 ±	0.70
COPRO /URO ratio*	2.01 ±	1.46	1.83 ±	1.11
ALA	1221.5 ±	266.7	1273.8 ±	303.0

Values represent mean $\pm$ SD

ALA: δ -aminolevulinic acid

URO: uroporphyrin

HEPTA: heptacarboxylporphyrin

HEXA: hexacarboxylporphyrin

PENTA: pentacarboxylporphyrin

COPRO: coproporphyrin

Rt: retention time (min)

U-peak: unknown peak

*: COPRO /URO ratio: COPRO (I + III) /URO (I + III) ratio

"Porphyrin" amounts of unknown peaks were calculated according to the formula used for the nearest porphyrin peak.

Fig. 1 Chromatograms of standard porphyrins (A), urine from a male control (B), urine from a female control (C), urine from a male patient (D), and urine from a female patient (E).

All abbreviations are the same as described in Table 3. Urine samples were oxidized with iodine prior to each assay.

positively correlated with As ($P \leq 0.05$) in the male patients, and some other porphyrins were also positively correlated with As ($P < 0.05$) in the female patients, and even in the controls of both sexes (Table 4). Nevertheless, there were no significant differences in concentrations of As, porphyrins and ALA between the patients and controls in males or females.

As shown in Fig. 2, the urinary concentrations of the principal porphyrins (URO I, COPRO I and COPRO III) and total porphyrins of the female patients and controls were generally higher than those of the corresponding males, and these sexual differences were statistically significant in some cases ($P < 0.05$).

5. Relationship between dermal injuries and urinary porphyrins or ALA

No significant correlation was found between the severity of As-caused dermal injuries and urinary porphyrins or ALA in the patients (data not shown).

Discussion

The village where the 16 patients lived was a typical severe endemic area of coal-caused chronic As poisoning, as described in the introduction of this paper. In this region, though many countermeasures have been taken to prevent this disease and domestic arsenical contamination has been generally alleviated, some local residents were still exposed to excessive As from coal.¹⁸⁻²⁰ In a village in this region, 32 of 219 villagers were diagnosed with chronic As poisoning during the period from 1991 to 1997.¹⁸

1. As in urine

Urinary As is often used as an indicator of human exposure to As. The average urinary As concentration of the patients was significantly higher than that of the controls, indicating that these patients were still exposed to As when the urine samples were collected. The average As concentration of the 16 controls (86.82

$\mu\text{g/g Cr.}$ or 0.064 ppm without adjusting with Cr.) was similar to the control value (0.07 ppm) in this area measured in 1991 by Zhou *et al.*¹⁵ The average As concentration of the 16 patients (184.40 $\mu\text{g/g Cr.}$ or 0.164 ppm) was lower than the average value (0.40 ppm) of the patients in the same area measured in 1991,¹⁵ suggesting that the level of exposure to As had reduced as compared with 9 years before. This value (184.40 $\mu\text{g/g Cr.}$) was similar to the previously reported value (257.6 and 129.0 $\mu\text{g/g Cr.}$ for the high exposure group and low exposure group, respectively) of some French smelter workers exposed to As,⁵ suggesting that the Chinese patients were exposed to As to a comparable extent as the French workers.

2. Porphyrins, ALA and their correlation with urinary As in urine

7-, 6-, 5- and 4-(COPRO) carboxylic porphyrins are successively formed as intermediates in the conversion from 8-carboxylic (URO) to 2-carboxylic (PROTO) porphyrin inside the human body, as porphyrin compounds are decarboxylated step by step. In this study, unknown peaks were detected as the precursors of these porphyrin intermediates, and the numbers of carboxylic acids were estimated by the retention time. In this HPLC separation system, porphyrin compounds with not more than 5 carboxylic acids (PENTA and COPRO) could be separated into isomers I and III, but those with more than 5 carboxylic acids (HEPTA, HEXA and URO) could not be separated.

After analyzing porphyrins and ALA in urine, there were two remarkable findings: (1) porphyrin concentrations in urine were higher in females than in males; (2) there were positive correlations between porphyrins and As concentrations, or between ALA and As concentrations, in the subjects, and the correlations were more obvious in the male patient group than in the other groups. The latter result suggests that xenobiotic As may influence the porphyrin pathway, and that males are more susceptible to this exposure.

This result seems in agreement with some other papers, which reported increases in urinary porphyrin^{2-11, 31} and ALA^{4, 32, 33} excretion by humans or animals exposed to As. However, in the present study, it is difficult to reach the conclusion that exposure to As can increase the urinary excretion of porphyrins and ALA, because the urinary porphyrin and ALA levels in the patients were not significantly higher than those in the controls (Table 3). It is conceivable that the As-caused increase of porphyrins may occur only when the body burden of As is over a certain threshold, which has not been clarified yet. In a study on a human population chronically exposed to As via drinking water in Mexico,²¹ urinary porphyrins increased only when urinary As exceeded 1000 $\mu\text{g/g Cr.}$, which was 5.4 times higher than in our

Table 4 Correlation analyses between urinary arsenic and urinary porphyrins and between urinary arsenic and urinary ALA in arsenic poisoning patients and controls

	Coefficient of correlation with urinary arsenic (r)			
	Male		Female	
	Patients (n=7)	Controls (n=8)	Patients (n=9)	Controls (n=8)
URO (I+III)	-0.4618	0.9875*	0.1329	0.3099
HEPTA (I+III)	-0.4863	0.8866*	0.1230	0.0975
HEXA (I+III)	-0.0533	ND	0.7725*	-0.1614
U-peak 1 (Rt=9.81)	-0.2841	-0.1219	-0.0121	0.7273*
U-peak 2 (Rt=11.42)	-0.4023	-0.2217	-0.0405	-0.2748
PENTA I	-0.1436	-0.5011	0.7253*	-0.1784
PENTA III	ND	0.7849*	-0.2045	-0.2551
U-peak 3 (Rt=14.37)	0.8617*	-0.4538	0.0083	-0.2435
COPRO I	0.4698	-0.0820	0.1061	0.1265
COPRO III	0.8290*	-0.3444	-0.2721	-0.2590
U-peak 4 (Rt=15.77)	-0.2248	0.0266	0.2297	0.8868*
Total porphyrins	0.9893*	-0.4734	-0.4304	-0.3555
COPRO III/COPRO I ratio	0.6894	-0.4500	-0.3188	-0.3109
COPRO/URO ratio	0.8778*	-0.4436	-0.3913	-0.5181
ALA	0.7504 ^Δ	0.0451	0.0208	0.7585*

*. $P < 0.05$, Δ : $P = 0.05$

ND: under detection limit

(All other abbreviations were the same as described in Table 3)

Fig. 2 Urinary porphyrins in male and female subjects

patients. The reason why urinary porphyrin and As levels in our patients were not higher than the controls is thought to be that the body burdens of As in these patients were below this threshold and hence the influence of arsenical exposure on porphyrin biosynthesis was not strong enough to cause the urinary porphyrin and ALA concentrations to increase significantly. It is conjectured that, when the body burden of As is not very heavy, the effect of As on porphyrin biosynthesis may appear as a correlation between As and some kinds of porphyrins, rather than an obvious increase in the concentrations of porphyrins or ALA in urine.

The results of the present study accords, in some respects, with the results of some previous reports.¹⁻⁵⁾ In a report by Garcia-Vargas *et al.*,¹⁾ no significant increase in urinary porphyrin excretion was found in 21 subjects chronically exposed to As via drinking water. In a report by Telolahy *et al.*,⁵⁾ the mean concentration of urinary URO of 84 As-exposed smelter workers was very similar to non-exposed controls.

The results of the present study that urinary COPRO/URO ratio was positively correlated with urinary As in male patients seemed to be in agreement with the results of a previous report,³⁾ which studied Mexican people exposed to As via drinking water. The above-mentioned sexual differences in urinary porphyrin levels and in the susceptibility of porphyrins to xenobiotic As should be taken into consideration in research on human porphyrins, particularly in studies concerning the effect of As.

3. Relationship between dermal injuries and urinary porphyrins or ALA
Why no positive correlation was found between dermal

injuries and urinary porphyrins or ALA in our patients is thought to be as follows: the dermal injuries were caused by long term exposure to As rather than recent exposure, and therefore may have had no correlation with urinary porphyrins or ALA, which may have changed due to recent exposure to As rather than long term exposure.

Conclusion

In the present study, a relationship between the exposure to As and increase in urinary excretion of porphyrins (total porphyrins, HEXA, PENTA I and COPRO III) and ALA appeared in patients, particularly male patients. This finding indicates that exposure to As influences porphyrin biosynthesis. In addition, the urinary porphyrin concentrations in females are generally higher than those of males.

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