Arch Toxicol (1999) 73: 90–95

INORGANIC COMPOUNDS

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Altered activity of heme biosynthesis pathway enzymes in individuals chronically exposed to arsenic in Mexico

Received: 21 December 1998 / Accepted: 14 January 1999

Abstract Our objective was to evaluate the activities of some enzymes of the heme biosynthesis pathway and their relationship with the profile of urinary porphyrin excretion in individuals exposed chronically to arsenic (As) via drinking water in Region Lagunera, Mexico. We selected 17 individuals from each village studied: Benito Juarez, which has current exposure to 0.3 mg As/l; Santa Ana, where individuals have been exposed for more than 35 years to 0.4 mg As/l, but due to changes in the water supply (in 1992) exposure was reduced to its current level (0.1 mg As/l), and Nazareno, with 0.014 mg As/l. Average arsenic concentrations in urine were 2058, 398, and 88 μg As/g creatinine, respectively. The more evident alterations in heme metabolism observed in the highly exposed individuals were: (1) small but significant increases in porphobilinogen deaminase (PBG-D) and uroporphyrinogen decarboxylase (URO-D) activities in peripheral blood erythrocytes; (2) increases in the urinary excretion of total porphyrins, mainly due to coproporphyrin III (COPROIII) and uroporphyrin III (UROIII); and (3) increases in the COPRO/URO and COPROIII/COPROI ratios. No significant changes were observed in uroporphyrinogen III synthetase (UROIII-S) activity. The direct relationships between enzyme activities and urinary porphyrins, suggest that the increased porphyrin excretion was related to PBG-D, whereas the increased URO-D activity would enhance coproporphyrin synthesis and excretion at the expense of uroporphyrin. None of the human studies available have reported the marked porphyric response and enzyme inhibition observed in rodents. In conclusion, chronic As exposure alters human heme metabolism; however the severity of the effects appears to depend on characteristics of exposure not yet fully characterized.

Key words Arsenic · Chronic exposure · Porphyrins · Porphobilinogen deaminase · Uroporphyrinogen decarboxylase

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Introduction

Arsenic (As) is a ubiquitous element present in low concentrations in air, soil and water. Arsenic compounds may represent a concern to environmental and occupational health becoming concentrated in the environment as a result of natural or anthropogenic sources (ATSDR 1993). The heme metabolism pathway is known to be highly susceptible to alterations induced by drugs and environmental chemicals, offering the opportunity to use these changes as indicators of damage caused by arsenic. Heme is mainly synthesized in bone marrow (85%) where it is required for hemoglobin formation, the remaining 15% is synthesized in liver and other organs where it is required for hemoprotein synthesis (Marks 1985). Animal studies have shown that subchronic exposure to 20–85 mg As/l in drinking water produced dose-related increases in uroporphyrin and coproporphyrin urinary excretion (Woods and Fowler 1978).

Changes in the activity of several enzymes of heme biosynthesis have also been reported in rodents after arsenic treatment. For example, decreases in hepatic ferrochelatase (Woods and Fowler 1978) and renal COPRO-OX (Woods and Southern 1989), and increases in hepatic 5-aminolevulinate synthetase activity (Cebrián et al. 1988). We have also reported a relationship between time-dependent porphyric responses and alterations in the hepatic and renal activities of porphobilinogen deaminase (PBG-D), uroporphyrinogen III synthetase (UROIII-S), uroporphyrinogen decarboxylase (URO-D) and COPRO-OX in rodents subchronically treated with arsenic via drinking water. The alterations in the profile of urinary porphyrins were

associated with decreases in URO-D and COPRO-OX activities (García-Vargas et al. 1995). However, no information on heme enzyme activities in erythrocytes was collected from those studies.

In contrast, the main alterations in the urinary porphyrin profile of individuals exposed to arsenic in drinking water (0.4 mg As/l), compared to individuals exposed to 0.02 mg As/l were: (a) significant reductions in coproporphyrin III (COPROIII) excretion, resulting in decreases in the COPROIII/COPROI ratio, and (b) small but significant increases in uroporphyrin excretion; both alterations were responsible for the decreased COPRO/URO ratio (García-Vargas et al. 1994). These alterations were interpreted as one step in the long process to develop chronic hepatic porphyria (CHP) described by Doss (1979). However, little information is available on the alterations of heme enzyme activities in chronically As-exposed humans. The objective of this work was to evaluate the activities of some enzymes of the heme biosynthesis pathway and their relationship with the profile of urinary porphyrin excretion in individuals exposed chronically to arsenic in drinking water in Region Lagunera, Mexico.

Materials and methods

Chemicals

Acetonitrile, methanol high-performance liquid chromatography (HPLC) grade, and dimethyl sulphoxide (analytical grade), were from Mallinkrodt (Paris, Ky, USA). Triton X-100 and sodium borohydride (suprapure grade) were from Merck (Darmstadt, Germany). Dithiothreitol (DTT), creatinine, sodium arsenate and dimethylarsenic acid (DMA) were from Sigma Chemical Co., (St. Louis, Mo, USA). Monomethylarsonic acid (MMA) was obtained from Vineland Co., (Vineland, NJ, USA). Uroporphyrin III, coproporphyrin III and a mixture of isomers I of 8-, 7-, 6-, 5- and 4-carboxylic porphyrins and mesoporphyrin were purchased from Porphyrin Products, (Logan, Utah, USA). Standard reference material for toxic metals (SRM 2670) and fluoride (SRM 2671a) in freeze-dried urine and water (SRM 1643c) were obtained from US National Institute of Standards and Technology (Gaithersburg, Md, USA). The remaining chemicals were analytical grade.

Group selection

The individuals for this study were selected from three villages with different exposure histories to As in drinking water. We interviewed ~25 individuals in each village but some refused to donate blood and were excluded from the study. Seventeen individuals (nine men and eight women) were selected from Santa Ana, Coahuila, where having been exposed for >35 years to 0.4 mg As/l, but due to changes in the drinking water supply (in 1992), the arsenic content was reduced to 0.05–0.13 mg As/l. Most of these individuals (16/17) participated in earlier studies (García-Vargas et al. 1994). Other individuals (ten men and seven women) were selected from Benito Juarez, Coahuila, with levels of 0.3 mg As/l in drinking water. The reference group consisted of seventeen individuals (five men and twelve women) from Nazareno, Durango, which had 0.014 mg As/l in drinking water. Most of these individuals (14/17) participated in earlier studies (García-Vargas et al. 1994). The uneven sex distribution was accidental.

All subjects were interviewed and blood and urine samples collected. The questionnaire included information about sociodemographic and occupation factors, medical conditions, alcohol and tobacco use. Individuals who were exposed to pesticides, hormonal contraceptives, and porphyrinogenic drugs in the 3 months before the study began were not included in the study. Those individuals exposed to alcohol in the last 5 days or suffering chronic alcoholism and clinical porphyria were not included. All individuals signed an informed consent form. The study was performed during 1995.

Blood samples were collected by venous puncture in heparinized tubes and centrifuged at 2000 *g* for 15 min. Plasma and leukocytes were discarded and erythrocytes were kept frozen at −20 °C until used. First void urine samples were collected and stored in plastic bottles at 4 °C, for no more than 12 h, then stored at −20 °C in stoppered polyethylene bottles and protected from light until the analysis of porphyrins was performed.

Porphyrin analysis

Urinary porphyrins were analysed by HPLC, according to García-Vargas et al. (1994). The HPLC system consisted of one pump series 200 (Perkin Elmer, Norwalk, Conn., USA). Detection was performed simultaneously with a Perkin Elmer diode array detector with an interference filter set at 365 nm and a Perkin Elmer LS-30 fluorescence detector set at excitation and emission wavelengths of 405 and 600 nm, respectively. Sample injection was through a Rheodyne 7125 injector (Cotati, Calif., USA) fitted with a 500 µl loop. The separation was performed on a 25 cm × 5 mm i.d ODS-Spherisorb column, 5 µm spherical silica chemically bonded with octadecyl sylyl groups (LDC Analytical, Norwalk, Conn., USA). The solvents for gradient elution were 10% (v/v) acetonitrile in 1 mol/l ammonium acetate, pH 5.16 (solvent A) and 10% (v/v) acetonitrile in methanol (solvent B). The column was equilibrated with 3% B-97% A (v/v) during 10 min before the sample was injected. Porphyrins were separated with a 5 min isocratic elution at 3% B-97% A, followed by a 30 min linear gradient from 3% B to 90% B, and by an isocratic elution at 90% B-10% A for further 10 min. The flow rate was 1 ml/min. Porphyrin recoveries were (mean ± SD of five samples): 94% ± 3 and coproporphyrin, 111% ± 1.9.

Enzyme assays

The activities of PBG-D and UROIII-S were assayed simultaneously according to Wright and Lim (1983). Uroporphyrins were eluted with 15% acetonitrile in 1 mol/l ammonium acetate buffer, pH 5.16, at a flow rate of 1 ml/min. The enzyme activities were expressed as nmoles of uroporphyrin/ml erythrocytes per hour. The activity of URO-D was assayed according to Lim et al. (1986). Coproporphyrins were eluted with 30% acetonitrile in 1 mol/l ammonium acetate buffer, pH 5.16, at a flow rate of 1 ml/min. The enzyme activity was expressed as nmoles of coproporphyrin I formed/ml of erythrocytes per hour. All assays were done in duplicate. Three blanks were analysed for each group of assays: the first contained the substrate without enzyme, the second contained the enzyme but no substrate, and the third contained both substrate and enzyme, but the reaction was terminated at zero time.

Arsenic analysis

Total arsenic (TAs) was determined by hydride generation atomic absorption spectrophotometry, using a Perkin Elmer 3100 equipped with a FIAS-200 flow injection atomic spectroscopy system. All measurements were made using an arsenic electrodeless discharge lamp and background corrector. Total arsenic in urine was determined in samples previously wet digested with nitric, sulphuric and perchloric acids (Cox 1980). Samples were pretreated with 0.5 ml of 10% (w/v) potassium iodide and 2.5% (w/v) ascorbic acid at room temperature 0.5 h before measurement. Separation of As species (Asi, MMA and DMA) was performed according to Crecelius et al. (1986). In the procedure, arsenicals were selectively reduced to the gaseous compounds arsine, methylarsine and

dimethylarsine by controlled pH using sodium borohydride as a reducing agent. Arsines were then trapped in a liquid nitrogen-cooled chromatographic trap, which upon warming allowed a separation of arsenic species based on boiling points.

Quality control for TAs included the analysis of freeze-dried urine standard reference material for toxic metals (SRM 2670) concurrently with urine samples from individuals. We attained an accuracy of 99% and variation coefficient of 4.2% ($n = 6$). Because standard urine containing known amounts of As species was not available commercially, the reliability of separation procedures was assessed by spiking urine samples with known amounts of Asi, MMA and DMA (20:20:60), hence three different concentrations (0.045, 0.42 and 1.15 mg/l) of TAs could be analysed (Vahter and Lind 1986). Recoveries ranged from 91 to 116% with coefficients of variation between 1 and 13%. Since TAs in urine may be highly influenced by ingestion of seafood arsenic, we determined both TAs after wet digestion (Cox 1980) and the sum of As species for each urine sample, without significant differences between their values being apparent. Analysis of TAs in water was carried out as reported previously (Del Razo et al. 1990). For quality control purposes, the standard reference water (SRM 1643c, NIST) was analysed at the same time as the collected water samples. The results obtained had an accuracy of 97 and 5% coefficient of variation.

Fluoride analysis

Fluoride concentrations in urine and water were determined by the ion selective electrode method (USEPA 1979; Orion Research 1983). Quality control for fluoride included the analysis of freeze-dried urine standard reference material for fluoride (SRM 2671a) concurrently with urine samples from individuals. The accuracy obtained was 97% and variation coefficient of 5.3%.

Creatinine in urine

Creatinine was measured by a colorimetric automated method using a Vitalab Eclipse Photometer. The reagent kit and the photometer were purchased from Merck Mexico, S.A., Mexico.

Statistical procedures

Data analysis was carried out using the SAS statistical program package (SAS Institute 1994). The significance of differences between exposed and control towns regarding total arsenic concentration in drinking water and urine, heme synthesis pathway enzymes and urinary porphyrin concentrations were analysed using Mann-Whitney *U*-test or Kruskal-Wallis test. Linear regression was used to assess the relationships between porphyrin concentrations, enzyme activities and urine arsenic concentrations.

Results

The individuals selected were between 15 and 60 years old, with similar age distribution between Santa Ana and Nazareno; however, individuals from Benito Juarez were slightly younger than those from Nazareno. Arsenic concentrations in the drinking water and urine from individuals living in the villages under study are shown in Table 1. Arsenic in water samples from Benito Juarez exceeded sixfold the maximum allowable concentration (50 μg As/l, WHO, 1981). The levels in Santa Ana indicated that changes in the water source had reduced exposure, but also that the inhabitants were still drinking water contaminated with arsenic at concentrations twice the mentioned limit. The data of Table 1 also show that the highest urinary arsenic concentrations were found in samples from Benito Juarez, which were 5- and 20-fold higher than those from Santa Ana or Nazareno respectively. Arsenic concentrations in urine from control individuals (Nazareno) were below the biomonitoring action level (100 μg/l) proposed by ATSDR (1993).

Table 1 Total arsenic concentration in drinking water and urine. Region Lagunera, Mexico

Village	*n*	Water (μg/l) (mean ± SD)	*n*	Urine (μg/l) (mean ± SD)
Nazareno	10	14.0 ± 3.1	17	88 ± 27
Santa Ana	20	116 ± 37	17	398 ± 258*
Benito Juarez	7	239 ± 88	17	2058 ± 833*†

* $P < 0.01$ vs Nazareno, † $P < 0.01$ vs Santa Ana, Mann-Whitney *U*-test

The prevalence of cutaneous signs of chronic arsenicism (hyperpigmentation, palmoplantar keratosis and papular keratosis) was 41% in Benito Juarez and 29% in Santa Ana. No cutaneous signs were observed in the less exposed individuals (Nazareno). Since fluorosis is also endemic in this area, fluoride concentrations were measured in both drinking water and urine from the studied individuals. Median fluoride concentrations in water were 4.9, 2.0 and 1.87 mg/l, values above the Mexican maximum allowable concentration (1.5 mg F/l). Median fluoride concentrations in urine samples were 5.47, 2.87 and 3.60 mg/l in Benito Juarez, Santa Ana and Nazareno, respectively. The exposed individuals showed small but significant increases in PBG-D and URO-D activities, as compared to control individuals; however, no significant changes were observed in UROIII-S activity (Table 2).

We also found a fourfold increase in the urinary excretion of total porphyrins in the exposed individuals, which was dependent on significant increases in coproporphyrin excretion, mainly COPROIII. There was a 2.5-fold increase in uroporphyrin III excretion ($P < 0.05$) in the exposed individuals from Santa Ana, but not in those from Benito Juarez. These changes resulted in increases in the COPRO/URO and

Table 2 Activities of heme synthesis pathway enzymes in chronic arsenic exposed individuals. Region Lagunera, Mexico. Data are of median (range)

Enzyme (nmoles/ml erythrocytes per h)	Nazareno	Santa Ana	Benito Juarez
Porphobilinogen deaminase	16.6 (12.0–31.3)	18.6* (11.8–23.9)	20.4* (13.6–31.5)
Uroporphyrinogen synthetase	102.8 (75.6–18.5)	101.4 (72.2–164.8)	112.9 (64.3–174.1)
Uroporphyrinogen decarboxylase	11.1 (7.5–14.0)	14.5* (9.0–19.4)	15.2* (8.0–19.3)

* $P < 0.05$ vs Nazareno, Mann-Whitney *U*-test

Table 3 Urinary porphyrins in chronic arsenic exposed individuals. Región Lagunera, México. Data are of median (range)

Porphyrins (pmoles/mg creatinine)	Nazareno	Santa Ana	Benito Juarez
Uroporphyrin-I	8.39 (1.92–52)	16.42 (3.44–112)	14.09 (1.43–20)
Uroporphyrin-III	2.25 (0.35–11)	5.65* (1.70–41)	2.12 (0.34–3.37)
Total uroporphyrins	10.64 (2.27–63)	22.07 (6.64–153)	16.21 (2.32–22)
Coproporphyrin-I (COPROI)	8.56 (2.63–47)	34.07* (3.38–159)	28.48* (5.85–59)
Coproporphyrin-III (COPROIII)	9.68 (4.37–51)	52.58* (7.18–437)	84.12* (17.11–295)
Total coproporphyrins	18.24 (7.10–182)	86.65* (10.56–493)	112.6* (23–337)
COPRO/URO	2.04	2.89*	7.66**
COPROIII/COPROI	1.13	1.54*	2.95**
Total porphyrins	31.80 (12.69–210)	156.04* (22.22–808)	134.15* (28–367)

* $P < 0.05$ vs Nazareno, Mann-Whitney U-test; ** $P < 0.01$ vs Nazareno, Mann-Whitney U-test

COPROIII/COPROI ratios in the exposed individuals (Table 3). There were no significant differences on the concentration of 7, 6 and 5-carboxylic porphyrins in urine, which were excreted in normal amounts.

We have found a small but significant direct relationship between PBG-D activity and the concentration of total porphyrins in urine ($r = 0.17$; $P < 0.05$), and between URO-D activity and coproporphyrins in urine ($r = 0.29$; $P < 0.05$). Similarly, we found a significant relationship between URO-D activity ($r = 0.41$, $P < 0.05$), total porphyrins in urine and the urinary concentration of total arsenic ($r = 0.53$, $P < 0.05$; Fig. 1). The relationship of heme parameters with arsenic species (Asi, MMA and DMA) was similar to that obtained for total arsenic. There was no apparent relationship between fluoride exposure and the heme parameters measured. In addition, multivariate analysis including age, alcohol and tobacco use did not significantly modify the aforementioned results.

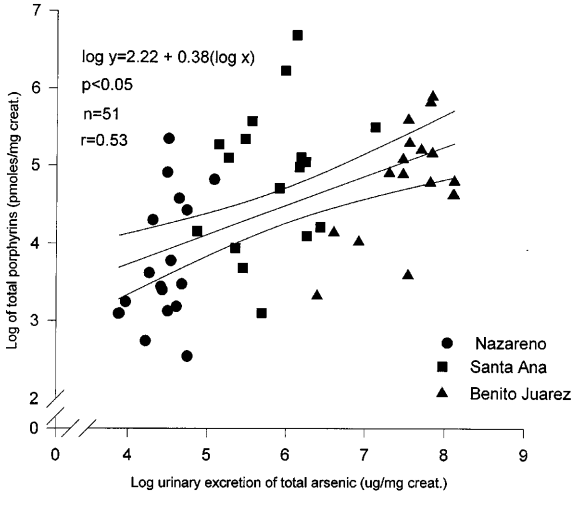

Fig. 1 Relationship between urinary excretion of total arsenic and total porphyrins in individuals of the Region Lagunera

Discussion

As expected, TAs in urine was increased in the exposed individuals and was even higher in those individuals currently exposed to the higher arsenic concentrations in drinking water at Benito Juarez. However, the increase appears to be disproportionate with respect to arsenic concentrations in drinking water, suggesting the need of assessing the relative contribution of other arsenic sources, such as food, towards total As intake. Quantitation of As species in urine (Asi, MMA and DMA) is the method of choice for monitoring workers exposed to inorganic As. The characteristics of occupational exposure and the short half time of As in the body, have led As in urine to be considered as an indicator of recent exposure. However, little information is available about the value for assessing exposure in cases of relatively constant and chronic exposure via drinking water, which may offer valuable information. In the villages under study, the changes in magnitude of exposure probably altered the balance among ingestion, storage and excretion of arsenic, since different ratios of TAs in urine to TAs in water were observed in Benito Juárez (8.6) and Santa Ana (3.4). The higher value was found in individuals currently exposed to ~240 μg As/l in drinking water at Benito Juarez, whereas individuals from Santa Ana were exposed to ~400 μg/l for a long period, but to lower concentrations (~100 μg/l) since 1992.

The more evident alterations in heme metabolism observed in the highly exposed individuals were: (1) small but significant increases in PBG-D and URO-D activities, (2) increases in the urinary excretion of total porphyrins, mainly due to COPROIII and UROIII, and (3) increases in the COPRO/URO and COPROIII/COPROI ratios. The direct relationships between enzyme activities and urinary porphyrins suggest that porphyrin excretion was related to PBG-D. URO-D activity, however, responsible for decarboxylation of uroporphyrins to coproporphyrins, would enhance

coproporphyrin synthesis and excretion at the expense of uroporphyrin, thus resulting in COPRO/URO increases. The wide range of excretion rates precluded statistical significance of the twofold increase in total uroporphyrin excretion in the As-exposed individuals, making it difficult to assess the biological significance. The direct relationships between the above-mentioned parameters of heme metabolism and total arsenic in urine suggest that arsenic exposure was strongly related to these alterations. However, the mechanisms are difficult to ascertain, as more information is required on the regulatory mechanism of the enzymes affected.

The increase in erythrocyte PBG-D activity found in this study is consistent with the increased hepatic and renal PBG-D activities found in rodents subchronically exposed to 20 or 50 mg As/l via drinking water (García-Vargas et al. 1995). In contrast to the increase in human URO-D activity reported here, rodent studies have shown that arsenic decreases hepatic URO-D activity in vitro and in rodents subchronically treated (Woods et al. 1981; Garcia-Vargas et al. 1995). There is little available information regarding increases in human PBG-D and URO-D activity resulting from exposure to environmental contaminants. Our impression is that this increase is attributable more to compensation in the heme biosynthetic pathway than to direct enzyme induction, although the latter possibility could not be ruled out. Although we did not measure COPRO-OX activity, a decrease in the activity could account for the increased urinary coproporphyrin excretion, since animal studies have shown inhibition of hepatic and renal COPRO-OX following subchronic arsenic exposure (Woods and Southern 1989; García-Vargas et al. 1995). Our results also suggest that alanine amino synthetase (ALA-S) may parallel the increased PBG-D activity. Although the largest amount of heme is produced in the erythroid tissue, experimental models have not yet quantitatively addressed the relative contribution of altered heme synthesis in other target organs towards metal-induced porphyrinuria. Hence, the relevance of alterations in erythrocyte heme enzymes to the changes in the profile of urinary porphyrins observed in this study is difficult to assess.

It is clear that chronic As exposure alters human heme metabolism, since it increases PBG-D and URO-D activities, producing uroporphyrinuria III and coproporphyrinuria. However, the severity of the effects appears to depend on characteristics of exposure not yet fully characterized. Studies on smelter workers exposed to arsenic trioxide via inhalation and ingestion have shown coproporphyrinuria without uroporphyrinuria or marked alterations in the COPRO/URO ratio (Telolahy et al. 1993). Studies on individuals exposed via drinking water in Santa Ana (~400 μg As/l), before exposure was reduced, have shown COPRO/URO inversion without significant increases in urinary porphyrins (García-Vargas et al. 1994). In contrast, we found no evidence to suggest that alterations described in the present study would progress to CHP or porphyria cutanea tarda, since in addition to the absence of COPRO/URO inversion, no inhibition of URO-D activity nor the presence of 7-Carboxylic porphyrins in urine was observed.

A likely explanation for the inconsistencies between both of our studies is that exposure was substantially reduced in Santa Ana and that individuals from Benito Juarez were younger and had shorter times of exposure. In addition, urinary coproporphyrin levels in the control subjects appeared to be in the low end of the normal range, therefore contributing towards the absence of the relative decrease in COPRO/URO ratio observed in our previous studies. None of the human studies available has reported the marked porphyric response and enzyme inhibition observed in rodents. However, exposure to higher concentrations and longer times of exposure could result in the porphyric response and URO-D inhibition found in rodents. In conclusion, chronic arsenic exposure via drinking water resulted in increased coproporphyrins and uroporphyrin III urinary excretion, and compensatory increases in erythrocyte PBG-D and URO-D activities.

Acknowledgements This work was partially supported by Consejo Nacional de Ciencia y Tecnología, México (CONACYT 0576P-M). We thank Rosalinda Flores M. for secretarial assistance and Eunice Vera A. for metal analysis.

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