BIOMEDICAL AND ENVIRONMENTAL SCIENCES **17**,101-108 (2004)

Arsenic Induced Inhibition of δ-aminolevulinate Dehydratase Activity in Rat Blood and its Response To Meso 2,3-dimercaptosuccinic Acid and Monoisoamyl DMSA

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Objective The objective of this study was to investigate arsenic induced changes in blood δ-aminolevulinic acid dehydratase (ALAD) after *in vitro* and *in vivo* exposure to this element and its response to co-administration of meso 2,3-dimercaptosuccinic acid (DMSA) and monoisoamyl DMSA (MiADMSA) either individually or in combination. **Methods** Rat whole blood was exposed to varying concentrations (0.1, 0.2 and 0.5 mmol/L) of arsenic (III) or arsenic (V), to assess their effects on blood ALAD activity. Varying concentrations of MiADMSA and DMSA (0.1, 0.5 and 1.0 mmol/L) were also tried in combination to determine its ability to mask the effect of arsenic induced (0.5 mmol/L) inhibition of blood ALAD *in vitro*. *In vitro* and *in vivo* experiments were also conducted to determine the effects of DMSA and MiADMSA either individually or in combination with arsenic, on blood ALAD activity and blood arsenic concentration. **Results** *In vitro* experiments showed significant inhibition of the enzyme activity when 0.1–0.5 mmol/L of arsenic (III and V) was used. Treatment with MiADMSA increased ALAD activity when blood was incubated at the concentration of 0.1 mmol/L arsenic (III) and 0.1 mmol/L MiADMSA. No effect of 0.1 mmol/L MiADMSA on ALAD activity was noticed when the arsenic concentration was increased to 0.2 and 0.5 mmol/L. Similarly, MiADMSA at a lower concentration (0.1 mmol/L) was partially effective in the turnover of ALAD activity against 0.5 mmol/L arsenic (III), but at two higher concentrations (0.5 and 1.0 mmol/L) a complete restoration of ALAD activity was observed. DMSA at all the three concentrations (0.1, 0.5 and 1.0 mmol/L) was effective in restoring ALAD activity to the normal value. **Conclusions** The results thus suggest that arsenic has a distinct effect on ALAD activity. Another important toxicological finding of the present study, based on *in vivo* experiments further suggests that combined administration of DMSA and MiADMSA could be more beneficial for reducing blood ALAD inhibition and blood arsenic concentration than the individual treatment.

Key words: Arsenic toxicity; δ-aminolevulinic acid dehydratase; *In vivo* and *in vitro* effects; Chelation; DMSA and MiADMSA; Combined effects

INTRODUCTION

Arsenic is a widespread environmental toxicant that may cause neuropathy, skin lesions, vascular lesions and cancer upon prolonged exposure[1-3]. It exists in inorganic and organic forms and in different oxidation states (-3, 0, +3, +5). In the case of environmental exposure,

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0895-3988/2004
CN 11-2816

toxicologists are primarily concerned with arsenic in the trivalent and pentavalent oxidation state. Arsenic compounds are environmental and industrial toxicants and exposure to them may cause acute or chronic effects on animals and humans.

Chemicals induced disturbances of the heme biosynthetic pathway have been utilized for many years as a class of biomarkers for detecting sub-lethal toxicity from organic and inorganic toxicants in mammals[4]. Much research has focused on the perturbation of heme metabolism by metals such as lead, mercury, nickel, and cadmium. The haem biosynthetic pathway plays an important role in all nucleated cells to provide chlorophyll and related structure[5]. In mammalian and avian tissues the principal product of this pathway is haem (ferro-protoporphyrin IX), an essential component of various biological functions including oxygen transport systems, mixed function oxidative reactions and other oxidative metabolic processes. δ-aminolevulinic acid dehydratase (ALAD), a sulfhydryl enzyme of the heme-biosynthesis pathway, has been implicated in the pathogenesis of heavy metal poisoning since various critical cellular processes are affected by a reduced concentration of heme. ALAD seems to be the principal lead binding protein in human erythrocyte[6]. The toxic effect of arsenic on ALAD has not been studied in detail but may involve protein synthesis, enzyme inhibition or enzyme activation.

ALAD inactivation may lead to an accumulation of delta aminolevulinic acid (substrate) that can cause an overproduction of reactive oxygen species, which in turn, could explain the toxic effect of arsenic. In view of the pro-oxidant effect of ALA, a study of the inhibition of ALAD by arsenic can contribute to a better understanding of the toxicology of this metalloid[7]. These processes may contribute to oxidative stress in cells and may be related to degenerative cellular mechanism[8,9].

The accepted treatment for poisoning with arsenicals is the administration of 2,3-dimercapto-1-propanol (BAL), a dithiol compound and a strong chelator of arsenic. However, recent advances in the treatment of arsenic toxicity have shown that meso 2,3-dimercaptosuccinic acid (DMSA) and sodium 2,3-dimercaptopropane 1-sulpfonate (DMPS) are orally effective, dithiol chelating agents useful for treating arsenic poisoning[10,11]. These chelators are less toxic than BAL and consequently both DMSA and DMPS can be administered in much higher doses than BAL[12,13]. Monoisoamyl DMSA (MiADMSA) is one of the most effective metal mobilizing agents of vicinal class[14,15]. Although, the compound is more toxic than the parent diacid DMSA, its structure features and few recent experimental evidences suggest that it might well be effective in chelating arsenic[16-18].

This study was undertaken to evaluate *in vivo* and *in vitro* effects of arsenic (III and V) on the activity of ALAD in blood of adult rat and further its response to the co-administration of meso 2,3-dimercaptosuccinic acid (DMSA) or monoisoamyl DMSA.

MATERIALS AND METHODS

Chemicals

δ-aminolevulinic acid (ALA), DMSA 5, 5′-dithiobis (2-nitrobenzoic) acid (DTNB) and p-dimethyaminobenzaldehyde (DMAB) were purchased from Sigma (St. Louis, MO)., MiADMSA was synthesized in our Synthetic Chemistry Division, by the controlled esterification of DMSA with the corresponding alcohol (isoamyl alcohol) in acidic medium[14]. The product was purified (purity 99.9%) and characterized using spectral and analytical methods before experimentation. The samples were stored, refrigerated in a

dessicator to avoid oxidation and thermal decomposition. Both the chelators were dissolved in saline. DMSA was dissolved in 10% sodium bicarbonate while MiADMSA was dissolved in 5% sodium bicarbonate solution. All the antidote solutions were prepared immediately before use. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or BDH Chemicals (Mumbai, India).

Animals and Treatments

Male adult albino rats weighing approximately 100 g from our own breeding colony were maintained in an air - conditioned room (20℃-25℃) under natural lighting conditions with free access to water and food (Amrut Feeds, Pranav Agro, New Delhi, metal contents of diet, in ppm dry weight Zn 45, Cu 10, Mn 55, Fe 70, Co 5). The Animal Use Committee of the DRDE, Gwalior, India, approved the protocols for the experiments. The rats were divided into 9 groups of 5 animals each and treated as below for 5 days.

Group 1 – Normal animals
Group 2 – Arsenic (III) , as sodium arsenite, 5 mg/kg, intraperitoneally
Group 3 – Arsenic (III), 5 mg/kg, i.p. + 0.1 mmol/L MiADMSA, orally
Group 4 – Arsenic (III), 5 mg/kg, i.p. + 0.5 mmol/L MiADMSA, orally
Group 5 – Arsenic (III), 5 mg/kg, i.p. + 1.0 mmol/L MiADMSA, orally
Group 6 – Arsenic (III), 5 mg/kg, i.p. + 0.5 mmol/L DMSA, orally
Group 7 – Arsenic (III), 5 mg/kg, i.p. +1.0 mmol/L DMSA, orally
Group 8 – Arsenic (III), 5 mg/kg, i.p.+ 0.2 mmol/L plus DMSA orally + 0.2 mmol/L MiADMSA, orally
Group 9 – Arsenic (III), 5 mg, i.p. + 0.5 mmol/L plus DMSA orally + MiADMSA, 0.5 mmol/L, orally

The rats were anesthetized with light ether prior to being killed. Whole blood was collected in tubes containing 1 mg/mL heparin until used.

The activity of blood δ-aminolevulinic acid dehydratase (ALAD) was assayed according to the procedure of Berlin and Schaller[19]. The assay system consisted of 0.2 mL of heparinized blood and 1.3 mL of distilled water. After 10 min of incubation at 37℃ for complete hemolysis, 1 mL of standard δ-aminolevulinic acid was added to the tubes and incubated for 60 min at 37℃. The reaction was stopped after 1 h by adding 1 mL of trichloroacetic acid (TCA). To the supernatant, an equal volume of Ehrlich reagent was added and the absorbance was recorded at 555 nm after 5 min.

Arsenic concentration in blood was measured after wet acid digestion using a microwave digestion system (CEM, USA, model MDS-2100). Arsenic was estimated using a hydride vapour generation system (Perkin Elmer model MHS-10) fitted with an atomic absorption spectrophotometer (AAS, Perkin Elmer model AAnalyst 100).

In vitro Experiments

Untreated mice were anesthetized with light ether. For the *in vitro* study, arsenic (III) and arsenic (V) solutions were prepared in deionised water, stored in plastic containers and used for a period of 1 week. ALAD activity was assayed according to the method of Berlin and Schaller[19] as described above. The reaction was started 10 min after the addition of 100 – 200 μL of whole blood which was previously hemolysed by diluting in an equal volume of water and carried out for 3 hours, at varying concentrations (0.1, 0.2 or 0.5 mmol/L) of arsenic (III) or arsenic (V). Blood ALAD activity was also determined by incubating whole blood with 0.1 mmol/L MiADMSA at varying concentrations of As (III or V) or 0.2 mmol/L

arsenic (III or V) at varying concentrations of MiADMSA (0.1, 0.2 or 0.5 mmol/L).

RESULTS

In vitro Inhibition of ALAD by Arsenic (III) and Arsenic (V) and Its Response to MiADMSA and DMSA

Arsenic (III) at mmol/L concentration caused a significant dose dependent inhibition in blood ALAD activity. Arsenic (V) too was able to inhibit the ALAD activity but the dose dependent effects were not prominent (Table 1). Addition of MiADMSA (0.1 mmol/L) was tested for its protection against varying doses of arsenic (0.1 to 0.5 mmol/L), on ALAD activity. MiADMSA 0.1 mmol/L provided a significant protection against the lower concentration of arsenic (0.1 mmol/L), but against the two higher arsenic concentrations (0.2 mmol/L and 0.5 mmol/L) no protection was observed (Table 2). Addition of MiADMSA alone at 0.1 mmol/L concentration did not have any significant effect on blood ALAD activity.

TABLE 1

Dose Dependent Effect of Arsenic (III) and Arsenic (V) on Blood δ-aminolevulinic Acid Dehydratase Activity *in vitro*

Normal	As (III), mmol/L			As (V), mmol/L		
Control	0.1	0.2	0.5	0.1	0.2	0.5
8.71±0.24*	2.92±0.11†	1.98±0.13†	0.62±0.04‡	2.82±0.19†	2.43±0.21†	1.85±0.19†

*Note.*Values are $\bar{x}\pm s$; n=5. *-‡Means with matching symbol notations in each column are not significant at 5% level of significance.

TABLE 2

Combined Exposure to Monoisoamyl DMSA with Varying Doses of Arsenic on Blood δ-aminolevulinic Acid Dehydratase Activity *in vitro*

	Blood ALAD nmol/min/mL RBC
Normal Control	6.61±1.07*
Arsenic (III),	
0.1 mmol/L	3.45±0.29†
0.2 mmol/L	1.86±0.21‡
0.5 mmol/L	0.96±0.21§
MiADMSA, 0.1 mmol/L	8.35±0.72*
Arsenic (III)	
0.1 mmol/L + MiADMSA, 0.1 mmol/L	6.25±0.63*
0.2 mmol/L + MiADMSA, 0.1 mmol/L	4.26±1.02†
0.5 mmol/L + MiADMSA, 0.1 mmol/L	3.13±0.66†

*Note.*Values are $\bar{x}+s$, n=5. *-§ Mean with matching symbol notations in each column are not significant at 5% level of significance.

When varying concentrations of MiADMSA (0.1, 0.5, and 1.0 mmol/L) were added to the arsenic (III) at a concentration of 0.5mmol/L, the inhibition caused by arsenic (III) was completely reversed (Table 3) at the concentrations of 0.5 mmol/L and 1.0 mmol/L, while the changes at 0.1 mmol/L were less significant. On the other hand, when varying concentrations of DMSA were added to arsenic (III), the inhibited ALAD activity was completely reversed (Table 3).

TABLE 3

Concomitant Exposure to Arsenic with Varying Doses of Monoisoamyl DMSA on Blood δ-aminolevulinic Acid Dehydratase Activity *in vitro*

	Blood ALAD nmol/min/mL RBC
Normal Control	6.61±1.07*
Arsenic (III), 0.5 mmol/L	1.12±0.12†
Arsenic, 0.5 mmol/L +	
MiADMSA, 0.1 mmol/L	3.02±0.29‡
0.5 mmol/L	6.89±0.21*
1.0 mmol/L	6.58±0.36*
Arsenic, 0.5 mmol/L +	
DMSA, 0.1 mmol/L	5.68±0.25*
0.5 mmol/L	8.18±0.59*
1.0 mmol/L	11.52±0.34§

Note.Values are $\bar{x}\pm s$; n=5. *-§ Mean with matching symbol notations in each column are not significant at 5% level of significance.

In vitro and In vivo Effects of Combined Addition of DMSA, MiADMSA and Arsenic on Blood ALAD Activity

In an interesting study, combined addition of DMSA, MiADMSA and arsenic (III) at a concentration was studied. Addition of 0.5 or 1.0 mmol/L DMSA and MiADMSA produced a complete recovery in arsenic induced inhibition of blood ALAD activity. ALAD activity in this condition was more pronounced compared to the control value (Table 4).

TABLE 4

Combined Administration of Arsenic (III), DMSA and MiADMSA on Blood δ-aminolevulinic Acid Dehydratase Activity *in vitro*

	ALAD nmol/min/mL Erythrocytes
Normal Control	8.29±0.45*
Arsenic, 0.5 mmol/L	1.12±0.12†
Arsenic, 0.5mmol/L + MiADMSA, 0.1 mmol/L +DMSA 0.1 mmol/L	8.20±0.57‡
Arsenic, 0.5mmol/L + MiADMSA, 0.5 mmol/L +DMSA 0.5 mmol/L	9.39±0.61*
Arsenic, 0.5mmol/L + MiADMSA, 1.0 mmol/L + DMSA 1.0 mmol/L	10.92±0.21*

Note. Values are $\bar{x}+s$; n=5. *-‡ Means with matching symbol notations in each column are not significant at 5% level of significance.

After the *in vitro* experiments, the blood ALAD and concentration of arsenic was also studied in animals intraperitoneally injected with arsenic at a dose of 5 mg/kg (0.066 mmol/L) for 5 days (Table 5). There was 40% inhibition of blood ALAD activity in the blood accompanied by a significant increase in blood arsenic concentration of animals exposed to arsenic. Co-administration of oral MiADMSA at a dose of 0.1, 0.2 and 0.4 mmol/L/kg and DMSA at a dose of 0.5 mmol/L produced a significant turnover in the ALAD activity and depletion of blood arsenic level but there was no dose dependent effect on blood ALAD while, blood arsenic concentration decreased dose dependently. Co-administration of

MiADMSA (0.1 mmol/L), DMSA (0.1 mmol/L) and arsenic led to a complete recovery in blood ALAD and a more pronounced depletion of blood arsenic (Table 5). Interestingly however, the effects were significantly less pronounced when the doses of DMSA and MiADMSA were increased to 0.2 mmol/L each (Table 5).

TABLE 5

Arsenic Induced Inhibition of δ-aminolevulinic Acid Dehydratase Activity and Its Response to Simultaneous Administration of DMSA, MiADMSA Either Individually or in Combination in Rat Blood *in vivo*

Groups	ALAD nmol/min/mL Erythrocyte	Blood As ng/mL
Untreated Animals	$6.69\pm0.76^{*}$	$1.11\pm0.21^{*}$
Arsenic 0.66 mmol/L/kg (control)	$4.04\pm0.16^{\dagger}$	$16.45\pm0.34^{\dagger}$
Arsenic + MiADMSA, 0.1 mmol/L, oral	$5.34\pm0.23^{\ddagger}$	$11.15\pm0.58^{\ddagger}$
Arsenic + MiADMSA, 0.2 mmol/L, oral	$5.89\pm0.31^{\ddagger}$	$7.13\pm0.92^{\S}$
Arsenic + MiADMSA, 0.4 mmol/L, oral	$5.45\pm0.14^{\ddagger}$	$5.23\pm0.65^{\S}$
Arsenic + DMSA, 0.5 mmol/L	$5.81\pm0.25^{\ddagger}$	$9.11\pm0.56^{\S}$
Arsenic + MiADMSA, 0.1 mmol/L, oral + DMSA, 0.1 mmol/L, oral	$6.94\pm0.27^{*}$	$4.45\pm0.21^{\S}$
Arsenic + MiADMSA, 0.2 mmol/L, oral + DMSA, 0.2 mmol/L, oral	$4.83\pm0.27^{\ddagger}$	$3.12\pm0.23^{\S}$

*Note.*Values are $\bar{x}\pm s$; $n=5$. *-§ Means with matching symbol notations in each column are not significant at 5% level of significance.

DISCUSSION

ALAD is a sulfhydryl-containing enzyme that catalyzes the asymmetric condensation of two molecules of ALA to porphobilinogen. This reaction is fundamental in the biosynthesis of tetrapyrroles (such as heme), the prosthetic group of various proteins[20]. ALAD is highly sensitive to the presence of heavy metals such as mercury, lead and selenium, which possess a high affinity for sulfhydryl group. Arsenic inhibited the activity of blood ALAD at mmol/L concentration, suggesting the sensitivity of this enzyme to both As (III) and As (V). This effect could be attributed to the attachment of arsenic to –SH group of the enzyme considering the strong affinity of arsenic for sulfhydryl groups[21,22]. Accordingly, in the present study both DMSA and MiADMSA, both strong thiol containing chelating agents reversed ALAD inhibition caused by arsenic. Interestingly there was a more pronounced increase in blood ALAD activity following co-administration of DMSA or in some case MiADMSA, which could be attributed to the availability of sulphydryl group and antioxidant effects of DMSA.

No effect of MiADMSA or DMSA individually on blood ALAD activity was observed, although some of the metal chelating agents such as 2,3-dimercaprol (BAL) and EDTA inhibited ALAD possibly by removing zinc from the site involved in maintaining residues in a reduced state[20,23]. ALAD inactivation may lead to an accumulation of δ-aminolevulinic acid (substrate) that can cause an overproduction of reactive oxygen species, which in turn could explain the toxic effects of arsenic. In view of the proxidant effect of ALA, a study of the inhibition of ALAD by arsenic can contribute to a better understanding of the toxicology of this metalloid. These processes may contribute to oxidative stress in cells and may be related to degenerative cellular mechanism. DMSA is one of the least toxic drug that could

be given orally, a less obvious benefit may also be derived as a result of DMSA's structural potential to serve as an antioxidant *in vivo*[24]. Use of DMSA has got some limitations. Hydrophilic and lipophobic properties of DMSA do not allow this chelator to pass through cell membranes. It was recently reported that the monoesters of DMSA might be a more effective chelating agent for metal poisoning than DMSA[14,25]. It has been observed that MiADMSA is more efficient in mobilizing brain lead than DMSA[26]. It is believed that while DMSA is a relatively efficient as a non-toxic chelator, MiADMSA should also be of great concern as a potential drug for chelation therapy in lead poisoning. DMSA and to some extent MiADMSA are known to have no adverse effects on body zinc concentration[18, 27,28]. It has been reported that DMSA tends to restore the inhibited blood ALAD activity after *in vivo* lead, arsenic and gallium arsenide intoxication[7,29,30]. This partial reactivation of blood ALAD was attributed to the chelating properties of the chelating agent(s) that help in removing metals from the binding sites. It can thus be concluded from the present study that ALAD may be a sensitive biochemical indicator for arsenic exposure and that combined administration of MiADMSA and DMSA may be a better treatment option than monotherapy. These findings however, require more detailed investigation using chronic *in vivo* exposure.

ACKNOWLEDGEMENT

Authors thank Mr. K. Sekhar, Director of the establishment for his support and encouragement.

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(*Received April* 5, 2003 *Accepted December* 30, 2003)