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Metallothionein does not sequester arsenic(III) ions in condition of acute arsenic toxicity

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ABSTRACT

The major cause of toxicity of trivalent arsenicals is due to their interaction with the sulfhydryl groups in proteins. Because of its high content, Metallothionein (MT) provides one of the most favorable conditions for the binding of As(III) ions to it. MT has long been anticipated for providing resistance in case of arsenic (As) toxicity with similar mechanism as in case of cadmium toxicity. The present study investigates whether the sequestration of As ions by MT is one of the mechanisms in providing protection against acute arsenic toxicity. A rat model study on the metal stoichiometric analysis of MT1 isoform isolated from the liver of arsenic treated, untreated and zinc treated animals has been carried out using the combination of particle induced X-ray emission (PIXE) and electrospray ionisation mass spectrometry (ESI–MS). The results revealed the absence of arsenic bound MT1 in the samples isolated from arsenic treated animals. Although, both Cu and Zn ions were present in MT1 samples isolated from all the treatment groups. Moreover, only partially metallated MT1 with varying number of Zn ions were observed in all the groups. These results suggest that the role of MT during acute arsenic toxicity is different from its already established role in case of cadmium toxicity.

1. Introduction

Arsenic and its compounds have been classified as human carcinogens by the International Agency for Research on Cancer (IARC) (2012). Several countries like Argentina, Bangladesh, Chile, China, India, Mongolia and Taiwan are severely affected due to the exposure of arsenic contaminated drinking water (Tseng, 2009). In India alone, arsenic contamination in ground water affects nearly 70 million people in 86 districts across 10 states (First Report Committee on Estimates, 2014). Chronic arsenic exposure of inorganic arsenic through drinking water causes skin lesions, hyperkeratosis, cancers, blackfoot disease, vascular diseases and diabetes (Tseng, 1977; Tseng et al., 2000; Kitchin, 2001; Wang et al., 2007a,b; Flora, 2011). The toxicity of arsenic compounds is highly dependent upon their oxidation state and chemical composition (Del Razo et al., 2001). Inorganic arsenate (As(V)) being a molecular analogue of phosphate, competes for binding to the phosphate anion transporters and replaces phosphate in some biochemical reactions. However, the toxicity of trivalent arsenicals is through their interaction with the sulfhydryl groups in proteins (Shen et al., 2013). Inorganic arsenite (As(III)) can inhibit a number of vital proteins (e.g. pyruvate dehydrogenase, glutathione reductase, glutathione S-transferase, thioredoxin reductase, and DNA ligases etc.) by binding directly to their thiol groups leading to manifestations of various diseases (Styblo et al., 1997; Snow et al., 1999; Chouchane and Snow, 2001; Wang et al., 2007a,b; Bergquist et al., 2009). In this context, the sulfhydryl rich protein, Metallothionein (MT), provides a favorable condition for the binding of As(III) ions to it. It has a significant role in heavy metal detoxification through various processes. In one of the processes, either the heavy element is directly sequestered by the apo-MT or it displaces Zn ions already bound to MT. Thus, the concentration of Zn in the free pool increases which leads to the binding of Zn to metal transcription factor 1 (MTF1), consequently initiating MT expression. Newly synthesized MT will further sequester the unbound heavy metal ions present in the cytosol. This mechanism is prevalent in case of acute toxicity of Cd and Ag (Suzuki and Yoshikawa, 1976; Klaassen et al., 2009). Other metals like Ni, Co and Mn do not directly interact with MT but induce oxidative stress in the cytosol (Haq et al., 2003). Thus, the concentration of Zn in the

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free pool increases, as the donor potential of MT increases under more oxidizing conditions, leading to MT induction (Maret and Vallee, 1998; Maret, 2011). In some cases, the metal ion binds directly to MTF1, which in turn initiates MT induction. For example arsenic (III) is directly sensed by the C-terminal cysteine cluster of MTF1, initiating the binding of MTF1 to metal response elements (MRE) and induction of *MT1* (He and Ma, 2009). Although, it has been demostrated that MT is induced in case of acute arsenic toxicity (He and Ma, 2009), there is no evidence for direct binding of As(III) ions with MT in such conditions. Various *in vivo* studies also provide evidence for the protective role of MT in case of chronic as well as acute arsenic toxicity (Kreppel et al., 1994; Liu et al., 2000; Park et al., 2001). One report shows that LD_{50} of As is 1.4 fold higher for wild type mice than MT null-mice (Park et al., 2001). Arsenic exposure is more toxic to MT1/II-null mice and also decreases GSH content and induces apoptotic factors such as caspase-3 (Liu et al., 2000). Pretreatment with zinc, an effective inducer of MT synthesis provides protection against As toxicity (Kreppel et al., 1994). The *in vitro* studies on binding of As to MT reveal that As(III) ions binds to MT in a ratio of 6:1 and the resulting complex is stable in a wide pH range (Toyama et al., 2002; Jiang et al., 2003; Ngu and Stillman, 2006; Ngu et al., 2008; Irvine et al., 2013; Garla et al., 2013).

The present study was designed to determine whether, like in case of cadmium, the sequestration of As(III) ions by MT is one of the mechanism for providing protection in case of acute arsenic toxicity. To address this, metal stoichiometric analysis of the isolated rat liver MT1 induced by acute arsenic toxicity has been carried out using the combination of particle induced X-ray emission (PIXE) and electrospray ionisation mass spectrometry (ESI–MS). Identical protocol was followed to analyse the MT samples isolated from untreated rats (*i.e.* animals without any metal supplementation) and Zn treated rats which serve as control and positive control respectively in this study. To confirm the expression of MT in various treatment groups, analysis of MT-1 mRNA expression was also carried out. Moreover, elemental analysis of the liver tissue samples was also performed to observe the accumulation of various heavy metals.

2. Materials and methods

2.1. Chemicals and reagents

Sodium arsenite ($NaAsO_2$) and Zinc sulfate ($ZnSO_4{\cdot}7H_2O$) were obtained from Central Drug House (P) Ltd, New Delhi, India; TRI-Reagent, one step with Platinum® Taq DNA Polymerase Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) kit was procured from Invitrogen, USA. All other reagents used for MT isolation and RNA analysis were of molecular biology grade from Sigma Chemical Co. St. Louis (USA).

2.2. Animal treatment

Male Wistar adult rats, weighing 200–250 g, procured from the Central Animal House, Panjab University, Chandigarh, India, were used for the present study. The rats were fed with standard pelleted diet and drinking water *ad libitum*. For the induction and isolation of MT, the rats were randomly divided into three groups of 10 animals each: Animals in Group I (control) were injected subcutaneously (sc) with saline. Animals in group II (Zn) were injected sc with 153 μmol of $ZnSO_4$/kg body weight in saline, once a day for two days. Group III (As) animals were injected sc with 75 μmol of $NaAsO_2$/kg body weight in saline once (Albores et al., 1992). All doses were prepared in isotonic saline. After 18 h of the last injection the animals were sacrificed by cervical dislocation under anesthesia and the livers were excised. The concentration of MT in liver, induced by metal supplementation through injection, has been observed to be maximum at 18 h post treatment (Albores et al., 1992). The experimental design and procedures were approved by the institutional Ethical Committee on Animal experiments, Panjab University, Chandigarh.

2.3. RNA isolation and RT-PCR

Total RNA was isolated from the liver tissue of various treatment groups using Tri-Reagent. The purity and quantity of RNA was determined by monitoring A_{260}/A_{280} ratio and absorbance at 260 nm (A_{260}). For the RT-PCR analysis, the following forward and reverse primers of MT-1 were used: forward "TGTGCCTGAAGTGACGAACAG" and reverse "TTCACATGCTCGGTAGAAAACG" (Liu et al., 2007). The PCR products were analyzed on 1.2% agarose gel electrophoresis and the densitometric analysis of bands was done using IMAGE J Software from National Institutes of Health, USA.

2.4. Statistical analysis

Statistical analysis software, SPSS (version 14) was employed for statistical processing of the data. Data are expressed as mean ± standard deviation. The statistical significance of the difference between various groups was determined using one-way analysis of variance (ANOVA). This was followed by multiple post-hoc least significant difference (LSD) test for comparison between different groups. The level of significance was set at $p \leq 0.05$ in all cases.

2.5. MT isolation

A combination of gel filtration and anion exchange chromatography was used for the isolation of MT from the rat liver of control and different metal supplemented groups by following the protocol reported earlier (Garla et al., 2013). The fractionation procedure was repeated multiple times to obtain the required amount of protein for both PIXE and ESI–MS studies. About 100–150 μg of MT was recovered from the liver of one animal.

2.6. PIXE

Elemental analysis of the MT samples isolated from rat liver was carried out using the PIXE facility at the Panjab University Cyclotron, Chandigarh, India (Puri et al., 2006). Details of the experimental procedure and calibration of the set up for the analysis of protein samples are described elsewhere (Hajivaliei et al., 1999; Garla et al., 2013). Briefly, 20 μL of MT solution was dispensed and dried on a Kapton foil. After drying, the sample appears as a circular spot of ~5 mm diameter. Three X-ray spectra from each sample were recorded by irradiating the sample spot at three different locations by 2.7 MeV protons. An average of three measurements represents the elemental concentration for each sample.

PIXE analysis of rat liver tissue was performed at the 3 UD pelletron accelerator at the Institute of Physics (IOP), Bhubaneswar, India (Hajivaliei et al., 1999; Balouria et al., 2011). The liver tissue samples were prepared by following the protocol reported by Balouria et al. (2011). Briefly, liver tissue was freeze dried and 200 mg of each sample was ground and mixed thoroughly with graphite powder (100 mg) with the help of Agate pestle and mortar. The mixture was further ground for the time, till it turned into a homogeneous fine powder. The resultant powder was made into pellets of 13 mm diameter with the help of a hydraulic press. These pellets were irradiated with 3 MeV protons and the emitted X-rays were detected using a Si(Li) detector positioned at 90° angle with respect to the beam. Calibration of the experimental facility