Arsenite induced oxidative damage in mouse liver is associated with increased cytokeratin 18 expression

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Abstract Cytokeratins (CK) constitute a family of cytoskeletal intermediate filament proteins that are typically expressed in epithelial cells. An abnormal structure and function are effects that are clearly related to liver diseases as non-alcoholic steatohepatitis, cirrhosis and hepatocellular carcinoma. We have previously observed that sodium arsenite (SA) induced the synthesis of CK18 protein and promotes a dose-related disruption of cytoplasmic CK18 filaments in a human hepatic cell line. Both abnormal gene expression and disturbance of structural organization are toxic effects that are likely to cause liver disease by interfering with normal hepatocyte function. To investigate if a disruption in the CK18 expression pattern is associated with arsenite liver damage, we investigated CK18 mRNA and protein levels in liver slices treated with low levels of SA. Organotypic cultures were incubated with 0.01, 1 and 10 μM of SA in the absence and presence of *N*-acetyl cysteine (NAC). Cell viability and inorganic arsenic metabolism were determined. Increased expression of CK18 was observed after exposure to SA. The addition of NAC impeded the oxidative effects of SA exposure, decreasing the production of thiobarbituric acid-reactive substances and significantly diminishing the up regulation of CK18 mRNA and protein. Liver arsenic levels correlated with increased levels of mRNA. Mice treated with intragastric single doses of 2.5 and 5 mg/kg of SA showed an increased expression of CK18. Results suggest that CK18 expression may be a sensible early biomarker of oxidative stress and damage induced by arsenite in vitro and in vivo. Then, during SA exposure, altered CK expression may compromise liver function.

Keywords Arsenic · Cytokeratin 18 · Oxidative damage · *N*-acetyl cysteine

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Introduction

Cytokeratins (CK) constitute a family of cytoskeletal intermediate filament proteins that are typically expressed in epithelial cells. CK associate to form heterodimers containing one member of each subfamily. Type I CK includes CK9 through CK20, while type II CK includes CK1 through CK8. In simple epithelia such as liver, exocrine pancreas, and intestine, the two major intermediate filament proteins are cytokeratin polypeptides 8 and 18 (CK8/CK18) with variable expression of CK19 and CK20 (Moll et al. 1982; Moll 1993; Calnek and Quaroni 1993). Traditionally, CK

were considered only as skeletal proteins providing mechanical stability, but lately evidence has shown that they also exert several non-skeletal functions (Omary et al. 2004). The presence of wild-type CK seems to protect hepatocytes from the transmittal of apoptotic stimuli under stress conditions (Caulin et al. 2000; Gilbert et al. 2001; Ku et al. 2003) by hyperphosphorylation of CK (Ku et al. 1998; Coulombe and Omary 2002) or possibly by overexpression of CK, as noted in mouse liver and gallbladder injury models (Cadrin et al. 2000; Denk et al. 2000; Fickert et al. 2002; Tao et al. 2003).

In humans, altered patterns of cytokeratin expression are observed in pathological conditions such as alcoholic liver, cirrhosis, chronic hepatitis and liver cancer (Rhodes and Oshima 1998). Also, chronic intoxication with griseofulvin or 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) in mice can induce cytoskeleton alterations highly similar to those seen in human alcoholic hepatitis (Zatloukal et al. 2000). The increase in cytokeratin expression in response to liver injury suggests that these proteins may behave as stress proteins, similar to heat shock proteins (hsp). This hypothesis is supported by the known physical and ATP-dependent association of hsp70 with CK8/CK18 that increases with heat shock (Liao et al. 1995).

Inorganic arsenic (iAs) is a known human carcinogen that contaminates well water worldwide, affecting large groups of people in many countries. Human exposure to iAs is associated with cancer and organ injury, including squamous and basal cell carcinomas in the skin, hepatocellular carcinoma, angiosarcoma, cirrhosis and hepatoportal sclerosis (IPCS 2001; Centeno et al. 2002; Patrick 2003). There is evidence suggesting that iAs toxicity involves oxidative damage (Izquierdo-Vega et al. 2006; Hughes and Kitchin 2006), mainly by the interaction of iAs with protein thiols that are central components of redox-sensitive proteins in redox signaling and control pathways (Hansen et al. 2006). Several studies have demonstrated that liver is the primary arsenic metabolizing organ (Hughes et al. 2003). Metabolic conversion of iAs into methylated products is a multistep process that yields mono–di and tri-methylated arsenic forms that have different toxic potential than that of the parent compound iAs.

Using the WRL-68 human hepatic cell line, we observe that sodium arsenite (SA) induces the synthesis of CK18 protein and promotes a dose-related disruption of cytoplasmic CK18 positive filaments (Ramírez et al. 2000). We speculate that these effects result from a stress-response to oxidative damage caused by SA.

To investigate whether the expression of CK18 could be associated with the oxidative damage induced by iAs in liver, organotypic cultures of mouse liver slices were treated with SA. Cells upregulated both CK18 mRNA and CK18 protein. This effect was inhibited when the antioxidant N-acetylcysteine (NAC) (Hirano et al. 2004) was added to cultures. Furthermore, mice treated systemically with 2.5 or 5 mg/kg of SA also showed an increase of CK18 in the liver. Our results suggest that CK18 induction occurs in response to the oxidative damage generated by the iAs and should be considered as an early indicator of iAs toxicity in the liver.

Materials and methods

Chemicals and solutions

SA, NAC, thiobarbituric acid, trichloroacetic acid, perchloric acid, DMEM culture media, antibiotics and the protease inhibitor cocktail were obtained from Sigma (St. Louis, MO).

Animals

Inbred male BALB/c mice (5–6 weeks old, weighing 22–25 g), obtained from the animal care facility of the Biomedical Research Institute of the National Autonomous University of Mexico (UNAM), were acclimated for 1 week before experiments were initiated.

Organotypic liver culture

SA solutions were prepared daily in deionized water. Livers from BALB/c mice were cut to obtain 250–500 μm thick slices of approximately 5,000 μm in diameter. Slices were weighed and placed in culture plates (two slices per well) containing DMEM culture media supplemented with 8% fetal bovine serum (Invitrogen, Carlsbad, CA), 10 μg/ml ampicillin and 1% streptomycin. Organotypic cultures were stabilized at 37°C with 5% carbon dioxide for 2 h. The liver slices were then exposed to 0.01, 1 or 10 μM SA for 3 h, followed by incubation with 1 or 2.5 mM NAC for 2 h. At the end of these treatments, tissues were washed in ice-cold phosphate saline buffer (PBS), pH 7.4, containing a protease inhibitor cocktail and were homogenized in the same buffer. The protein concentrations were determined using the Bradford method (BioRad Laboratories, Hercules CA) using albumin as standard (Bradford 1976). For polyacrylamide gel electrophoresis (PAGE) and immunoblotting, tissues were homog-

enized in ice-cold 0.1 M PBS, pH 7.4. Samples were then aliquoted and frozen by immersion in liquid nitrogen.

Viability of liver slices

The viability of liver slices after treatment was determined by measuring intracellular K^+ levels using the method described by Azri et al. (1990) with some modifications. Briefly, slices were washed in ice-cold PBS and homogenized. An aliquot of tissue homogenate was added to 0.02 ml of concentrated perchloric acid. The mixture was gently shaken and centrifuged at 12,000 rpm for 10 min at 4°C. K^+ levels in the supernatant fractions were then analyzed by air-acetylene flame atomization using an atomic absorption spectrophotometer (Perkin Elmer 3100) at 766.5 nm and reported as μmol per gram of tissue.

Animal exposure

BALB/c mice received a single dose of 0, 2.5 or 5.0 mg of SA per kg of body weight via the intragastric route and were sacrificed 24 h later by cervical dislocation. The dose of SA, in this study, represents one-eighth and one-fourth of the LD_{50} dose for mice, respectively (ATSDR 2000). Livers were extracted and washed in ice-cold isotonic saline solution to remove debris and blood. For PAGE and immunoblotting, tissues were homogenized in ice-cold 0.1 M PBS, pH 7.4, in the presence of a proteinase inhibitor cocktail, aliquoted and frozen by immersion in liquid nitrogen.

Determination of arsenic species

We measured the levels of arsenic species in tissue homogenates by hydride-generation atomic absorption spectroscopy after column chromatographic separation of inorganic arsenic and its metabolites: monomethylarsenic (MMA), dimethylarsenic (DMA) and trimethylarsenic (TMAO), as described by Hughes et al. (2003).

Western blot analysis

Normalized homogenates were separated by standard procedures (Laemmli 1970) on 10% acrylamide gels under reducing conditions [25 mM β-mercaptoethanol (BioRad) or 10 mM dithiothreitol (BioRad)]. The proteins were visualized by western blotting using primary mouse monoclonal antibodies against mouse CK18 (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary peroxidase-coupled antibodies against mouse IgG (Santa Cruz). Immunoblot analysis was performed using a Kodak Gel Logic 100 Imaging System (Rochester, NY).

Lipid peroxidation assay

Lipid peroxidation by liver slices was determined based on the formation of thiobarbituric acid-reactive substance (TBARS) as described elsewhere (Buege and Aust 1978). Briefly, 0.5 ml of liver homogenate were added to a reaction mixture containing 2 ml of 2.5% trichloroacetic acid (pH 1.0) and 1 ml of 0.6% thiobarbituric acid, followed by 30 min heating at 95°C. After cooling, the chromogen was read spectrophotometrically at 532 nm against a "blank" reaction mixture lacking homogenate but subjected to the entire procedure. To correct for background absorption, absorbance values at 572 nm were subtracted from those at 532 nm, the latter representing the absorption maximum of the 2:1 TBA:MDA adduct. The concentration of TBARS was expressed in μmol per gram of tissue.

RT–PCR

CK18 mRNA levels were quantified by RT–PCR. Total RNA was isolated using the TRIZOL method (Invitrogen). RNA samples were transcribed into first strand cDNA using RT–MLV retrotranscriptase (Invitrogen). The cDNA was amplified by PCR with the following primers: 5′-GACGCTGAGACCACACT and 5′-TCCATCTGTGCCTTGTAT (Zhong et al. 2003). Actin primers were used as an internal reference. Image analysis was performed using a Kodak Gel Logic 100 Imaging System.

Statistical analysis

A total of three samples from each of the five mice was used for each treatment dose for both the in vitro and the in vivo experiments. Values always represent mean ± SD of the five triplicate liver slices. Data were analyzed by ANOVA followed by a Dunn's post hoc test. These statistical tests were performed using the software Stata 8.0 (Stata Corp., College Station, TX). Differences between treatments were considered significant when $P < 0.05$.

Ethics

The experiments reported in this article were carried out following the guidelines stated in "Principles of Laboratory Animal Care" (NIH publication #85–23, revised 1985) and the 'Norma Oficial Mexicana de la

Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA)" titled "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" (Clave NOM-062-ZOO-1999) (published in August 2001).

Results

To test the hypothesis that SA induces expression of the cytokeratin CK18, we used an organotypic liver culture model. Culturing liver slices in the presence of SA did not affect cell viability, since K^+ levels remained above 100 μmol K^+/g of tissue (Table 1). Liver slices were able to uptake SA and metabolize it to MMA and DMA metabolites in the presence and absence of the reduced thiol NAC, which has antioxidant activity

Table 1 Intracellular K^+ as an indicator of cell viability

SA (μM)	K^+ (μmol/g) Mean ± SD
0	107.0 ± 13.5
0.01	101.5 ± 11.3
1.0	115.5 ± 11.5
10	106.4 ± 14.2

(Table 2). Consistent with our hypothesis, incubation of liver tissue with different concentrations of SA resulted in a dose-dependent increase in CK18 mRNA levels. This induction was statistically significant at 1 and 10 μM SA, which caused a three to sevenfold increase in basal expression over control cultures (Fig. 1a). Furthermore, the upregulation of CK18 mRNA correlated with increased levels of the protein (Fig. 2a). NAC inhibited the induction of CK mRNA
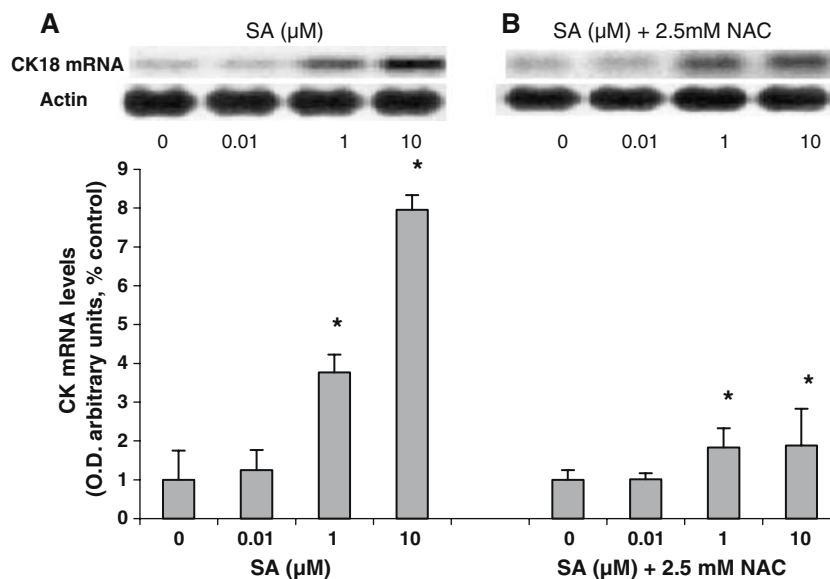
Table 2 Arsenic methylated species present in the organotypic cultures after 3 h of SA and SA + 2.5 mM NAC treatments (mean ± SD)

	IAs (ng/g)	MMA (ng/g)	DMA (ng/g)	Total As (ng/g)
SA (μM)				
0	7.22 ± 0.67	<1	<1	7.22 ± 0.67
0.01	11.4 ± 1.37	<1	<1	11.4 ± 1.37
1	181.81 ± 17.55	12.73 ± 1.61	20.02 ± 5.20	206.97 ± 10.74
10	1,052.40 ± 54.41	49.08 ± 7.89	176.23 ± 56.75	1,277.72 ± 35.60
SA (μM) + 2.5 mM NAC				
0	4.03 ± 0.43	<1	<1	2.03 ± 0.43
0.1	8.25 ± 0.78	<1	<1	8.25 ± 0.78
1	113.99 ± 1.71	<1	<1	113.99 ± 1.71
10	908.02 ± 85.03	45.24 ± 8.53	24.33 ± 5.87	977.61 ± 93.37

Fig. 1 Semi-quantitative RT–PCR of CK18 mRNA levels in mouse liver organotypic cultures treated with 0, 0.01, 1 and 10 μM of SA (**a**) and with the same concentrations of SA but in the presence of 2.5 mM NAC (**b**). Densitometric evaluation of the agarose gel images was performed using β-actin mRNA as loading control. CK18 mRNA levels are expressed as a percentage of loading control. Bars represent mean ± SD of triplicate cultures. *Asterisks* indicate significance ($P < 0.05$) compared to control cultures according to Dunn's post hoc test
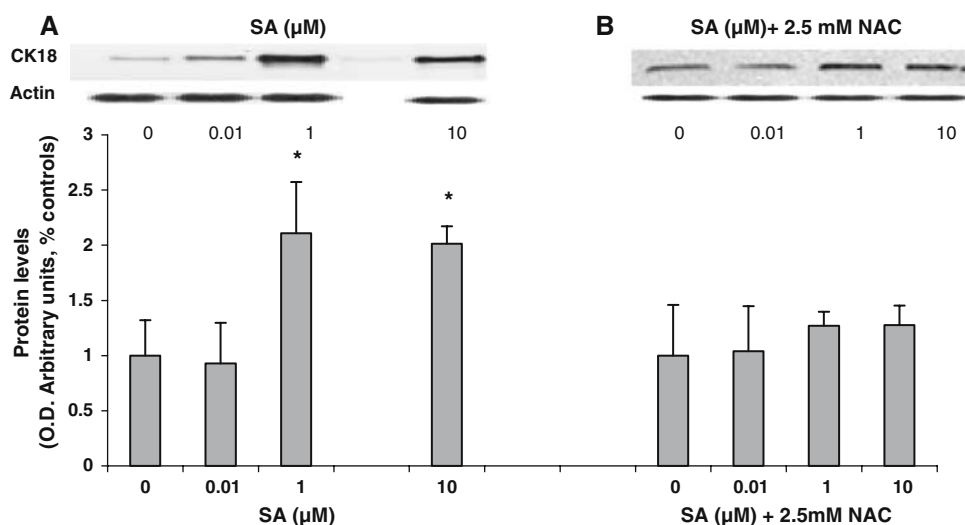
and protein by SA but did not modulate the basal expression of CK18 mRNA or protein (Figs. 1b, 2b).

We speculated that the increase in CK18 resulted from oxidative damage caused by SA. To test this idea, we treated liver slices with SA and then with 2.5 mM of NAC, a GSH agonist with antioxidant effects.

The presence of intracellular arsenic clearly induced CK18 mRNA, and this effect was modulated by the addition of NAC (Table 2; Fig. 3). A significant dose-related effect was demonstrated by a quadratic regression using CK18 mRNA levels and iAs concentrations in liver slices exposed to SA and to SA in the presence of NAC. The slope values of the positive relationships were 0.015 and 0.0087, respectively (Fig. 3).

We also observed that the total arsenic was slightly reduced by NAC treatment, similar to what was

observed by Santra et al. (2007) reducing the amount of methylated species formation.

Our model postulates that SA induces oxidative damage, which induces CK18 expression. We therefore measured oxidative damage following SA treatment by measuring the formation of TBARS during lipid peroxidation. We found that arsenite treatments indeed induced the TBARS production indicative of oxidative damage. Furthermore, the addition of 2.5 mM of NAC in the incubation media significantly prevented the production of TBARS, as expected (Fig. 4).

Finally, we tested our model in vivo. Similar to what was observed in vitro, 24 h after mice were given 2.5 or 5 mg/kg of SA orally, their livers showed statistically significant increased levels of CK18 mRNA and protein (Fig. 5).
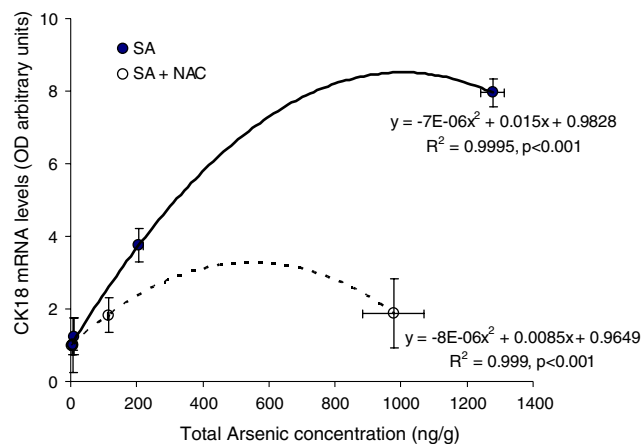
Discussion

CK participate in the network of cytoskeletal intermediate filaments, performing a variety of important cellular functions including cell division, motility, maintenance of cellular mechanical integrity, stress responses and vesicle transport. Importantly, CK also play an essential "guardian" role in the liver that is unmasked after exposure to environmental stresses (Omary et al. 2004) such as iAs exposure. In this paper, we tested the hypothesis that arsenite treatment of liver would generate oxidative damage that would in turn induce the expression of the major type I cytokeratin found in hepatocytes CK18.

Cultured mouse liver slices in fact showed a dose-dependent modulation of CK18 gene expression following SA treatment. By measuring both CK18 mRNA and protein levels (Figs. 1a, 2a), we found that

Fig. 3 Relationship between CK18 mRNA levels (O.D. arbitrary units as a percentage of loading control) and intracellular arsenic (ng/g of tissue) in liver organotypic cultures treated with 0, 0.01, 1 and 10 μM SA in the absence or presence of 2.5 mM NAC. The data represent mean ± SD of triplicate cultures
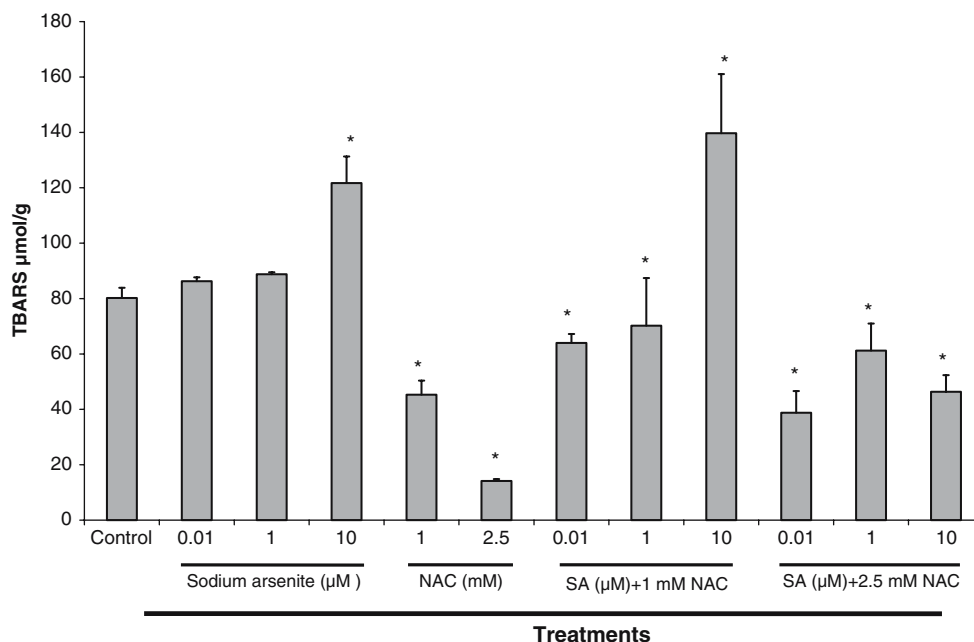
Fig. 4 Thiobarbituric acid-reactive substance (TBARS) production as a measure of oxidative damage in mouse liver organotypic cultures treated with different concentrations of SA and NAC. Bars represent mean ± SD of triplicate cultures. Asterisks represent Dunn's post hoc test, $P < 0.05$

the increased transcription of the gene results in increased translation into protein in the hepatocyte cytoplasm. We reported similar results in the WRL-68 human hepatic cell line, where we also observed alterations in the distribution pattern of the CK filaments after treatment with SA (Ramírez et al. 2000). The increased levels of CK18 mRNA were clearly related

to the intracellular levels of arsenic (Fig. 3), even at the low level of 1 μM.

N-acetylcysteine (NAC) inactivates free radical species thereby protecting cells against oxidative damage (De Flora et al. 2001). The induction of CK18 was significantly inhibited (by approximately twofold) by the presence of NAC. We also observed (Table 2; Fig. 3)

Fig. 5 CK18 mRNA (**a**) and CK18 protein (**b**) levels in mouse liver 24 h after being treated with 0, 2.5, and 5.0 mg/kg of sodium arsenite. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and β-actin protein were used as loading controls for **a** and **b**, respectively. Both molecules are expressed as a percentage of loading control. Bars represent mean ± SD of five animals in duplicate determinations after densitometric analysis. *Asterisks* represent Dunn's post hoc test, $P < 0.05$
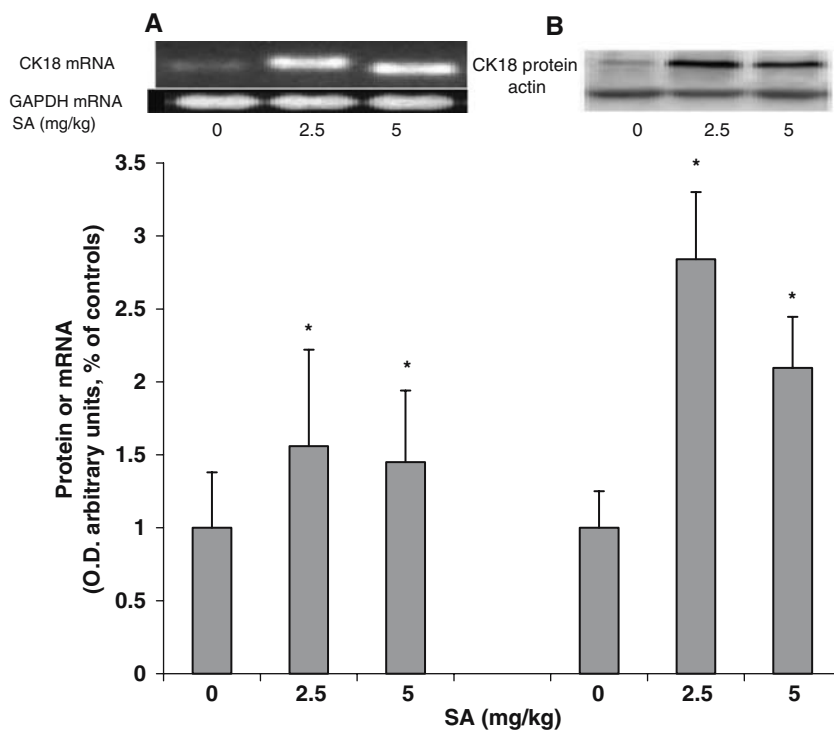
that the total arsenic burden in liver slices was slightly reduced by NAC reducing the amount of methylated species formation. The key point in the metabolism of arsenic is the repetitive reduction of pentavalent forms of arsenic and oxidative methylation of trivalent forms of arsenic (Thomas et al. 2004). There is ample evidence demonstrating in these series of reactions, the generation of reactive oxidative species that cause cell and tissue damage (Yamanaka and Okada 1994; Kitchin and Ahmad 2003). Thus, a reduction in the formation of methylated species would also reduce the formation of reactive damaging species. Taken together, these results strongly support the idea that oxidative stress could be modulating the expression of CK18 in mouse hepatocytes. In this regard, several studies have proposed oxidative stress as a mechanism of action of arsenic toxicity (Hansen et al. 2006).

Arsenic compounds have high affinity for thiol groups such as those present in NAC. It is therefore possible that some arsenite may be trapped by NAC so that its delivery to liver cells may be impaired. However, the cysteine analog is readily taken up by cells and can directly scavenge ROS (Kelly 1998; De Flora et al. 2001). Moreover, a number of studies have demonstrated that NAC is able to reduce or counteract oxidative damage. Electron spin resonance and confocal microscope studies showed that As (III) stimulated ROS generation and Hsp70 expression in human pulmonary epithelial and MDA231 cells, and these effects were inhibited by NAC (Han et al. 2005; Kim et al. 2005). In addition, NAC was able to inhibit the cytotoxicity of the iAs metabolites, monomethylarsonous acid (MMAIII), dimethylarsinic acid (DMAV), dimethylarsinous acid (DMAIII) and trimethylarsine oxide (TMAO) in rat bladder cells (Wei et al. 2005).

It is important to mention that NAC did not modulate basal CK18 expression (Figs. 1, 2) although 2.5 mM of NAC was able to reduce significantly TBARS formation (Fig. 4). Similar results were obtained while TBARS formation and antioxidant enzyme activities (Sudheer et al. 2007) or gene expression modulation were evaluated (Chen et al. 2005) in the presence or absence of NAC.

Our observations together with the effect observed in the animals after SA administration suggest that CK18 expression could be an early biomarker of the oxidative stress and damage induced by iAs in vitro and in vivo. Consistent with this idea, the overexpression of CK18 was dramatically elevated in the hepatocellular carcinomas that developed in adult mice exposed transplacentally to arsenic during gestation (Liu et al. 2004). In humans, accumulation of CK18 is a histopathological feature seen in hepatocellular carcinoma, a tumor type associated with iAs exposure (Centeno et al. 2002). It is also well documented that arsenic accumulates in tissues with high keratin content such as hair and skin. If this accumulation is due to sequestration of arsenic by CK, the induction of CK18 by iAs in hepatocytes would increase the accumulation of arsenic in the liver.

Our in vitro and in vivo results (Ramírez et al. 2000 and this paper) indicate that the increased presence of CK18 protein could be considered as an early indicator of iAs liver toxicity. CK18 synthesis in liver cells is tightly correlated with differentiation programs and with several cellular processes such as apoptosis and cell proliferation. In addition, CK18 seems to be a substrate for a variety of protein kinases involved in mitosis, apoptosis and stress (Caulin et al. 2000; Gilbert et al. 2001; Omary et al. 2004). Thus during chronic iAs exposure, the altered CK18 expression could modify differentiation patterns in liver, compromising the cellular physiology by impairing the protective role of CK18 and inducing hepatic susceptibility to further toxic injury (Ku et al. 2003; Omary et al. 2004).

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