

Arsenite-induced activation of JNK1/2 and p38 MAP kinases in ELM-1 murine erythroleukemia cells

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SUMMARY. Arsenite stimulates different mitogen-activated protein kinase (MAPK) pathways in the murine erythroleukemia cell line ELM-I-1. The activation of JNK1/2 and p38 was determined by Western blotting with antibodies specific for phosphorylated, activated forms of these kinases. Our data indicate that arsenite stimulates rapid phosphorylation of JNK1/2 and p38 MAPKs in ELM-I-1 cells. In the concentration range of 12.5-500 μ M arsenite stimulates the stress response kinases JNK1/2 and p38. Maximal JNK1/2 and p38 activation was observed at 50 μ M. This concentration produces a 7-40 fold increase in the activity of the JNK1/2, and a 4.5-7.5 fold increase in the activity of the p38 MAPK. The arsenite effects were time dependent: maximal activation of the stress response kinases JNK1/2 and p38 were observed after a 60 min exposure to arsenite.

Keywords: arsenite, erythroleukemia cells, JNK1/2, MAPK, p38

Introduction

Arsenic is an important environmental toxicant (Bernstam and Nriagu, 2000; Ventura-Lima *et al.*, 2011), and is a major health concern for 200 million people worldwide (Ellinsworth, 2015). Human exposure to drinking water contaminated with arsenic is a serious global health concern and predisposes to cardiovascular disease states, such as hypertension, atherosclerosis, and microvascular disease (Ellinsworth, 2015). Epidemiological studies have established a close association between exposure to arsenic and increased incidences of cancer in arseniasis-endemic areas of the world including Taiwan, Mexico, Chile, Argentina, Thailand, India, Canada and the USA (Lau *et al.*, 2004). Arsenic is carcinogenic to humans, and targets in particular the urinary bladder, liver, kidney, skin, lung, prostate and other internal sites (Bode

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and Dong, 2002; Plataniias, 2009). The Agency for Toxic Substances and Disease Registry ranks arsenic as number one on their Priority List of Hazardous Substances (<http://www.atsdr.cdc.gov/spl/>), and it is also classified as a Group I carcinogen by the International Agency for Research on Cancer (Ellinsworth, 2015).

The mechanism by which arsenic mediates carcinogenesis remains a subject of debate, with evidence supporting several plausible etiologies, including disruption of signaling cascades (Ventura-Lima *et al.*, 2011), elevated levels of oxidative stress (Ellinsworth, 2015), chromosomal aberrations and epigenetic changes (Faita *et al.*, 2013; Reichard and Puga, 2010).

Despite the adverse effects of arsenicals, they show great promise in the chemotherapy of certain types of human cancer (Bode and Dong, 2002; Plataniias, 2009; Wang, 2001). In this regard, arsenic is an effective treatment for acute promyelocytic leukemias (Plataniias, 2009; Wang, 2001). Thus, it appears that arsenic, a known human carcinogen, is also an effective chemotherapeutic (Bode and Dong, 2002; Plataniias, 2009). Therefore the study of the mechanism of arsenic on the mitogen activated protein kinases could have important implications in both cancer causation and cancer chemotherapy.

The various effects of arsenite may be mediated through activation of a MAP kinase cascade (Bode and Dong, 2002; Huang *et al.* 1999; Porter *et al.*, 1999; Ventura-Lima *et al.*, 2011). Previous investigations have demonstrated that arsenite activates members of the MAP kinase family, transcription factors such as activator protein-1 (AP-1), and immediate early genes, including c-jun, c-fos and c-myc, which help to regulate the expression of transforming oncoproteins and growth factors (Bode and Dong, 2002; Li *et al.*, 2006; Plataniias, 2009). Each of the three major mitogen-activated protein kinase (MAPK) pathways (ERK, JNK, p38) transduce a variety of extracellular signals that lead to diverse cellular responses such as cell growth, differentiation, proliferation, apoptosis and stress responses to environmental stimuli (Kyriakis and Avruch, 2012; Schaeffer and Weber, 1999; Tibbles and Woodgett, 1999).

The purpose of this study is to examine for the first time the MAPK signal transduction pathways induced by arsenic in our established *in vitro* murine erythroleukemia cell line (ELM-I-1).

Materials and methods

Chemicals and reagents

Anti-(p38) rabbit polyclonal IgG, anti-(JNK1/2) goat polyclonal IgG, anti-(phosphorylated-JNK1/2), anti-(phosphorylated-p38) mouse monoclonal IgG, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-(rabbit IgG) and anti-(mouse IgG) peroxidase conjugates, Bradford reagent and protease inhibitors, sodium m-arsenite (NaAsO₂) were obtained from Sigma (St. Louis, MO, U.S.A.);

α -minimal essential medium without nucleotides, Dulbecco's modified Eagle medium, horse serum, fetal calf serum were purchased from Life Technologies (Gaithersburg, MD, U.S.A.), analytical grade chemicals from Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany).

Cells and culture conditions

Erythropoietin-responsive murine erythroleukemia cells, line ELM-I-1 (Schaefer *et al.*, 2004) was kindly provided by Prof. W. Ostertag, Heinrich Pette Institute for Experimental Virology and Immunology (Hamburg, Germany). ELM-I-1 cells were grown in α -minimal essential medium without nucleosides, supplemented with 10 % (v/v) horse serum and with 2 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin at 37 °C in a humidified air/CO₂ (19:1) atmosphere. In the experiments, exponentially growing cells were plated at (1-2) x 10⁵ cells/ml and at (4-8) x 10⁴ cells/ml. Approximately 16 h later the cells were treated with arsenite. At the indicated time points, cells were harvested and analysed.

Western blot analysis

Cells were collected, rapidly washed in ice cold phosphate-buffered saline (PBS) and suspended in RIPA-buffer [5 mM Tris-HCl (pH 7.4), 15 mM NaCl, 100 mM EGTA, 1% Nonidet P-40, 10 % deoxycholic acid] containing 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM Na₃VO₄, 1 mM p-nitrophenylphosphate and 10 mM sodium pyrophosphate. The samples were lysed on ice for 30 minutes and insoluble material was removed by centrifugation at 12000 x g at 4 °C for 12 min. Protein content in the supernatants was determined using the Bradford reagent, the samples were then diluted with 3x Laemmli sample buffer [180 mM Tris-HCl (pH 6.8), 6% (w/v) SDS, 300 mM dithiothreitol, 45 % (v/v) glycerol, 22.5 mM EDTA, 0.0015% (w/v) bromphenol blue] 3 : 1 and heated to 100 °C for 5 min. 40 to 60 μ g of total protein were separated on 10 % (w/v) sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A) using the Mini-Protean II System of Bio-Rad (Hercules, CA, U.S.A.). The membranes were blocked with 5 % (w/v) dry milk in Tris-buffered saline [TBS: 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl] containing 0.1 % (v/v) Tween-20 and probed with anti-(p38) and anti-(JNK1/2) polyclonal antibodies as well as with anti-(phosphorylated-JNK1/2) and anti-(phosphorylated-p38) monoclonal antibody at room temperature for 1h. After washing in TBS-Tween-20, the membranes were incubated with horseradish peroxidase-conjugated second antibodies, and visualised using the ECL Western blotting system of Amersham (Buckinghamshire, U.K.). When the same membrane was sequentially probed with antibodies of different species origin, the membrane was treated with 0.5 % (v/v) H₂O₂ in TBS at room temperature for 25 min, extensively washed in TBS, then blocked and analysed with the next first and peroxidase-conjugated second antibodies as described above.

Results and discussion

Although inorganic arsenic is clearly carcinogenic in humans (Bode and Dong, 2002; Plataniias, 2009), it is unclear if it acts through genotoxic or epigenetic mechanisms. Arsenic is usually assumed to act principally *via* an epigenetic effect (Bode and Dong, 2002; Plataniias, 2009). An important epigenetic mechanism could be the inappropriate activation or inactivation of signal transduction pathways leading to the activation of transcription factors, which in turn modulates the gene expression (Bode and Dong, 2002; Porter *et al.*, 1999; Ventura-Lima *et al.*, 2011).

To determine whether arsenite exposure affects the activity of specific factors involved in murine erythroleukemia cell signaling, we used antibodies against phospho-p38 and phospho-JNK1/2 to examine the effect of arsenite on MAPK pathways by Western blot.

Concentration and time-dependent effects of arsenic treatment on JNK1/2 kinase activity

The JNK pathway has been implicated in the regulation of apoptosis induced by various stimuli (Kyriakis and Avruch, 2012; Muscarella and Bloom, 2002; Plataniias, 2009; Qu *et al.*, 2002; Tibbles and Woodgett, 1999). To determine if JNK signal pathway may be involved in arsenic-induced effects, ELM-I-1 cells were subjected to different concentrations (1-500 μM) of arsenite for 60 min.

Dual phosphorylation of JNKs at Thr183/Tyr185 is essential for kinase activity and phosphorylation at this site is an excellent marker of JNK activity (Kyriakis and Avruch, 2012; Tibbles and Woodgett, 1999). Thus, to confirm JNK activation, the levels of phosphorylated JNK1/2 were determined by Western blot analysis (Fig. 1A) and analyzed by scanning densitometry (Fig. 1B).

The immunoblot data revealed that sodium arsenite enhanced the JNK1/2 signal transduction pathway, as indicated by the increase in activated JNK1/2 (Fig. 1). Although at 1 μM concentration arsenite has no detectable effect, our data show that at low concentration of 12.5 μM it can produce an ~3.5-4 fold increase in the phosphorylated form of the JNK1/2. Maximal activation (7-40 fold increase over basal level) were obtained with 50 μM arsenite.

In our experiments the membranes used to define phosphorylated forms of MAPKs were stripped and then reprobed with an antibody that detects each kinase regardless of its phosphorylation state, to ensure equal loading of each lane and to serve as an internal control for subsequent quantitation. As shown in Figure 1A (bottom panel), no major differences were observed between the treated and control cells in the levels of total JNK1/2, so the increase of the phosphorylated form (Fig. 1 A, upper panel) is not the result of an arsenite-induced JNK1/2 protein overexpression.

In the following time-dependent experiments we used 50 μM sodium arsenite (Fig. 2). Phospho-JNK1 and 2 were detected 2 min after arsenite addition and maintained for at least 4 hours following exposure. Maximal JNK1/2 activation was observed after 60 min of incubation with arsenite.

ARSENITE-INDUCED ACTIVATION OF JNK1/2 AND p38 MAP KINASES IN ELM-1 CELLS

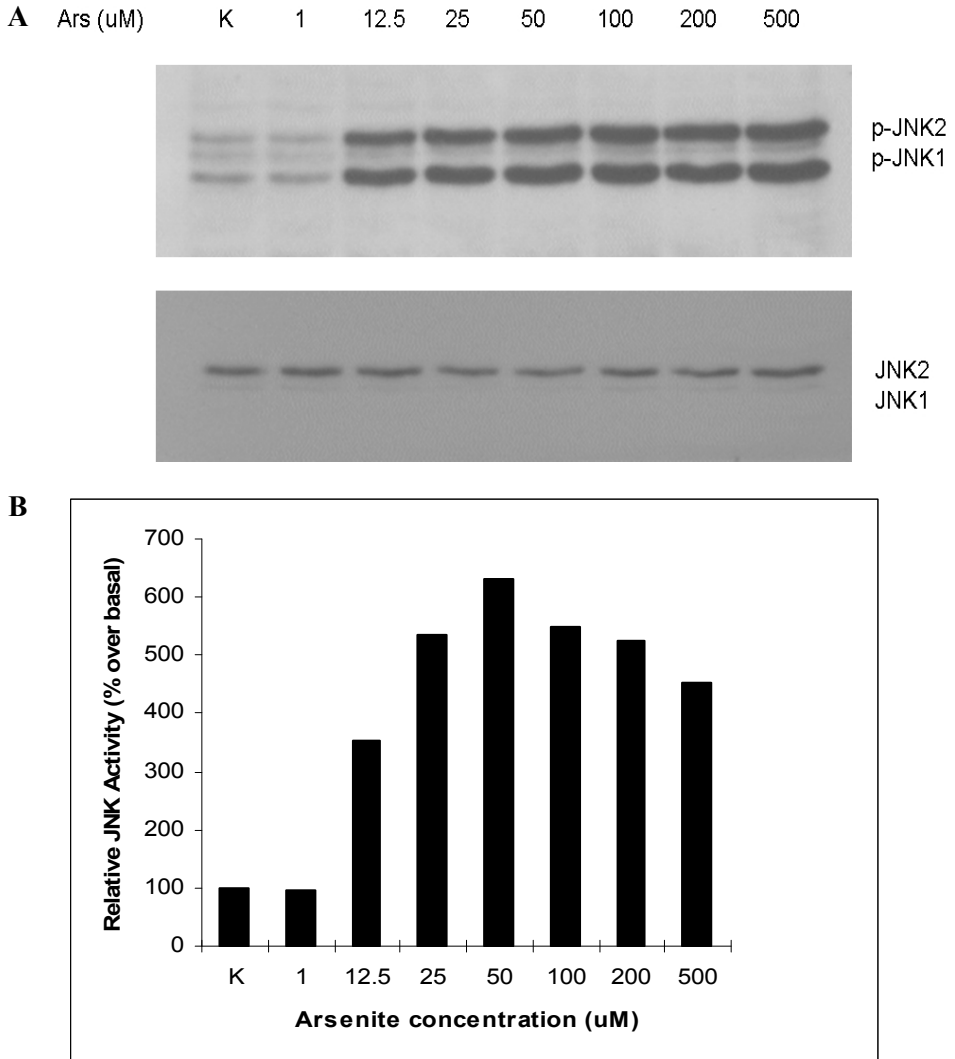


Figure 1. Dose response curve of JNK1/2 activation by arsenite (cells were treated with different concentrations of arsenite for 1 h; K - control). JNK1/2 MAP-kinase phosphorylation was determined by Western blotting (A, upper lane) and the activity is expressed graphically as fold stimulation over basal, quantified using a scanning densitometry system (B). The results shown in A are representative of three independent experiments. In the bottom lane (A) Western blots were reprobated with antibodies against JNK1/2 that recognize the non-phosphorylated JNK1/2 forms to assess the total amount of JNK1/2

At present, we do not know which of these potentially mechanism is primarily responsible for activation of JNK1/2 in the ELM-I-1 cell line.

Effects of arsenic treatment on p38 kinase activity

We next explored whether arsenite influences p38 MAPK activation in ELM-I-1 cells. To determine if p38 signaling pathway may be involved in arsenic-induced effects, ELM-I-1 cells were subjected to different concentrations (1-500 μ M) of arsenite for 60 min. Maximal activation were obtained with 50 μ M arsenite (data not shown).

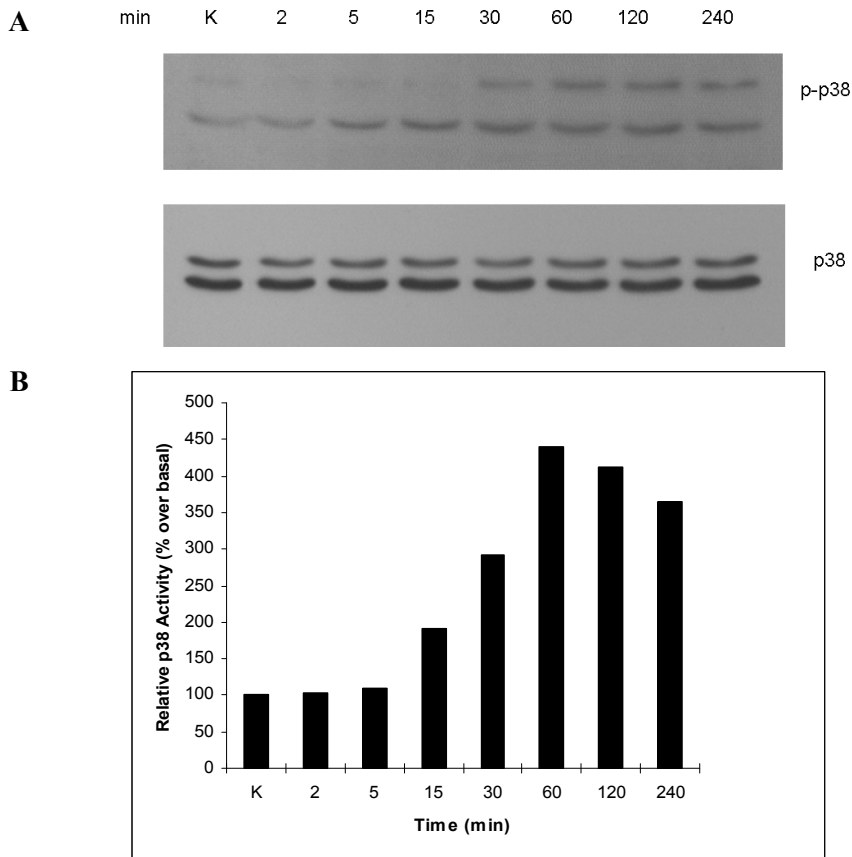


Figure 3. Time course of p38 activation by arsenite in ELM-I-1 cells (cells were treated with 50 μ M arsenite for the times indicated; K - control). p38 MAP-kinase phosphorylation was determined by Western blotting (A, upper lane) and the activity is expressed graphically as fold stimulation over basal, quantified using a scanning densitometry system (B). The results shown in A are representative of three independent experiments. In the bottom lane (A) Western blots were reprobbed with antibodies against p38 that recognize the non-phosphorylated p38 forms to assess the total amount of p38

For the time-dependent experiments we used 50 μM sodium arsenite. Figure 3A,B shows that arsenite (50 μM) increased ~ 2 fold the level of phospho-p38 after a 15 min arsenite treatment. Maximal stimulation (~ 4.5 -7.5 fold over the basal level) was measured after 60 min of arsenite incubation. The phosphorylated form of the p38 was observed for at least 4 h after arsenite exposure.

Figures 3A, bottom panel, show that the level of non-phosphorylated p38 following arsenite remained the same, indicating that changes in p38 phosphorylation were not due to alterations in steady-state protein level.

Conclusions

Our data indicate that arsenite rapidly stimulates the phosphorylation of JNK1/2 and p38 MAPKs in ELM-I-1 cells. In the concentration range of 12.5-500 μM arsenite stimulates the stress responses kinases JNK1/2 and p38. Maximal JNK1/2 and p38 activation was observed at 50 μM . This concentration produces an 7-40 fold increase in the activity of the JNK1/2, and an 4.5-7.5 fold increase in the activity of the p38 MAPK. The arsenite effects were time-dependent: maximal activation of the stress response kinases JNK1/2 and p38 was observed after 60 min exposure to arsenite.

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REFERENCES

- Bernstam, L., Nriagu, J. (2000) Molecular aspects of arsenic stress, *J. Toxicol. Env. Health Part B.*, **3**, 293-322
- Bode, A. M., Dong, Z. (2002) The paradox of arsenic: molecular mechanisms of cell transformation and chemotherapeutic effects, *Critical Reviews in Oncology/Hematology* **42**, 5-24
- Ellinsworth, D. C. (2015) Arsenic, Reactive Oxygen, and Endothelial Dysfunction, *J Pharmacol Exp Ther* **353**, 458 – 464
- Faita, F., Cori, L., Bianchi, F., Andreassi, M. G. (2013) Arsenic-Induced Genotoxicity and Genetic Susceptibility to Arsenic-Related Pathologies, *Int. J. Environ. Res. Public Health* **10**, 1527-1546
- Huang, C., Ma, W.-Y., Li, J., Goranson, A., Dong, Z. (1999) Requirement of ERK, but Not JNK, for Arsenite-induced Cell Transformation, *J. Biol. Chem.*, **274**(21), 14595-14601

- Kyriakis, J. M., Avruch, J. (2012) Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update, *Physiol Rev* **92**, 689–737
- Lau, A. T. Y., Li, M., Xie, R., He, Q. -Y., Chiu, J. -F. (2004) Opposed arsenite-induced signaling pathways promote cell proliferation or apoptosis in cultured lung cells, *Carcinogenesis* **25**(1), 21-28
- Li, J. -P., Lin, J. -C., Yang, J. -L. (2006) ERK Activation in Arsenite-Treated G1-Enriched CL3 Cells Contributes to Survival, DNA Repair Inhibition, and Micronucleus Formation, *Toxicological Sciences* **89**(1), 164–172
- Meriin, A. B., Yaglom, J. A., Gabai, V. L., Mosser, D. D., Zon, L., Sherman M. Y. (1999) Protein-Damaging Stresses Activate c-Jun N-Terminal Kinase via Inhibition of Its Dephosphorylation: a Novel Pathway Controlled by HSP72, *Mol. Cell. Biol.* **19**(4), 2547-2555
- Muscarella, E., Bloom, S. E. (2002) Differential Activation of the c-Jun N-Terminal Kinase Pathway in Arsenite-Induced Apoptosis and Sensitization of Chemically Resistant Compared to Susceptible B-Lymphoma Cell Lines, *Toxicological Sciences* **68**, 82–92
- Platanias, L. C. (2009) Biological Responses to Arsenic Compounds, *J. Biol. Chem.* **284**, 18583-18587
- Porter, A. C., Fanger, G. R., Vaillancourt, R. R. (1999) Signal transduction pathways regulated by arsenate and arsenite, *Oncogene* **18**, 7794-7802
- Reichard, J. F., Puga, A. (2010) Effects of arsenic exposure on DNA methylation and epigenetic gene regulation, *Epigenomics* **2**(1), 87–104
- Qu, W., Bortner, C. D., Sakurai, T., Hobson, M. J., Waalkes, M. P. (2002) Acquisition of apoptotic resistance in arsenic-induced malignant transformation: role of the JNK signal transduction pathway, *Carcinogenesis* **23**(1), 151-159
- Schaefer, A., Kósa, F., Bittorf, T., Magócsi, M., Rosche, A., Ramirez-Chávez, Y., Marotzki, S., Marquardt, H. (2004) Opposite Effects of Inhibitors of Mitogen-activated Protein Kinase Pathways on the Egr-1 and β -Globin Expression in Erythropoietin- responsive Murine Erythroleukemia Cells, *Cellular Signaling* **16**(2), 223-234.
- Schaeffer, H. J., Weber, M. J. (1999) Mitogen-Activated Protein Kinases: Specific Messages from Ubiquitous Messengers, *Mol. Cell. Biol.* **19**(4), 2435-2444
- Theodosiou, A., Ashworth, A. (2002) Differential effects of stress stimuli on a JNK-inactivating phosphatase, *Oncogene* **21**(15), 2387-2397
- Tibbles, L. A., Woodgett, J. R. (1999) The stress-activated protein kinase pathways, *Cell. Mol. Life Sci* **55**, 1230-1254
- Ventura-Lima, J., Bogo, M. R., Monserrat, J. M. (2011) Arsenic toxicity in mammals and aquatic animals: A comparative biochemical approach, *Ecotoxicology and Environmental Safety* **74**, 211–218
- Wang, Z. -Y. (2001) Arsenic compounds as anticancer agents, *Cancer Chemother Pharmacol* **48**(1), S72-S76

