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Enhanced HSP30 and HSP70 accumulation in *Xenopus* cells subjected to concurrent sodium arsenite and cadmium chloride stress

Imran Khamis, John J. Heikkila *

Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada

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ABSTRACT

Heat shock proteins (HSPs) are molecular chaperones that aid in protein folding, translocation and in preventing stress-induced protein aggregation. The present study examined the effect of simultaneous sodium arsenite and cadmium chloride treatment on the pattern of HSP30 and HSP70 accumulation in A6 kidney epithelial cells of the frog, *Xenopus laevis*. Immunoblot analysis revealed that HSP30 and HSP70 accumulation in concurrent stressor treatments were significantly higher than the sum of HSP30 or HSP70 accumulation in individual treatments. This finding suggested a synergistic action between sodium arsenite and cadmium chloride. KNK437 inhibitor studies indicated that the combined stressor-induced accumulation of HSPs may be regulated, at least in part, at the level of transcription. Immunocytochemistry revealed that simultaneous treatment of cells with the two stressors induced HSP30 accumulation primarily in the cytoplasm in a punctate pattern with some dysregulation of F-actin structure. Increased ubiquitinated protein accumulation was observed with combined sodium arsenite and cadmium chloride treatment compared to individual stressors suggesting an impairment of the ubiquitin proteasome degradation system. The addition of a mild heat shock further enhanced the accumulation of HSP30 and HSP70 in response to relatively low concentrations of sodium arsenite plus cadmium chloride.

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1. Introduction

Heat shock protein (HSP) synthesis in response to elevated temperature or exposure to chemical stressors, such as sodium arsenite or cadmium chloride, is an almost universal phenomenon of prokaryotic and eukaryotic cells (Feige et al., 1996; Morimoto, 1998, 2008; Heikkila, 2010). HSPs have been classified, primarily by size, into small HSPs (sHSPs), HSP40, HSP60, HSP70 and HSP90. They can be expressed constitutively, strictly stress-inducible or constitutive and stress-inducible. Furthermore, HSP accumulation patterns can differ between tissues, developmental stages or species. Under normal conditions, HSPs are involved in protein folding and translocation. During stress conditions both constitutive and stress-inducible HSPs act as molecular chaperones to bind denatured proteins, suppress their aggregation or misfolding and aid in their refolding after resumption of normal conditions. sHSPs are evolutionarily divergent except for an α-crystallin domain, a conserved 80–100 amino acid region near the C-terminus (Arrigo, 1998; MacRae, 2000; Van Montfort et al., 2001). The sHSP family forms multimeric structures, which are essential for their molecular chaperone function in vivo. A number of cellular functions have been proposed for sHSPs including resistance against apoptosis, actin capping/decapping activity, acquisition of thermotolerance, cellular differentiation and modulation of redox parameters. Also, synthesis or mutation of sHSPs has been associated with desmin-related muscle myopathy, Parkinson's and Alzheimer's diseases (Sun and MacRae, 2005). One of the most intensively investigated HSPs is cytoplasmic HSP70, an evolutionarily conserved stress-inducible HSP70 family member that inhibits, in an ATP-dependent manner, the aggregation of unfolded protein as well as facilitating protein folding, protein translocation across cell membranes and degradation of damaged protein (Katschinski, 2004; Daugaard et al., 2007).

Stress-induced accumulation of HSPs, which is regulated, at least in part, at the transcriptional level, is mediated by the interaction of the *trans*-acting regulatory factor, heat shock factor 1 (HSF1), and the heat shock element (HSE) found in the promoter regions of *hsp* genes. Normally HSF1 is present as an inactive monomer bound to HSP90. In response to heat shock, which causes an increase in the cellular levels of denatured protein, HSP90 is recruited to inhibit their aggregation permitting HSF1 monomers to form hyperphosphorylated active trimers that relocate to the nucleus, bind to the HSE, and facilitate the transcription of *hsp* genes by RNA polymerase II (Morimoto, 1998; Voellmy, 2004; Gidalevitz et al., 2011). Similarly, with chemical stressors such as sodium arsenite or cadmium chloride, it was suggested that the accumulation of damaged or misfolded protein within the cell leads to HSF1 activation and the expression of *hsp* genes (Zou et al., 1998; Voellmy, 2004).

Sodium arsenite and cadmium chloride are two widespread environmental contaminants that have been associated with various forms of cancer in humans as well as causing cellular injury in a number of organs including kidney, liver and reproductive tissues (Del Razo et al., 2001; Waisberg et al., 2003; Barbier et al., 2004). At the cellular

* Corresponding author at: Department of Biology, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada. Tel.: +1 519 885 1211x33076; fax: +1 519 746 0614.
E-mail address: heikkila@uwaterloo.ca (J.J. Heikkila).

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level, sodium arsenite and cadmium chloride can cause cell cycle arrest, disruption of the cytoskeleton, oxidative stress, apoptosis, dysregulation of gene expression, DNA damage and the accumulation of denatured and/or abnormal protein (Chou, 1989; Del Razo et al., 2001; Waisberg et al., 2003; Blechinger et al., 2007; Mendez-Armenta and Rios, 2007; Mouchet et al., 2007; Woolfson and Heikkila, 2009). Amphibians, like other aquatic animals, are sensitive to the presence of sodium arsenite or cadmium chloride in their habitat. For example, during growth and development of the frog, *Xenopus laevis*, cadmium was observed to have toxic and genotoxic effects including thyroid gland activity, ocular anomalies, bent notochord and heart, kidney, ovary and gut dysplasia (Sunderman et al., 1991; Plowman et al., 1994; Mouchet et al., 2006; Sharma and Patino, 2008). Additionally, sodium arsenite caused malformations during *Xenopus* development, altered thyroid hormone-induced tail resorption during metamorphosis and induced alterations in F-actin cytoskeletal structure in cultured cells (Gornati et al., 2002; Gellalchew and Heikkila, 2005; Davey et al., 2008). Finally, treatment of *Xenopus* cells with either sodium arsenite or cadmium chloride induced the accumulation of HSP30, a small HSP family, HSP70, and ubiquitinated protein, which was indicative of proteasomal inhibition (Darasch et al., 1988; Ali et al., 1996; Beasley et al., 1997; Gordon et al., 1997; Lang et al., 2000; Gornati et al., 2002; Fernando et al., 2003; Heikkila, 2004; Gellalchew and Heikkila, 2005; Gauley and Heikkila, 2006; Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009; Young et al., 2009; Heikkila, 2010; Brunt et al., 2012).

While the aforementioned studies are useful in the analysis of the cellular and molecular responses to single stressors, it is important to note that amphibians in their habitat may encounter multiple stressors simultaneously. Previously, we reported that treatment of *Xenopus* cells with either cadmium chloride or sodium arsenite plus a concurrent mild heat shock resulted in an enhanced accumulation of HSPs that was greater than found with the sum of the individual stressors (Woolfson and Heikkila, 2009; Young et al., 2009). These responses were regulated, at least in part, at the level of *hsp* gene transcription, since pretreatment of cells with an HSF1 activation inhibitor, KNK437, repressed HSP accumulation. While a mild heat shock can potentiate the effect of either sodium arsenite or cadmium chloride, individually, no information, to the best of our knowledge, is available on the effect of these two chemical stressors simultaneously on HSP accumulation in aquatic animals. However, two studies were carried out with mammalian cultured cells. In a qualitative study, it was determined that simultaneous treatment of rat kidney cells with sodium arsenite and cadmium chloride produced dose-dependent increases in HSP70 levels at 10-fold lower concentrations than observed with the individual chemicals alone (Madden et al. 2002). Furthermore, equimolar solutions of cadmium chloride plus sodium arsenite induced strong accumulations of HSP70 and HSP25 in mouse cells compared to the stressors individually (Eichler et al. 2005).

In the present study, we investigated the effect of simultaneous sodium arsenite and cadmium chloride treatment of *X. laevis* A6 kidney epithelial cells on HSP30, HSP70 and ubiquitinated protein accumulation by immunoblot analysis. Additionally, we determined the effect of KNK437, an HSF1 inhibitor, on HSP30 and HSP70 accumulation in cells treated simultaneously with sodium arsenite and cadmium chloride. Immunocytochemistry permitted an analysis of the intracellular localization of HSP30 and F-actin structure in cells subjected to the combined stressors. Finally, we examined the effect of concurrent mild heat shock, sodium arsenite and cadmium chloride treatment on the accumulation of HSP30 and HSP70.

2. Materials and methods

2.1. Maintenance and treatment of cultured cells

X. laevis A6 kidney epithelial cells (CCL-102; American Type Culture Collection, Rockville, MD, USA) were grown at 22 °C in 70% Leibovitz L-15 media containing 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Sigma-Aldrich, Oakville ON, Canada) in T75 cm^2 BD Falcon tissue culture flasks (VWR International, Mississauga, ON, Canada). Flasks containing A6 cells were treated with 1, 2.5, 5, or 10 μM sodium arsenite (Sigma-Aldrich; stock solution of 100 mM was dissolved in sterile water) or 10, 50 or 100 μM cadmium chloride (Sigma-Aldrich; stock solution of 100 mM was dissolved in sterile water) for various periods of time ranging from 4 to 24 h at 22 or 30 °C. Additionally, some cells were treated with 30 μM MG132 (carbobenzoxy–L-leucyl–L-leucyl–L-leucinal) (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) at 22 °C, as a positive control for proteasomal inhibition. In HSF1 inhibitor studies, cells were pretreated with 100 μM of KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam (dissolved in DMSO); Calbiochem, Gibbstown, NJ, USA), for 6 h at 22 °C before stressor treatments. KNK437 was left in these culture flasks during the experiments (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008). Following the various treatments, cells were rinsed using 65% Hanks balanced salt solutions (HBSS) and removed by means of scraping in 1 mL of 100% HBSS. These cells were transferred to a 1.5 mL microfuge and collected via centrifugation at 16,000 g for one min. The pellets were then frozen and stored at −80 °C.

2.2. Protein isolation and immunoblot analysis

The protocol for the isolation of total protein from A6 cells was previously described (Young et al., 2009). Protein concentrations were obtained by means of a bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Pierce, Rockford, IL, USA). SDS-PAGE was executed using a BioRad Mini Protean III gel system (BioRad, Mississauga, ON, Canada) with 10% (for ubiquitinated protein) and 12% acrylamide gels. Proteins were then transferred to nitrocellulose membranes (BioRad) by means of a Trans-Blot Semi-dry Transfer Cell (BioRad). Staining the membrane with Ponceau-S (Sigma-Aldrich) ensured equal loading and efficiency of transfer for each sample. Immunodetection was performed using either the polyclonal rabbit anti-*Xenopus* HSP30 (Fernando and Heikkila, 2000; 1:1000 dilution), the polyclonal rabbit anti-*Xenopus* HSP70 (Gauley et al., 2008; 1:350 dilution), the polyclonal rabbit anti-actin antibody (Sigma-Aldrich; 1:200) or the mouse anti-ubiquitin antibody (Invitrogen Molecular Probes, Carlsbad, CA, USA; 1:150 dilution). Membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (BioRad) at a 1:3000 dilution, or goat anti-mouse secondary antibody at a 1:1000 dilution. Finally, membranes were immersed in alkaline phosphatase detection buffer (50 mM Tris, 50 mM NaCl, 25 mM $MgCl_2$, pH 9.5) with 0.33% nitroblue tetrazolium chloride and 0.17% 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt. Densitometric analyses (within the range of linearity) of the immunoblots were performed using ImageJ (1.44q) Java-based image processing software obtained from the National Institutes of Health. Most experiments were repeated at least 3 times, with the densitometric values being expressed as a percentage of the maximum signal within a particular experiment unless otherwise indicated. Statistical analysis was performed on normalized data using analysis of variance followed by Tukey's multiple comparison post-test to determine if statistically significant differences existed between samples.

2.3. Immunocytochemistry and laser scanning confocal microscopy

A6 cells were grown on base-washed (0.2 M NaOH solution), flame sterilized glass coverslips in sterile petri plates for 24 to 48 h at 22 °C. A6 cells were then incubated with 10 μM sodium arsenite, 10 μM, 50 μM or 100 μM cadmium chloride or 10 μM sodium arsenite combined with 10 μM, 50 μM or 100 μM cadmium chloride at 22 °C for 16 h. Following these treatments, cells were rinsed with phosphate buffered saline (PBS) and fixed in 3.7% paraformaldehyde (BDH, Toronto, ON, Canada) for 15 min and then rinsed 3 times with PBS. The A6 cells were then

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