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Simultaneous exposure of *Xenopus* A6 kidney epithelial cells to concurrent mild sodium arsenite and heat stress results in enhanced *hsp30* and *hsp70* gene expression and the acquisition of thermotolerance

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ABSTRACT

In this study, we examined the effect of concurrent low concentrations of sodium arsenite and mild heat shock temperatures on *hsp30* and *hsp70* gene expression in *Xenopus* A6 kidney epithelial cells. RNA blot hybridization and immunoblot analysis revealed that exposure of A6 cells to 1–10 μM sodium arsenite at a mild heat shock temperature of 30 °C enhanced *hsp30* and *hsp70* gene expression to a much greater extent than found with either stress individually. In cells treated simultaneously with 10 μM sodium arsenite and different heat shock temperatures, enhanced accumulation of HSP30 and HSP70 protein was first detected at 26 °C with larger responses at 28 and 30 °C. HSF1 activity was involved in combined stress-induced *hsp* gene expression since the HSF1 activation inhibitor, KNK437, inhibited HSP30 and HSP70 accumulation. Immunocytochemical analysis revealed that HSP30 was present in a granular pattern primarily in the cytoplasm in cells treated simultaneously with both stresses. Finally, prior exposure of A6 cells to concurrent sodium arsenite (10 μM) and heat shock (30 °C) treatment conferred thermotolerance since it protected them against a subsequent thermal challenge (37 °C). Acquired thermotolerance was not observed with cells treated with the two mild stresses individually.

1. Introduction

Heat shock proteins (HSPs) are molecular chaperones that are involved in the folding and translocation of cellular proteins (Feige et al., 1996; Morimoto, 1998; Westerheide and Morimoto, 2005; Morimoto, 2008). During stress conditions, such as heat shock, both constitutive and stress-inducible HSPs bind to and inhibit irreversible aggregation or misfolding of denatured protein and then facilitate their refolding once normal cellular conditions have been re-established. Stress-inducible *hsp* gene expression involves the activation of heat shock factor 1 (HSF1) which interacts with the heat shock element (HSE) found in the 5′ upstream regions of *hsp* genes (Feige et al., 1996; Morimoto, 1998; Katschinski, 2004; Voellmy, 2004). HSF1, which preexists in the cell as an inactive monomer, forms a trimer upon heat or chemical stress enabling it to bind to the HSE and facilitate *hsp* gene transcription. The activation of HSF1 appears to be initiated by the presence of unfolded, misfolded or damaged protein (Voellmy, 2004).

There are six main families of HSPs, grouped according to size, which include HSP100, HSP90, HSP70, HSP60, HSP40, and the small HSPs (sHSPs) (Feige et al., 1996; Morimoto, 1998, 2008). The HSP70 family includes the cytoplasmic stress-inducible HSP70 which has the ability to act as a molecular chaperone to protect unfolded proteins from aggregation as well as assisting their folding into a correct functional conformation (Morimoto et al., 1994; Feige et al., 1996; Morimoto, 1998, 2008). The sHSPs (16–42 kDa) are quite divergent except for a conserved α-crystallin domain consisting of 80–100 amino acids (Buchner et al., 1998; MacRae, 2000; Van Montfort et al., 2002; Heikkila, 2004). SHSPs can form highly polymeric structures that may be necessary for function within the cell. In vivo functions that have been proposed for sHSPs include the acquisition of thermotolerance, resistance against apoptosis, actin capping/decapping activity, cellular differentiation, and modulation of redox parameters (Arrigo, 1998; MacRae, 2000; Van Montfort et al., 2002). Synthesis or mutation of sHSPs have been associated with a variety of diseases including muscle myopathy, cataracts, multiple sclerosis, Alzheimer's disease and a number of other neuropathologies (Quinlan and Van Den Ijssel, 1999; Bova et al., 1999; Irobi et al., 2004).

Sodium arsenite and other arsenic compounds can induce the accumulation of HSPs in eukaryotic cells (reviewed in Del Razo et al., 2001). Arsenic is a toxic environmental pollutant that results in various renal, cardiovascular, and hepatic diseases including cancer (Del Razo et al., 2001). It has been estimated that 100 million people worldwide have an elevated cancer risk due to the presence of arsenic contamination in drinking water. At the cellular level, arsenic was reported to cause cell cycle arrest, cytoskeletal injury, and apoptosis (Chou, 1989; Li and Chou, 1992; Liu et al., 2001; Bode and Dong, 2002;

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Del Razo et al., 2001). Arsenic can also induce oxidative damage of cellular protein since it generates reactive oxygen species and free radicals like hydrogen peroxide (Del Razo et al., 2001). It is likely that the accumulation of damaged protein produced by sodium arsenite leads to the activation of HSF and the expression of *hsp* genes (Zou et al., 1998; Voellmy, 2004).

Most of the studies that examined sodium arsenite-induced *hsp* gene expression have focused on the effect of this stressor, alone, at normal culture temperatures. However, organisms or tissues may encounter multiple stressors simultaneously (e.g. sodium arsenite plus elevated temperature, heavy metals, or chemical agents). In rat kidney cells, simultaneous treatment with arsenic and cadmium produced elevated levels of HSP70 at approximately 10-fold lower doses than single element exposures alone, although this expression was dose-dependent (Madden et al., 2002). Also, in C6 rat glioma cells, sodium arsenite-induced accumulation of HSP27 and αB crystallin was markedly enhanced by concurrent treatment with diethyl maleate or buthionine sulfoximine, agents that lower cellular levels of glutathione (Ito et al., 1998). Previously, in a preliminary report, we found that the simultaneous treatment of *Xenopus* A6 cells with a low concentration of sodium arsenite plus a mild heat shock resulted in enhanced *hsp* gene expression that was much greater than found with each stress individually (Heikkila et al., 1987).

In general, the study of the effect of simultaneous multiple stresses on *hsp* gene expression has been quite limited. In mammalian cultured cells, enhanced *hsp* gene expression with dual stresses compared to the stressors individually was reported with ethanol and heat shock in lymphocytes, iron and aluminum in neural cells, bimoclomol and heat shock in NIH 3T3 cells as well as acidic pH and NaCl in kidney cells (Rodenheiser et al., 1986; Alexandrov et al., 2005; Neuhofer et al., 1999; Torok et al., 2003). In aquatic animals, which are particularly susceptible to multiple environmental toxicants or stresses, potentiated *hsp* gene expression was described with the following concurrent stresses: heat shock and non-ionic pollutants in freshwater sponge; wound stress and hypoxia in sea star; pesticide and viral infection in salmon and elevated temperature plus herbimycin A, hydrogen peroxide or cadmium chloride in *Xenopus* kidney cells (Muller et al., 1995; Briant et al., 1997; Muller et al., 2004; Eder et al., 2007; Holm et al., 2008; Woolfson and Heikkila, 2009).

Additional insight into our understanding of multiple stressors on *hsp* gene expression is of importance given the considerable interest in the modulation of the heat shock response in the potential treatment of human diseases of protein conformation (Westerheide and Morimoto, 2005). In the present study, we have fully characterized *hsp30* and *hsp70* gene expression in *Xenopus laevis* A6 kidney epithelial cells exposed simultaneously to various low sodium arsenite concentrations and different mild heat shock temperatures. The present research builds on a preliminary study, which examined only a single set of stress parameters on *hsp* gene expression in A6 cells (Heikkila et al., 1987). In the present study, we have also employed immunocytochemical analysis to monitor the intracellular localization of HSP30 induced by the combined stresses. Additionally, we examined whether the HSF1 activation inhibitor, KNK437, inhibited the accumulation of HSP30 and HSP70 in A6 cells exposed simultaneously to mild sodium arsenite and heat shock treatment. Finally, we compared the ability of concurrent mild sodium arsenite and heat shock treatment to induce thermotolerance in A6 cells.

2. Materials and methods

2.1. Maintenance and treatment of X. laevis A6 kidney epithelial cells

A6 cells (CCL-102; American Type Culture Collection) were grown at 22 °C in 55% 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 of A6 cells, destined for protein or mRNA analysis, were immersed in water baths maintained at temperatures of 26, 28, 30 or 33 °C for 1.5 to 24 h. After the heat shock treatments, A6 cells used for protein analysis were placed at 22 °C for 2 h before cell harvesting. Accuracy of the heat shock temperatures was verified by means of a Traceable digital thermometer (Model No. 15-077-8; Fisher Scientific, Ottawa, ON, Canada). A6 cells were also treated with sodium arsenite at concentrations of 1, 5, 7.5 or 10 μM (stock solution of 10 mM was dissolved in sterile water) at 22, 26, 28 or 30 °C for periods of time ranging from 1.5 to 24 h followed by a 2 h recovery period at 22 °C in the absence of sodium arsenite. Following the different treatments, cells were rinsed with 65% Hanks balanced salt solution (HBSS; Sigma) followed by the addition of 1 mL of 100% HBSS. Cells were removed using a rubber scraper, transferred to a 1.5 mL microfuge tube, and pelleted by centrifugation for 1 min at 16,000 *g* in a microcentrifuge. Harvested A6 cell pellets were frozen and stored at −80 °C.

2.2. RNA isolation and northern blot analysis

RNA was isolated from A6 cells using the QIAgen RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) as detailed in the manufacturer's instructions. RNA concentration and integrity were assessed by spectrophotometry and electrophoresis with ethidium bromide staining. For northern blot analysis, total RNA was electrophoresed on 1.2% (w/v) formaldehyde/agarose gels (Lang et al., 1999; Sambrook and Russell, 2001) and then transferred onto a positively charged nylon membrane (Roche Diagnostics, Laval, QC, Canada). RNA was UV cross-linked to the membrane with an ultraviolet cross-linker (UltraLum Inc., Claremont, CA, USA). Equal loading and quality of transfer of the RNA were confirmed by staining the membrane with 1× Reversible Blot Stain (Sigma-Aldrich). Preparation of digoxigenin (DIG)-labeled antisense *hsp30* and *hsp70* riboprobes was described previously (Lang et al., 1999, 2000). The RNA blot pre-hybridization, hybridization, and washing procedures were outlined in Lang et al. (1999). Chemiluminescent detection was performed according to the manufacturer's instructions (Roche Diagnostics, Mississauga, ON, Canada). Images were visualized using a DNR chemiluminescent imager (DNR Bio-Imaging Systems Ltd., QC, Canada). Densitometric analyses within the range of linearity were conducted using Image J (Version 1.38) software on the data obtained from at least 3 separate experiments.

2.3. Protein isolation and immunoblot analysis

A6 cell total protein isolation was performed as described by Phang et al. (1999). Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). SDS-PAGE was performed using a BioRad Mini Protean III gel system (BioRad, Mississauga, ON, Canada) and 12% polyacrylamide gels. Protein samples were transferred to nitrocellulose membranes (BioRad) using a Trans-Blot Semi-dry Transfer Cell (BioRad). Equal loading and efficiency of transfer for each sample were determined by staining the membrane with Ponceau-S (Sigma-Aldrich). Immunodetection was carried out using either the polyclonal rabbit anti-*Xenopus* HSP30 antibody (Fernando and Heikkila, 2000; 1:2000 dilution) or the polyclonal rabbit anti-*Xenopus* HSP70 antibody (Gauley et al., 2008; 1:150 dilution). Membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (BioRad; 1:3000 dilution). For detection, the blots 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 of appropriately stained blots to maintain the signals within