Examination of sodium arsenite- and cadmium chloride-induced HSP accumulation and inhibition of proteasome activity in *Xenopus laevis* A6 kidney epithelial cells

by

Jara Brunt

A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Master of Science
in
Biology

Waterloo, Ontario, Canada, 2011

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

Sodium arsenite (NA) and cadmium chloride ($CdCl_2$) are two relatively abundant environmental toxicants that have numerous detrimental effects on living organisms. At the cellular level, NA and $CdCl_2$ produce reactive oxygen species which cause protein damage. Recent studies, in mammalian systems, have suggested that NA and $CdCl_2$ can inhibit the ubiquitin-proteasome system (UPS). The UPS is the major degradation route for the elimination of damaged protein. This process involves two successive steps: the addition of ubiquitin to damaged protein and subsequent degradation by the 26S proteasome. Previously our laboratory determined that inhibition of the UPS can induce the accumulation of heat shock protein (HSPs) in *Xenopus laevis* A6 cells. HSPs are molecular chaperones, which bind to unfolded proteins to prevent aggregation and assist in protein refolding. The goal of this study was to examine the effect of NA and $CdCl_2$ on the UPS of *Xenopus laevis* cells and to investigate any possible association between HSP accumulation and proteasomal activity. In the present study, treatment of A6 cells with NA or $CdCl_2$ caused an increase in HSP30 and HSP70 accumulation, as well as in protein ubiquitination. In fact, treatment with 30 μM NA or 200 μM $CdCl_2$ resulted in a 1.7- and 2-fold increase, respectively, in the relative levels of ubiquitinated protein compared to control. Examination of the relative levels of ubiquitinated protein over time revealed significant increases in cells treated up to at least 24 h after exposure of A6 cells with 30 μM NA or 200 μM $CdCl_2$. To further examine the effect of NA and $CdCl_2$ on proteasome activity, a cell-based assay measuring proteasomal chymotrypsin (CT)-like activity was employed. Treatment with 20 or 30 μM NA caused a 40 % decrease in the relative levels of chymotrypsin (CT)-like activity in A6 cells compared to control

cells. The CT-like activity of A6 cells treated with 100 or 200 μM $CdCl_2$ decreased by 40 % and 75 %, respectively, compared to control. The increase in ubiquitinated protein and a decrease in CT-like activity suggest that NA and $CdCl_2$ can inhibit the UPS in A6 cells. In order to examine any possible association between HSP accumulation and proteasome activity an inhibitor of HSP accumulation, KNK437, was employed. In A6 cells pretreated with KNK437 followed by NA or $CdCl_2$ exposure a decrease in the relative levels of HSP30 and HSP70 and ubiquitinated protein was noted compared to cells treated with NA alone. However, the CT-like activity of cells pretreated with KNK437 prior to NA or $CdCl_2$ showed no significant difference compared to cells treated with NA or $CdCl_2$ without pretreatment. The findings of this study suggest that NA and $CdCl_2$ inhibit proteasomal activity in A6 cells and that there is a possible association between HSP accumulation and the mechanism by which damaged proteins are ubiquitinated.

Acknowledgements

First and foremost I want to thank my supervisor Dr. Heikkila for giving me this opportunity. I am very grateful for your guidance, patience, experience and support throughout the completion of my degree. I have learned so much over the last two years and have become a more independent scientist. I would also like to thank my committee members Dr. Glick, Dr. Marsden and Dr. B. Dixon for their time and comments. I would like to thank Dale Weber for his help with confocal microscopy. My years in the Heikkila lab would not have been as enjoyable if not for the lifelong friends I met along the way. To Saad, Ashley, AJ, Shantel, Imran, Danny and Charles thanks for the memories and for helping me along the way. Last but not least I want to thank my family and friends for their encouragement and support throughout my degree.

Table of Contents

List of Figures

List of Abbreviations

ANOVA	Analysis of variance
APS	Ammonium persulfate
ATCC	American type culture collection
BCA	Bicinchonic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BiP	Immunoglobulin-binding protein
BSA	Bovine serum albumin
C	Control
$CdCl_2$	Cadmium chloride
CHIP	Carboxyl terminus of Hsc70-interacting protein
CT	Chymotrypsin
DAPI	4,6-diamidino-2-phenylindole
DBD	DNA binding domain
DMSO	Dimethylsulphoxide
EDTA	Ethylene-diamine-tetraacetic acid
FBS	Fetal bovine serum
GRP78	Glucose-regulated protein 78
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIP	HSP70 interacting protein
HOP	HSP70-HSP90 organizing protein
HR	Hydrophobic repeat
HSC	Heat shock cognate
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
hsp	Heat shock protein gene
HSR	Heat shock response

KNK437	N-formyl-3, 4-methylenedioxy-benzylidene-γ-butyrolactam
L-15	Leibovitz-15 media
LSCM	Laser scanning confocal microscopy
MG132	Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal
MHC	Major histocompatibility complex
NA	Sodium arsenite
NBT	4-nitro blue tetrazolium
PBS	Phosphate buffered saline
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sHSP	Small heat shock protein
siRNA	Small interfering RNA
Suc-LLVY-aminoluciferin	Suc-Leu-Leu-Val-Tyr-aminoluciferin
TBS-T	Tris buffered saline solution – Tween 20
TEMED	tetramethylethylenediamine
Tris buffer	Tris(hydroxymetyl)aminomethane
TRITC	Rhodamine-tetramethylrhodamine-5-isothiocyanate phalloidin
UPS	Ubiquitin proteasome system

1. Introduction

Stress can have adverse effects on organisms, tissues and cells. At the cellular level, stress, both environmental and physiological, can cause protein to unfold, misfold and aggregate which can cause a disruption of normal function. In response to stress, cells have protective mechanisms which involve a change in gene expression which includes increased transcription of heat shock protein genes (*hsp)* and proteasomal degradation (Morimoto, 2008).

1.1 Heat Shock Proteins

Heat shock proteins (HSPs) are a large group of molecular chaperones that were first discovered in 1962 by Ritossa in the salivary glands of *Drosophila*. HSPs are expressed in response to a number of signals such as environmental stressors, non-stress conditions, such as growth and development, pathophysiological state and disease states (Morimoto, 1998; 2008). As molecular chaperones, HSPs play an important role in regulating protein conformation by binding to unfolded or abnormal proteins maintaining them in a folding competent state until normal physiological conditions return and refolding can occur (Morimoto, 1998; 2008). Furthermore, HSPs are involved in assembly and disassembly of macromolecular complexes, regulating translocation and facilitating posttranslational folding (Morimoto, 2008). Some HSPs are constitutively expressed, while others are inducible. There are six families of HSPs based on molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSPs (sHSPs).

1.2 Heat Shock Protein 70

HSP70 molecular chaperones are a highly conserved and abundant family involved in regulating protein folding during normal and stress conditions (Katschinski,

2004). In eukaryotic cells, the HSP70 family includes cytoplasmic stress-inducible HSP70, cytoplasmic constitutively expressed heat shock cognate 70 (HSC70), mitochondrial HSP70 and immunoglobulin-binding protein (BiP; also referred to as glucose-regulated protein 78 or GRP78), which is found in the endoplasmic reticulum (Morimoto, 1998; 2008). HSP70 proteins have highly conserved amino acid sequences and are found in every organism in multiple cellular components (Daugaard et al., 2007). The HSP70 domain structures include an ATPase domain, a region with protease sensitive sites, a peptide binding domain and C-terminal region that is G/P-rich (Daugaard et al., 2007). The C-terminal region contains an EEVD-motif which enables HSP70 to bind other HSPs and co-chaperones and is required for chaperone function (Daugaard et al., 2007). Co-chaperones such as Bag-1, carboxyl terminus of Hsc70-interacting protein (CHIP), HSP40/DnaJ, HSP70 interacting protein (HIP) and HSP70-HSP90 organizing protein (HOP) bind to HSP70 to regulate its activity and accomplish its diverse functions including the protection of cells from aggregation of unfolded protein and assist in the folding or refolding of these proteins (Boorstein et al., 1994; Katschinski, 2004). In addition, HSP70 proteins have been shown to inhibit apoptosis and are involved in the degradation of abnormal or misfolded protein, translocation of proteins between cellular compartments and protein complex degradation (Mosser et al., 2000; Katschinski, 2004; Daugaard et al., 2007).

1.3 Small Heat Shock Proteins

The sHSPs are a group of molecular chaperones which range in size from 10 to 42 kDa. They are a highly divergent family with a conserved α-crystallin domain of 80-100 amino acids and are found in nearly every cellular compartment in almost every organism

(Franck et al., 2004; Nakamoto and Vigh, 2007). SHSPs are involved in numerous cellular processes such as cellular differentiation, chaperone activity, actin capping/decapping and modulation of redox perameters (MacRae, 2000; Van Montfort et al., 2001). SHSPs have the ability to aggregate into large, highly polymeric structures that are necessary for function within the cell (Arrigo and Landry, 1994; MacRae, 2000; Van Montfort et al., 2001; Heikkila, 2004).

Although the sHSPs are a diverse group of stress proteins, most contain two or three functional domains. The first domain is the previously mentioned α-crystallin domain located near the carboxy-terminus. This domain consists of β–strands organized into β–sheets and is responsible for dimer formation (Kim et al., 1998, van Montfort et al., 2001). The amino-terminal is thought to be involved in oligomerization while the carboxy-terminal extension stabilizes oligomers and is essential for chaperone function (MacRae, 2000; Fernando and Heikkila, 2000). Studies indicate that the carboxyl region is required to inhibit stress-induced aggregation and to maintain secondary structure for chaperone function (Fernando and Heikkila, 2000).

Most sHSPs assemble into large oligomeric complexes that are between 9 and 30 subunits in size (Kim et al., 1998; Nakamoto and Vigh, 2007). These oligomeric complexes, and the dissociation of them, are necessary for the chaperone function of sHSPs and are important in their regulation (van Montfort et al., 2001; Stromer et al., 2003; Nakamoto and Vigh, 2007). As molecular chaperones, sHSP bind with non-native proteins, holding them in a folding competent state, in order to prevent their aggregation. Once the stress has been removed, sHSPs associate with ATP-dependent HSPs, like HSP70, which will assist in refolding the protein (MacRae, 2000). Phosphorylation

removes sHSPs from the complex therefore changing the secondary structure and inhibiting oligomerization (Fernando et al., 2003).

1.4 Heat Shock Response

The heat shock response, also known as the stress response, is a protective mechanism that functions to initiate recovery and adaptation to stress (Morimoto, 2008). Regulation of stress-induced *hsp* gene expression in eukaryotes is controlled primarily at the level of transcription by heat shock factor (HSF1). Evidence of HSF1 involvement in HSP accumulation was shown using small interfering RNA (siRNA) to knockdown HSF1. This knockdown caused a decrease in HSP70 and HSP40 accumulation (Song et al., 2010). HSF1 is a member of the family of transcription factors called HSFs, which also includes HSF2, HSF3 and HSF4, and specifically binds to heat shock elements (HSEs) in the promoters of *hsp* genes. A single HSF protein is expressed in lower eukaryotes such as *Drosophila, Caenorhabditis elegans* and yeast (Shamovsky & Nudler, 2008). *Xenopus laevis* expresses at least two distinct HSFs, mammalian cells have three different HSFs while plants have been shown to possess more than twenty unique HSF proteins (Shamovsky & Nudler, 2008). HSF1 is the stress-responsive member of the HSF family and is activated by a number of stressors that cause the accumulation of unfolded protein including heat shock, sodium arsenite (NA) and cadmium chloride ($CdCl_2$) treatment (Voellmy, 2004; Morimoto, 2008).

HSF1 is a member of the family of "winged" helix-turn-helix transcription factors (Shamovsky & Nudler, 2008). There is little homology between different species, but the domain organization is conserved. The N-terminal DNA binding domain (DBD) is followed by an oligomerization domain, which contains two hydrophobic repeat domains

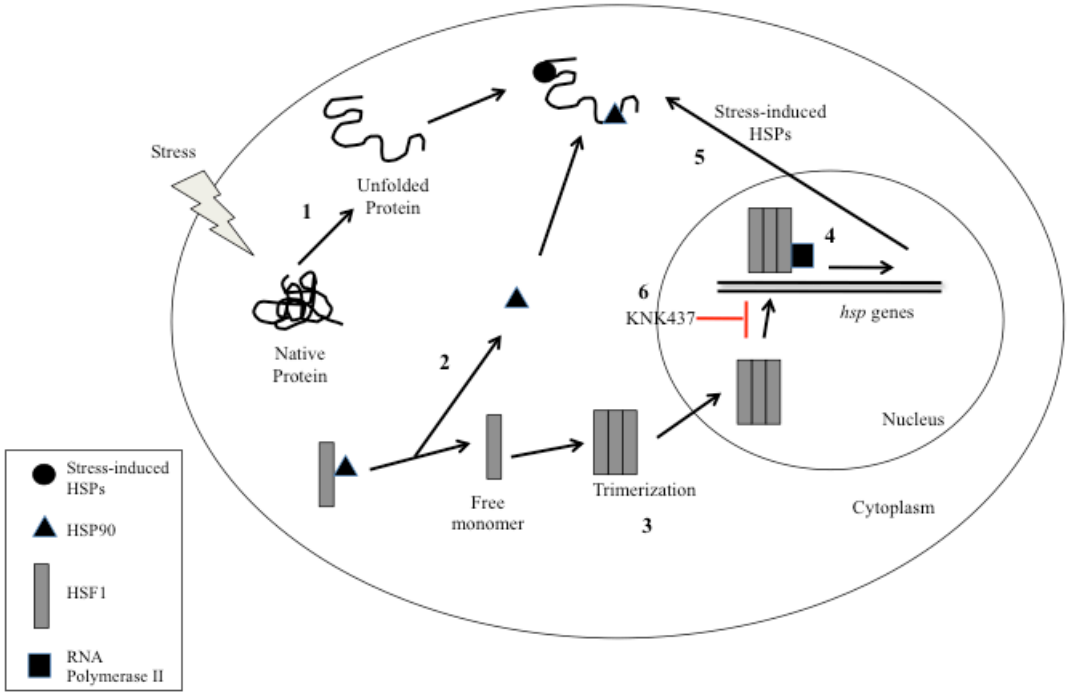
(HR-A/B), and by a regulatory domain. An additional hydrophobic repeat, HR-C, and a transactivation domain are located further downstream (Shamovsky & Nudler, 2008).

The DBD is the region of HSF1 that interacts with the HSE sequences in the promoters of *hsp* genes (Voellmy, 2004). The hydrophobic repeats of the oligomerization domain and HR-C at the C-terminal are leucine zipper domains. Intramolecular interactions of HR-A and HR-B with HR-C maintain HSF1 in a monomeric form by suppressing trimerization (necessary for binding to HSE). Upon cellular stress, the intramolecular interactions maintaining HSF1 in its monomeric state are disrupted allowing the newly former trimer to bind to the HSE (Rabindran et al., 1993; Shamovsky and Nudler, 2008). The regulatory domain is the location for both constitutive and inducible serine phosphorylation and regulates the transactivation domain which is located at the C-terminal (Pirkkala, 2001).

As summarized in Figure 1, inactive HSF1 is found in the cytoplasm as a monomer bound to HSP90. Under stressful conditions, HSF1 trimerizes, translocates to the nucleus and binds to the HSE in the 5' promoter region of these genes and facilitates transcription of *hsp* genes by RNA polymerase II (Feige et al., 1996; Voellmy, 2004; Heikkila, 2010). Once these proteins are produced they bind to unfolded and denatured proteins and maintain them in a folding competent state until the stress is no longer present. Following the removal of the stressor, the cell stops producing excess HSPs and their levels return to pre-stress conditions (Heikkila, 2003).

The HSE is conserved among all eukaryotes and is composed of multiple inverted repeats of the five nucleotide motif nGAAn (Amin et al., 1988, Xiao & Lis, 1988; Schuetz et al., 1991). This motif is considered the fundamental unit of recognition and

Figure 1. Stress-induced activation of HSR. (1) External stressful stimuli cause native proteins in the cell to unfold. (2) HSP90 is bound to HSF1 under normal conditions in the cell and upon stress is recruited to prevent aggregation of unfolded proteins. (3) This allows HSF1 monomers to trimerize and translocate to the nucleus. (4) The HSF1 trimer binds to the heat shock element at the 5' promoter of *hsp* genes initiating transcription by RNA polymerase II. (5) Stress-induced HSPs are recruited to the unfolded protein to maintain it in a folding competent state. (6) KNK437 inhibits the activation of HSF1.


Stress
Unfolded Protein
1
Native Protein
2
Free monomer
Trimerization
3
4
5
Stress-induced HSPs
6
KNK437
hsp genes
Nucleus
Cytoplasm
Stress-induced HSPs
HSP90
HSF1
RNA Polymerase II

each subunit of HSF trimer interacts with one of three nGAAn repeats within the complete HSE (Perisic et al., 1989). At least one HSE is present within all promoters of stress-inducible *hsp* genes, mediating the response to heat shock as well as other stressors, such as agents that alter protein conformation (Mosser et al., 1990; Shamovsky and Nudler, 2008).

KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam) is a benzylidene lactam compound that has been used to inhibit HSP accumulation (Yokota et al., 2000). Ohnishi et al. (2004) determined that KNK437 suppresses binding of HSF1 to the HSE which is required for *hsp* gene expression. The effects of KNK437 on HSP accumulation are similar to results found in studies employing siRNA for HSF1. For example, Song et al. (2010) showed a similar decrease in HSP70 and HSP40 accumulation when either KNK437 or HSF1-siRNA was used. In previous studies, our laboratory has shown that in *Xenopus laevis* A6 cells, KNK437 inhibits *hsp* gene expression induced by heat shock, NA, $CdCl_2$, herbimycin A, MG132, celastrol and curcumin (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008; Walcott and Heikkila, 2010; Khan and Heikkila, 2011). The inhibition of HSP accumulation by KNK437 was also shown in human cells, as was a suppression of the acquisition of thermotolerance in cells (Yokota et al., 2000; Ohnishi et al., 2004)

1.5 *Xenopus laevis*

The South African clawed frog, *Xenopus laevis,* is an entirely aquatic organism that has been used in developmental research for numerous years. A great deal of information on eukaryotic cell biology and the regulation of gene expression has been obtained from this system. Furthermore, *Xenopus* cultured cells are available as

amphibian model systems including the A6 kidney epithelial cell line which was developed by Rafferty in 1969. This continuous cell line is easy to culture and has quick cell generation and growth. It has been used for numerous studies including examination of proliferation, differentiation and signal transduction pathways, and the regulation of epithelial sodium channels, to name a few (Matsumoto et al., 1993; Tanaka et al., 2003; Guerra et al., 2004; Bjerregaard, 2007; Yu et al., 2008).

1.5.1 *Xenopus* HSP70

Bienz (1984) first isolated 4 members of the stress-inducible *hsp70* gene family. In *Xenopus* four *hsp70* genes have been isolated (A-D), however the full gene for *hsp70A* is the only complete sequence available. Additionally, our laboratory has isolated and characterized the constitutively expressed *hsc70.I* and *hsc70.II* genes and *BiP* gene (Ali et al., 1996a; 1996b; Miskovic et al., 1997). The *hsp70* genes are intronless and in the 5' region there are *cis*-acting elements which include a TATA and CCAAT box and 3 HSEs (Heikkila, 2010). *Hsp70* gene expression was induced in A6 cells by a variety of stressors such as heat shock, herbimycin A, hydrogen peroxide, NA, $CdCl_2$, celastrol and curcumin (Darasch et al., 1988; Briant et al., 1997; Muller et al, 2004; Woolfson and Hekkila, 2009; Young et al., 2009; Walcott and Heikkila, 2010; Khan and Heikkila, 2011).

1.5.2 *Xenopus* HSP30

HSP30 is a member of the sHSP family that has been extensively examined in *Xenopus laevis*. To date, five isoforms of the *Xenopus* HSP30 genes have been isolated, *hsp30A-E.* Of these isoforms, *hsp30A* and *hsp30B* were found to be non-functional, due to an insertional mutation in the coding region of *hsp30A* while *hsp30B* was a

pseudogene (Bienz, 1984). *Hsp30C* and *hsp30D* encoded fully functional 24 kDa proteins that can act as molecular chaperones and contain the conserved α-crystallin domain found in other sHSPs (Krone et al., 1992). *Hsp30E* was only partially sequenced. *Hsp30* genes lack introns and have an AT rich 3' end which contains a mRNA instability sequence and a polyadenylation sequence (Krone et al., 1992). Analysis of the 5' region of the *hsp30C gene* revealed multiple *cis*-acting elements: a CCAAT box, 2 TATA boxes and 3 HSEs (Krone et al., 1992). Various cellular stresses including heat shock, NA, herbimycin A, hydrogen peroxide, $CdCl_2$, celastrol and curcumin were shown to induce *hsp30* gene expression in *Xenopus laevis* cultured cells (Briant et al., 1997; Muller et al., 2004; Young et al., 2009; Woolfson and Heikkila, 2009; Walcott and Heikkila, 2010; Khan and Heikkila, 2011). The intracellular localization of HSP30 in *Xenopus* A6 cells, as determined by immunocytochemistry, was found to be primarily in the cytoplasm in a granular pattern (Gellalchew and Heikkila, 2005). Additionally, HSP30 displays molecular chaperone activity by preventing heat-induced aggregation of proteins and maintaining them in a folding competent state (Fernando and Heikkila, 2000; Abdulle et al., 2002).

1.6 Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) is the major degradation route for the elimination of cellular proteins. This process is important in maintaining homeostasis during a number of biological processes such as cell cycle progression, development and differentiation, as well as eliminating damaged or misfolded proteins. The UPS is responsible for 80-90% of cellular degradation (Lee and Goldberg, 1998). Degradation of cellular proteins by the UPS is an ATP-dependent process which involves two

successive steps: the addition of ubiquitin to proteins targeted for degradation and subsequent degradation by the 26S proteasome.

Ubiquitination involves multiple ubiquitinating enzymes denoted E1 thru E4. Initially, ubiquitin activating enzymes (E1) activate ubiquitin in an ATP-dependent process which involves adenylation of ubiquitin (Lehman, 2009). Once activated, ubiquitin is transferred to a cysteine residue on a ubiquitin conjugating enzyme (E2). The E2 bound ubiquitin is subsequently transferred to an ubiquitin ligase (E3), which is capable of recognizing proteins targeted for degradation. E3 enzymes bind ubiquitin to the target protein by creating an isopeptide bond between the carboxy-terminal glycine of ubiquitin and a lysine residue of the protein (Lehman, 2009). For recognition of target damaged proteins by the 26S proteasome a minimum of four ubiquitin molecules are required. A fourth ubiquitin enzyme (E4) has been shown to facilitate the conjugation of additional ubiquitin molecules to create a polyubiquitin chain.

The 26S proteasome is responsible for enzymatic degradation of polyubiquitinated proteins. Structurally, it consists of a 20S core surrounded by two 19S regulatory particles on either end. The 19S regulatory regions recognize ubiquitinated protein and in the presence of ATP pass the protein to the 20S core (Lee and Goldberg, 1998). The 19S particles contain deubiquitinating enzymes that release free ubiquitin as damaged proteins enter the 26S proteasome (Lee and Goldberg, 1998). The 20S core is made up of four α or β heptameric stacked rings in an $\alpha\beta\beta\alpha$ conformation. The α subunits surround a small opening for entrance of polypeptide substrates, whereas β subunits contain proteolytic sites that function together in protein degradation (Lee and Goldberg, 1998). The β-rings in eukaryotes have catalytic sites with chymotrypsin-like,

trypsin-like and caspase-like activity (Lee and Goldberg, 1998). Exopeptidases break down most peptide products into amino acids, but some products are transported to the cell surface for antigen presentation after binding to major histocompatibilty complex (MHC) class I molecules in the endoplasmic reticulum (Bogyo et al., 1997; Lee and Goldberg, 1998).

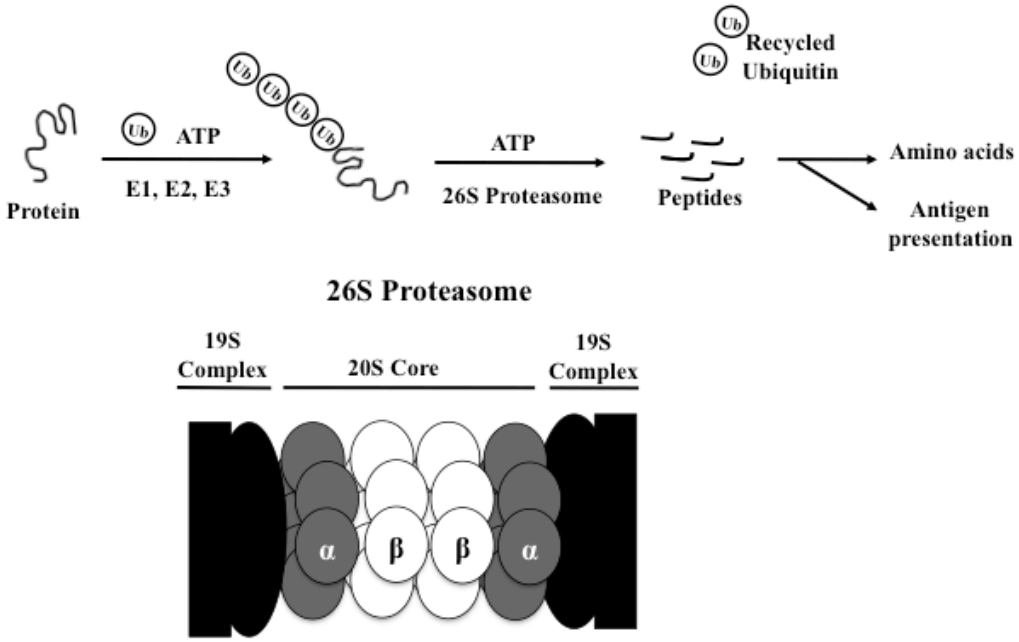
A schematic summary of the UPS can be found in Figure 2. Proteins destined for degradation are tagged with ubiquitin by the ubiquitinating enzymes. These tagged proteins are recognized by the 19S regulatory subunits of the 26S proteasome which pass the damaged protein to the 20S core, releasing ubiquitin, for enzymatic degradation. The tagged proteins are broken down into peptides by the catalytic sites in the 20S core.

1.6.1 Proteasomal Inhibition

Inhibition of the proteasome can have a number of effects on cells including a decrease in the rate of protein breakdown and disruption of normal levels of proteolysis. Along with the accumulation of regulatory proteins, such as the cyclin-dependent kinases p21 and p27, proteasome inhibition can lead to aggregation of damaged protein and apoptosis (Yang et al., 2008). Numerous disease states, such as Alzheimer's, Huntington's and Parkinson's disease, have been associated with the impairment of the UPS (Masliah et al., 2000; Ross and Pickart, 2004). In *Xenopus* and other eukaryotic organisms, inhibition of the proteasome has also been associated with an increase in *hsp* gene expression (Bush et al., 1997; Stangl et al., 2002; Pritts et al., 2002; Le Goff et al., 2004; Awasthi and Wagner, 2005; Lundgren et al., 2005; Noonan et al., 2007; Young and Heikkila, 2010; Walcott and Heikkila, 2010; Khan and Heikkila, 2011). There are several chemical inhibitors of proteasome activity such as carbobenzoxy-L-leucyl-L-

Figure 2. Schematic diagram of cellular protein degradation by the UPS.

Polypeptides that are destined for degradation are tagged by the addition of ubiquitin molecules by the ubiquitinating enzymes in the presence of ATP. Polyubiquitinated proteins are recognized by the 19S regulatory particle of the 26S proteasome. These proteins are degraded within the 20S core resulting in small peptides. Some of these peptides are further degraded into amino acids, while others are transported to the cell surface for antigen presentation (Adapted from Lee and Goldberg, 1998).


Ub
Recycled
Ubiquitin
Ub
Ub
Ub
Ub
Ub
ATP
E1, E2, E3
Protein
ATP
26S Proteasome
Peptides
Amino acids
Antigen
presentation
26S Proteasome
19S
Complex
20S Core
19S
Complex
α
β
β
α

leucyl-L-leucinal (MG132), lactacystin, celastrol and curcumin which have been shown to inhibit proteasome activity and induce *hsp* gene expression in *Xenopus* A6 cells (Young and Heikkila, 2010; Walcott and Heikkila, 2010; Khan and Heikkila, 2011).

1.6.2 CHIP: an E3 ubiquitin ligase

An interaction between molecular chaperones and E3 ubiquitin ligases has been suggested in the process of ubiquitination. The main substrate recognition factor of the carboxyl terminus of Hsc70-interacting protein (CHIP), an E3 ubiquitin ligase, has been suggested to be HSP70 (Esser et al., 2004). CHIP can bind HSP70 or HSC70 through its N-terminal tetratricopeptide domain and it contains a U-box domain at the C-terminal that binds to E2 conjugating enzymes (Esser et al., 2004). A number of studies have suggested that CHIP preferentially ubiquitinates HSP70-bound substrates (Meacham et al., 2001; Murata et al., 2001; Peng et al., 2004).

1.6.3 Folding versus degradation

The fate of HSP70 bound substrates is determined by the interaction of HSP70 with various cofactors. An intracellular competition occurs for the binding sites of HSP70 which determines whether the substrate undergoes degradation or refolding. Cofactors such as HIP and HOP promote protein folding whereas an interaction with CHIP and Bag-1 leads to degradation of the polypeptide. CHIP and HOP bind the same site of HSP70 at the carboxy-terminus of HSP70, whereas Bag-1 and HIP compete for binding at the amino-terminal (Hohfeld and Jentsch, 1997; Scheufler et al., 2000; Stankiewicz et al., 2010).

1.7 Arsenic

Arsenic is an abundant environmental stressor and exposure is commonly due to contaminated drinking water and industrial emissions (Del Razo et al., 2001). It primarily accumulates in water and soil (Del Razo et al., 2001). Arsenic exposure can lead to various renal, cardiovascular and hepatic diseases and has been associated with a number of cancers affecting major organs such as the liver, lungs, kidneys, bladder and skin (Del Razo et al., 2001). At the cellular level, arsenic can cause metabolic abnormalities, inhibition of growth, cytoskeletal collapse and apoptosis (Chou, 1989; Liu et al., 2001; Bode and Dong, 2002; Del Razo et al., 2001).

Toxicity of arsenic depends on the oxidation state and composition and it has been shown that inorganic arsenicals, specifically trivalent inorganic arsenic species, such as NA, are the most toxic forms (Del Razo et al., 2001). It has been suggested that arsenic causes toxicity by substituting for phosphate which affects processes such as DNA synthesis and the synthesis of ATP (Del Razo et al., 2001). Also, the high affinity of arsenite for sulfhydryl groups in protein was also reported as a mechanism of arsenic toxicity (Chen et al., 1998; Liu et al., 2001; Del Razo et al., 2001). Indirect toxicity by arsenic compounds is the production of reactive oxygen species and free radicals which can induce oxidative damage of cellular proteins (Del Razo et al., 2001).

Previously in our laboratory it has been shown that NA induces *hsp* gene expression (Ohan et al., 1998; Gauley and Heikkila, 2006; Young et al., 2009). The effect of NA on the UPS has been examined primarily in mammalian systems. It was shown that NA inhibits proteasomal activity as determined by an increase in the relative

levels of ubiquitinated protein and decreases in the chymotrypsin-like activity compared to control cells (Tsou et al., 2005; Medina-Diaz et al., 2009; Kirkpatrick, 2003).

1.8 Cadmium

Cadmium is a toxic heavy metal that accumulates primarily in the kidney, reproductive tissues and liver (Barbier et al., 2004; Mouchet et al., 2006). Cadmium has been associated with a number of cancers such as lung, prostate, kidney and pancreas (Waisberg et al., 2003). At the cellular level, cadmium toxicity causes dysregulation of gene expression, DNA damage, inhibition of DNA repair, apoptosis and oxidative stress (Waisberg et al., 2003; Mouchet et al., 2007; Mendez-Arementa and Rios, 2007; Blechinger et al., 2007). Within cells, cadmium reacts with thiol groups or substitutes for zinc in proteins which results in the formation of denatured or abnormal proteins (Waisberg et al., 2003; Galazyn-Sidorczuk et al., 2009).

In *Xenopus* A6 cells, $CdCl_2$ was shown to induce HSP30 and HSP70 accumulation (Woolfson and Heikkila, 2009). The effect of $CdCl_2$ on proteasome activity has primarily been examined in mammalian cells with the exception of studies examining marine bivalves, mussels and claims employing mussel gill and digestive glands (Thevenod and Friedmann, 1999; Figueiredo-Pereira and Cohen, 1999; Othumpangat et al., 2005; McDonagh and Sheehan, 2006; Chora et al., 2008; Li et al., 2008; Yu et al., 2008; McDonagh, 2009; Yu et al., 2011).

1.9 Objectives

The main goal of this thesis was to examine the effects of NA and $CdCl_2$, on proteasome activity and HSP accumulation in *Xenopus laevis* A6 kidney epithelial cells. Furthermore, this study investigated the possibility of an association between HSP

accumulation and proteasome activity. While a large amount of information regarding the effects of NA and $CdCl_2$ on proteasome activity in mammals, there is no available data in amphibians. NA and $CdCl_2$ are abundant environmental stressors that cause numerous effects on organisms. *Xenopus* is an ideal organism to examine the effects of environmental contamination due to the fluctuating conditions it experiences in an entirely aquatic environment. The specific objectives of this research are as follows:

- To determine the pattern of HSP30 and HSP70 accumulation in cells exposed to various treatment times and concentrations of NA and $CdCl_2$.
- To determine the effect of NA and $CdCl_2$ on the intracellular localization of HSP30 by immunocytochemistry and laser scanning confocal microscopy.
- To examine the effect of NA and $CdCl_2$ on proteasome activity in A6 cells by analyzing the relative level of ubiquitinated protein and chymotrypsin-like activity.
- To determine the effect of the inhibition of NA- and $CdCl_2$-induced HSP30 and HSP70 accumulation on proteasome activity by employing KNK437, an inhibitor of HSP accumulation.

2. Materials and Methods

2.1 Maintenance and Treatment of A6 Cells

Xenopus laevis A6 kidney epithelial cells were acquired from the American Type Culture Collection (CCL-102) (ATCC, Rockville, MD). A6 cells were cultured at 22 °C in 55% Leibovitz (L)-15 media (Sigma, Oakville, ON) supplemented with 1% penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively; Sigma) and 10% fetal bovine serum (FBS: Sigma) in T75 cm^2 BD Falcon tissue culture flasks (VWR International, Mississauga, ON). Once the cells became confluent, old media was removed by aspiration and 2 mL versene [0.02% (w/v) KCl, (0.8% (w/v) NaCl, 0.02% (w/v) KH_2PO_4, 0.115% (w/v) Na_2HPO_4, 0.02% (w/v) Na_2EDTA] was added for 2 min and then aspirated. A second rinse with 1 mL of versene for one minute followed and then aspirated. Subsequently, 1 mL of 1X trypsin (Sigma) in 100% Hank's balanced salt solution (HBSS; Sigma) for 1 min was used to remove cells from the surface of the flask. Cells were then resuspended in fresh media and distributed evenly into additional culture flasks.

Flasks that reached approximately 90-100% confluency were used for treatments. Heat shock treatments were performed in water baths set at 33 °C or 35 °C for 2 h followed by a 2 h recovery at 22 °C. NA (Sigma) was dissolved in distilled sterile water to make a stock solution of 100 mM and this stock solution was used to create a working solution of 1 mM concentration. $CdCl_2$ (Sigma) treatments were performed using a 100 mM working solution. MG132 (Sigma) and KNK437 (Calbiochem; Gibbstown, NJ) were dissolved in dimethylsulphoxide (Sigma) to prepare 5 mg/mL stock solutions. Working solutions of NA and $CdCl_2$ were used to create dilutions of 20 or 30 µM NA or

100 or 200 μM $CdCl_2$. Treatments with MG132 and KNK437 employed concentrations of 30 μM MG132 and 100 μM KNK437. To create these dilutions, the appropriate volumes of each chemical were added to 10 mL L-15 media. Treatments with KNK437 were performed 2 h prior to treatment with NA or $CdCl_2$. After the various treatments, media was removed from flasks and cells were rinsed with 2 mL of 65% HBSS and then 1 mL of HBSS was added. A cell scraper was used to remove cells from the flask which were subsequently transferred to a 1.5 mL microcentrifuge tube. Cells were pelleted by centrifugation at 14, 000 rpm for 1 min, the supernatant was removed and cells were stored at -80 ˚C until protein isolation.

2.2 Protein Isolation and Quantification

Total protein was isolated by resuspending cells with 200 - 400 μL lysis buffer (200 mM sucrose, 2 mM EGTA, 1 mM EDTA, 40 mM NaCl, 30 mM HEPES, pH 7.4) containing 1 % (w/v) SDS, 1 % (w/v) protease inhibitor cocktail (Roche; Laval, QC). For ubiquitinated protein detection 10 mM N-ethylmaleimide (Sigma) was added to the lysis buffer to inhibit ubiquitin conjugating enzymes. A sonic dismembranator (Model 100, Fisher Scientific; Waltham, MA) was used to sonicate samples on ice for 20 bursts followed by centrifugation at 14,000 rpm for 1 h at 4 °C. The supernatant was transferred to a new 1.5 mL microcentrifuge tube and stored at -20 ˚C until use.

The bicinchoninic acid (BCA) method was used for protein quantification according to the manufacturer's instructions (Pierce, Rockford, IL). A protein standard was created using a bovine serum albumin (BSA; Bioshop; Burlington, ON). BSA was diluted in distilled water to concentrations ranging from 0 to 2 mg/mL. Protein samples were diluted to a concentration of 1:2 in distilled water. BSA standards and protein

samples in 10 μL volumes were loaded into each well, in triplicate, into a 96 well polystyrene plate. To each standard and protein sample 80 μL of BCA reagent A and B (Pierce), 50:1, was added and the plate was incubated at 37 °C for 30 minutes. Subsequently, the plate was read at 562 nm using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, California) and Softmax Pro software. A standard curve was created using the BSA protein standards which was used to determine the concentration of the protein samples.

2.3 Western Blot Analysis

Proteins were separated by SDS-PAGE using 12% gels for HSP30, HSP70 and actin and 10% gels for ubiquitinated protein. Separating gels [10-12% (w/v) acrylamide, 0.32% (v/v) n'n'-bis methylene acrylamide, 0.375 M Tris pH 8.8, 1% (w/v) SDS, 0.2% (w/v) ammonium persulfate (APS), 0.14% (v/v) tetramethylethylenediamine (TEMED)] were prepared and left for 20 min to polymerize after 100% ethanol was layered over the gel. Once the separating gel solidified, ethanol was poured off and the stacking gel [4% (v/v) acrylamide, 0.11% (v/v) n'n'-bis methylene acrylamide, 0.125 M Tris pH 6.8, 1% (w/v) SDS, 0.4% (w/v) APS, 0.21% (v/v) TEMED] was added. Combs were inserted to create lanes and the gel was left to polymerize for 20 min. As gels polymerized, protein samples were prepared using 20 or 60 μg of protein, depending on the primary antibody used, and added to loading buffer [0.0625 M Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.00125% (w/v) bromophenol blue]. Samples were boiled for 10 minutes, cooled, pulse spun and loaded in the gel. Gels were electrophoresed with 1 X running buffer [25 mM Tris, 0.2 M glycine, 1 mM SDS] at 90 V until samples reached the separating buffer at which point the voltage was increased to

130 V for ubiquitinated protein or 160 V for HSP30, HSP70 and actin until the samples reached the bottom of the gel. As the gel was running, nitrocellulose membranes (BioRad, Mississauga, ON) and filter paper (BioRad) were cut to a size of 5 X 8.5 cm. Membranes were soaked for 30 min in 10% transfer buffer [25 mM Tris, 192 mM glycine, 10% (v/v) methanol], for ubiquitin blots, or 20% transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol], for HSP30, HSP70 and actin. Once electrophoresis was finished, the stacking gel was removed and the remainder of the gel was soaked in transfer buffer for 15 min. Protein was transferred to the nitrocellulose membrane with a Trans-Blot Semi-dry Transfer cell (BioRad) for 25 min at 20 V. Once the transfer was complete, membranes were stained with Ponceau-S stain [0.19% (w/v) Ponceau-S, 5% (v/v) acetic acid] for 15 min to determine the success of the transfer. Blots were rinsed with milliQ water and scanned. Membranes were then incubated with 5% blocking [20 mM Tris pH 7.5, 0.1% Tween 20 (Sigma), 300 mM NaCl, 5% (w/v) Nestle® Carnation skim milk powder] solution for 1 h to prevent non-specific binding. Once the blocking solution was removed, the membranes were incubated with rabbit anti-*Xenopus* HSP30 (1:1000), rabbit anti-*Xenopus* HSP70 (1:350; Abgent, San Diego, CA) or rabbit anti-actin (1:200; Sigma) polyclonal antibodies diluted in 5% blocking solution for at least 1 h. For ubiquitinated protein, membranes were incubated overnight with a mouse anti-ubiquitin (1:150; Invitrogen, Carlsbad, CA) monoclonal antibody diluted in 5% bovine serum albumin (BSA; Fisher Scientific). After incubation with primary antibody, membranes were washed three times, once for 15 minutes and twice for ten minutes, with 1X TBS-T [20mM Tris, 300 mM NaCl, (pH 7.5), 0.1% (v/v) Tween 20]. Membranes were then incubated with secondary antibody in blocking solution for 1 h.

AP-conjugated goat-anti-rabbit (BioRad) at a dilution of 1:3000 dilution for HSP30, HSP70, actin and AP-conjugated goat-anti-mouse (BioRad) at a 1:1000 dilution were used. Following incubation with secondary antibody, TBS-T was used to wash the membranes, once for 15 minutes and twice for 5 minutes. Blots were then immersed in alkaline phosphatase detection buffer [50 mM Tris, 50 mM NaCl, 25 mM $MgCl_2$ pH 9.5, 0.3% 4-nitro blue tetrazolium (NBT; Roche) and 0.17% 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP; Roche)] until bands or ubiquitinated protein lanes were visible and then detection buffer was removed and the blots were rinsed with distilled water. The blots were then scanned.

2.4 Densitometry and Statistical Analysis

Image J software (Version 1.44; National Institute of Health) was used to perform densitometric analysis on all blots performed in triplicate. The average densitometric values were expressed and graphed as a percentage of the maximum HSP30 or HSP70 bands, lane of ubiquitinated protein or as a percentage of treatment without pretreatment with KNK437. The standard error is represented by vertical error bars. To determine if any statistically significant differences existed between samples, a one-way ANOVA with a Tukey's post-test were performed on the data. Confidence levels used were 95% ($p<0.05$;*) and 85% ($p<0.15$; ∇).

2.5 Laser Scanning Confocal Microscopy

A6 cells were grown on flame sterilized 22 x 22 mm base-washed glass coverslips in sterile Petri dishes. Coverslips were washed with base solution [49.5% (v/v) ethanol, 0.22 M NaOH] in small staining jars (Thomas Scientific Apparatus, Philadelphia, PA) for 30 min and then rinsed with distilled water for 3 h.

After treatment, L-15 media was removed and cells were rinsed twice with phosphate buffered saline (PBS; 1.37 M NaCl, 67 mM Na_2HPO_4, 26 mM KCl, 14.7 mM H_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ pH 7.4). Once washed, coverslips were transferred to small Petri dishes and fixed with 3.7 % paraformaldehyde (BDH, Toronto, ON) for 15 min. A6 cells were rinsed with three 5 min each washes with PBS and then permeabilized using 0.3 % Triton X-100 (Sigma) for 10 min. After an additional three washes with PBS, A6 cells were incubated with 3.7 % (w/v) bovine serum albumin fraction V (BSA fraction V; Fischer Scientific) for 1 h or overnight at 4 °C. Subsequently, coverslips were incubated with affinity-purified rabbit anti-*Xenopus* HSP30 antibody (1:500) in 3.7 % BSA for 1 h. After three washes for 3 min each with PBS, indirect labeling of cells was carried out with a fluorescent-conjugated secondary antibody, goat anti-rabbit Alexa Fluor 488 (Invitrogen Molecular Probes) at a 1:200 dilution in 3.7 % BSA for 30 min in the dark. Coverslips were then incubated with rhodamine-tetramethylrhodamine-5-isothiocyanate phalloidin (TRITC; Invitrogen Molecular Probes) for 15 min at a 1:60 in 3.7 % BSA in the dark in order to visualize the actin cytoskeleton. Coverslips were dried and mounted on a glass microscope slide with Vectashield (Vector Laboratories Inc., Burlingame, CA) containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc.) to stain nuclei followed by three washes of 5 min each. Clear nail polish was used to permanently attach coverslips to glass slides and the slides were stored at 4 °C. Slides were examined using a Zeiss Axiovert 200 confocal microscope with LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, ON).

2.6 Proteasome Assay

To examine the chymotrypsin (CT)-like activity of A6 cells treated with NA and $CdCl_2$, the Promega Proteasome-Glo™ Chymotrypsin-like cell based luminescent assay kit was used (Promega, Madison, WI). The Proteasome-Glo™ Cell-Based Regeant contains a specific luminogenic substrate for CT-like activity, Suc-Leu-Leu-Val-Tyr-aminoluciferin (Suc-LLVY-aminoluciferin) in a buffer for cell permeabilization, proteasome activity and luciferase activity. Cleavage of the proteasome generates an aminoluciferin substrate that is consumed by luciferase to produce a luminescent signal which is proportional to CT-like activity. To detect the CT-like activity, the Proteasome-Glo™ Reagent was produced by equilibrating the Proteasome-Glo™ Cell-Based Buffer and Luciferin Detection Reagent to room temperature and mixing them with the flurogenic substrate Suc-LLVY-AMC.

A6 cells were treated with NA or $CdCl_2$ and following treatment old media was removed and flasks were washed with 2 mL of versene and then aspirated. A second rinse with 1 mL of versene for one minute followed and then aspirated. Cells were then detached from flasks using 1 X trypsin and resuspended in 9 mL of L-15 media. The cell suspension was then transferred to a 15 mL falcon tube and centrifuged at 5000 rpm for 3 min at 4 °C. Media was removed and pellets were rinsed with 5 mL of fresh L-15 media. Again, media was removed and 5 mL of fresh L-15 was added to resuspend A6 cells. The total number of cells per mL was determined for each sample using a Bright-Line haemocytometer (Hausser Scientific, Horsham, PA) and aliquots of each sample were prepared to 15 000 cells per 100 µL of L-15 media. Each aliquot was added to a white-walled 96 well plate including a L-15 blank (L-15 media + Proteasome-Glo™ Reagent).

Proteasome-Glo™ Reagent was added to each sample in the plate at a 1:1 ratio (100 µL reagent). The plates were mixed for 2 min on a shaker and then incubated at room temperature in the dark for 30 min before detection of luminescence. The Victor^3 luminometer (PerkinElmer Inc., Waltham, MA) was used to detect luminescence of each sample with a filter set at 340/480 nm. Measurements were repeated 5 times and the values were compared to the L-15 blank and to control cells maintained at 22 °C.

3. RESULTS

3.1 Effect of NA and $CdCl_2$ on HSP accumulation

Previously in our laboratory and in the present study it was shown that a mild heat shock or treatment with NA or $CdCl_2$ induced HSP30 and HSP70 accumulation in A6 cells (Ohan et al., 1998; Phang et al., 1999; Fernando and Heikkila, 2000; Gellalchew and Heikkila, 2004; Young et al., 2009; Woolfson and Heikkila, 2009). For example, cells incubated at 33 and 35 °C for 2 h with a 2 h recovery at 22 °C produced an increase in the relative levels of HSP30 and HSP70 in comparison to control (Fig. 3). The multiple HSP30 bands found in Figure 3 (top panel) may represent the various family members that were detected by the anti-HSP30 antibody which was prepared against the entire coding sequence of HSP30C (Fernando and Heikkila, 2000). This was expected given the high amino acid sequence identity among the HSP30 family members (Krone et al., 1992). In these experiments, actin levels were relatively unaffected by the heat shock treatments. The effect of two different concentrations of NA is shown in Figure 4. Treatment with 20 and 30 μM NA for 16 h caused an increase in HSP30 and HSP70 accumulation in A6 cells. Densitometric analysis revealed an enhanced accumulation of HSP30 after treatment with 30 μM NA. The relative levels of HSP70 increased 10-fold with 20 μM NA and 7-fold after treatment with 30 μM NA. Similarly, A6 cells treated with 100 or 200 μM $CdCl_2$ for 18 h had elevated levels of HSP30 and HSP70 as shown in Figure 5. Maximal accumulation of HSP30 and HSP70 was detected in cells treated with 200 μM $CdCl_2$ for 18 h.

Figure 3. Heat shock-induced HSP30 and HSP70 accumulation in A6 cells. A6 cells were incubated at 33 °C and 35 °C for 2 h with a 2 h recovery at 22 °C. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis employing a polyclonal rabbit anti-*Xenopus* HSP30, anti-*Xenopus* HSP70 or polyclonal rabbit anti-actin antibodies as described in the Materials and methods. The anti-HSP30 antibody detects multiple family members of the HSP30 family. These data are representative of three separate experiments.

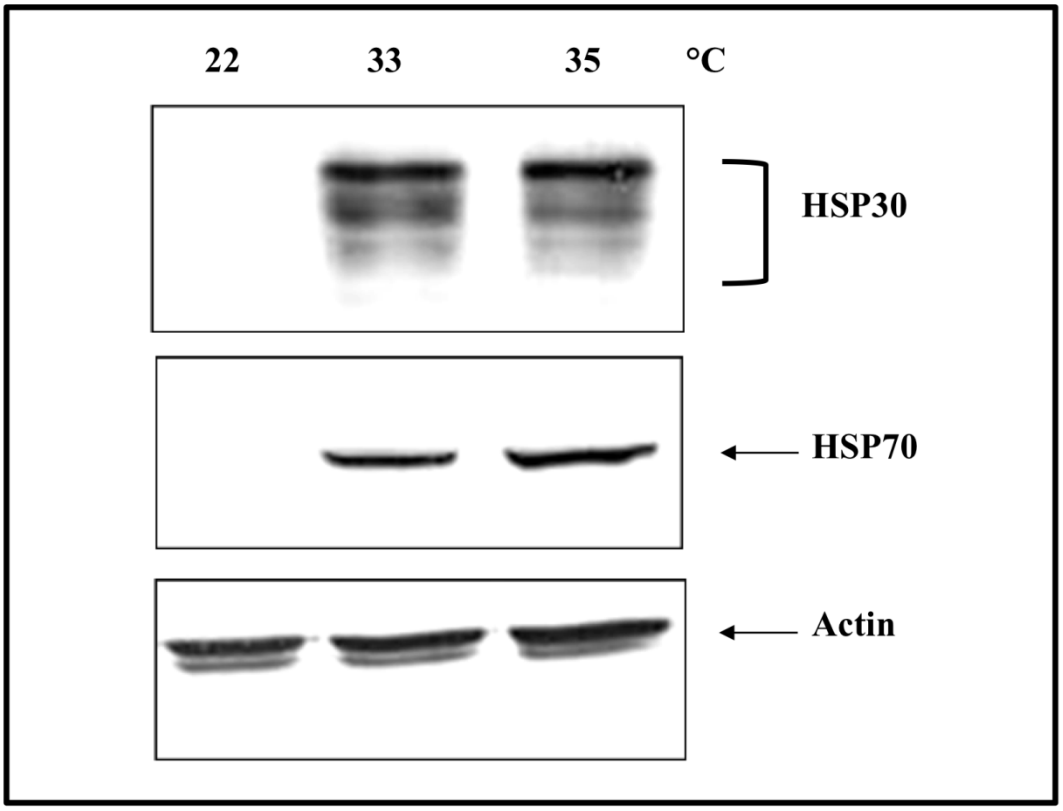

22
33
35
°C
HSP30
HSP70
Actin

Figure 4. NA-induced HSP30 and HSP70 accumulation in A6 cells. A) Cells were maintained at 22 °C (C) or exposed to 20 or 30 μM NA for 16 h at 22 °C. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis. B) Image J (Version 1.44) software was used to carry out densitometric analysis of HSP30 (black bars) and HSP70 (white bars) bands in the western blot images as described in Materials and methods. Results are expressed as a percentage of the maximum signal acquired for each protein. Vertical error bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control and 20 or 30 μM NA are indicated as * ($p<0.05$). These results are representative of at least three different experiments.

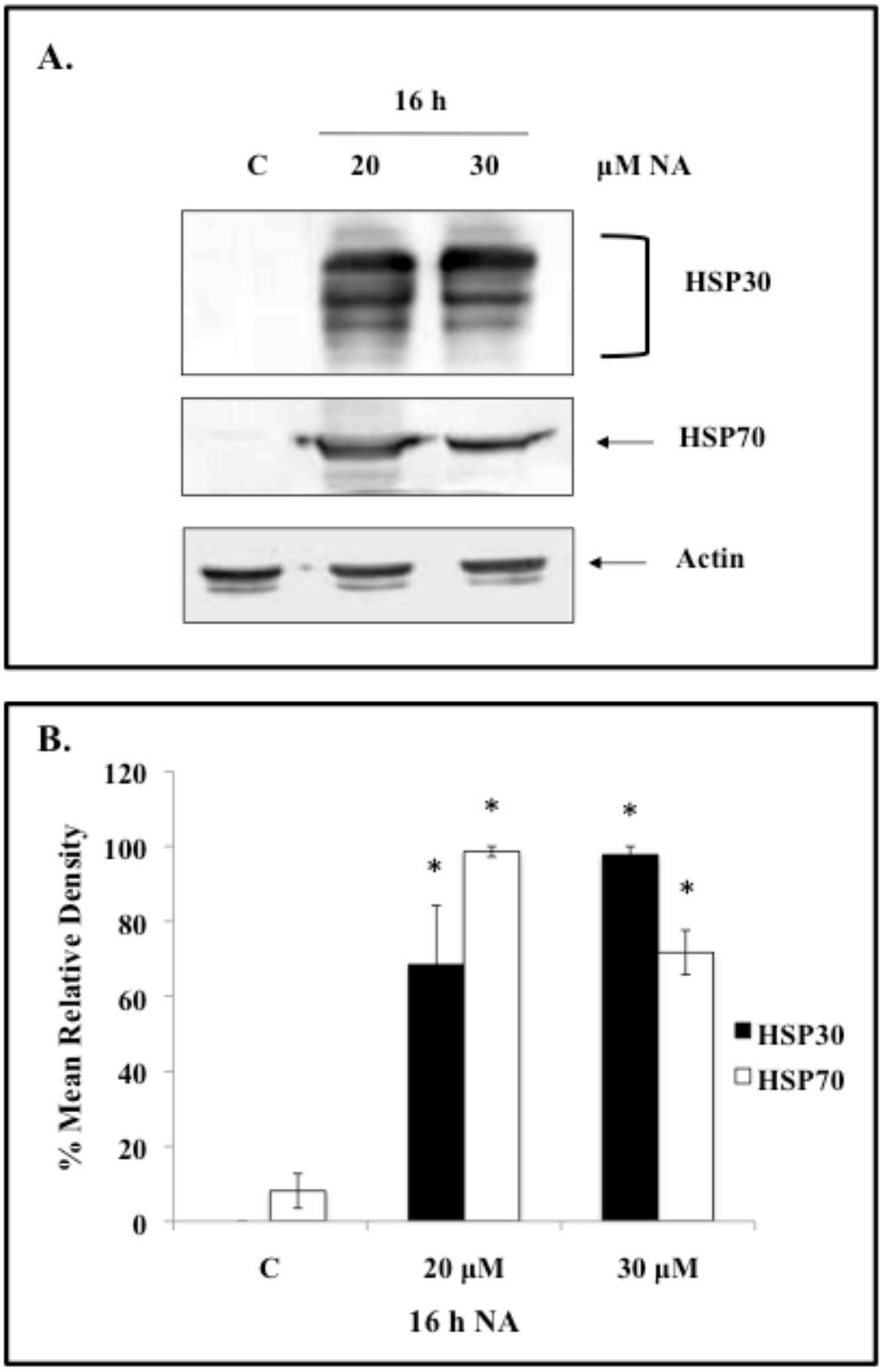
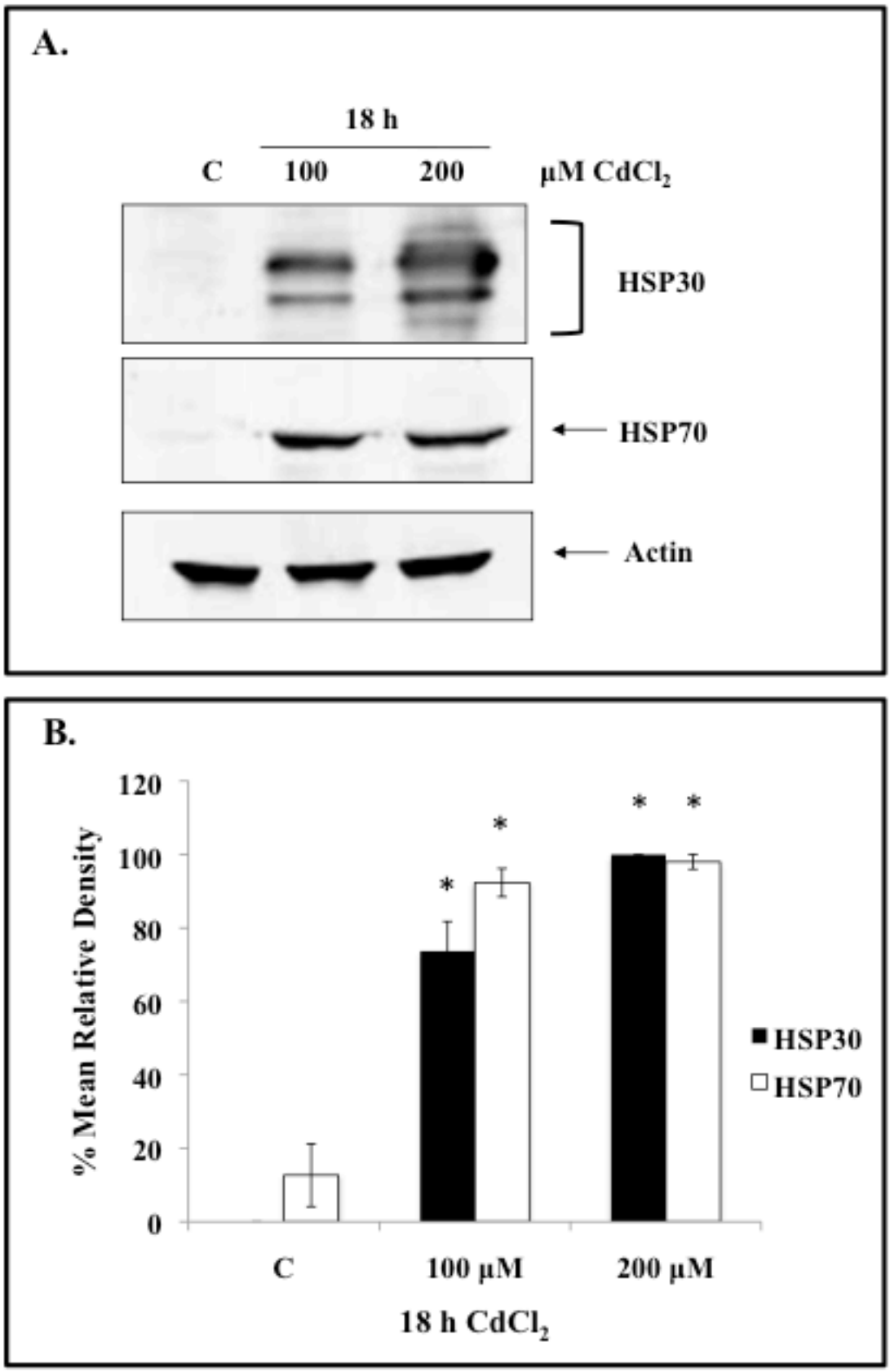

A.
16 h
C
20
30
μM NA
HSP30
HSP70
Actin
B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
*
*
HSP30
HSP70
C
20 μM
30 μM
16 h NA

Figure 5. The effect of $CdCl_2$ on the accumulation of HSP30 and HSP70 in A6 cells. A) Cells were maintained at 22 °C (C) or treated with $CdCl_2$ concentrations of 100 or 200 μM for 18 h. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis of the signal intensity for HSP30 (black) and HSP70 (white) protein of the immunoblots was performed using Image J software. The results are expressed as a percentage of the maximum band while the standard error is represented by vertical error bars. One-way ANOVA with a Tukey's post-test was used to calculate the level of significance of the differences between samples. Significant differences between control (C) and cells exposed to $CdCl_2$ for 18 h are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A.
18 h
C
100
200
μM CdCl2
HSP30
HSP70
Actin
B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
*
*
HSP30
HSP70
C
100 μM
200 μM
18 h CdCl2

3.2 Localization of HSP30 in NA and $CdCl_2$ treated A6 cells

The accumulation and intracellular localization of HSP30 induced by NA or $CdCl_2$ treatment was verified by immunocytochemistry and laser scanning confocal microscopy (LSCM). The localization of HSP70 was not investigated since the anti-HSP70 antibody, which was used successfully in immunoblot experiments, was unable to specifically detect HSP70 by immunocytochemistry. As shown in Figures 6 and 7, accumulation of HSP30 was not detected in control cells maintained at 22 °C. However, treatment with 20 and 30 μM NA for 16 h resulted in the accumulation of HSP30 in approximately 90% and 95% of cells, respectively (Fig. 6). Furthermore, in A6 cells treated with 100 and 200 μM $CdCl_2$, HSP30 accumulated in approximately 60% and 95% of cells, respectively (Fig. 7). After both stressors HSP30 was detected primarily in the cytoplasm in a granular pattern. Examination of the actin cytoskeleton revealed that treatment with 30 μM NA and 200 μM $CdCl_2$ resulted in the presence of relatively large dense actin structures and a loss of the actin stress fibres pattern that was observed in control cells.

3.3 Temporal pattern of NA- and $CdCl_2$-induced HSP accumulation.

In time course studies, immunoblot analysis revealed enhanced HSP30 and HSP70 levels from 8 to 24 h of exposure to 30 μM NA (Fig. 6). A significant increase ($p<0.05$) in HSP30 and HSP70 accumulation was detected after 8 h exposure and accumulation increased up to 24 h. A6 cells treated with 200 μM $CdCl_2$ showed a gradual increase in the relative accumulation of HSP30 and HSP70 from 10 to 24 h (Fig. 7). Significant increases in HSP30 and HSP70 accumulation were observed after 10 h

Figure 6. Localization of HSP30 accumulation in A6 cells treated with NA. Cells were cultured on glass coverslips and maintained at 22 °C or incubated with 20 or 30 μM NA for 16 h. Actin and nuclei were stained directly with phalloidin conjugated to TRITC (red) and DAPI (blue), respectively. HSP30 was indirectly detected with an anti-HSP30 antibody and a secondary antibody conjugated to Alexa-488 (green). The 20 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of three separate experiments.

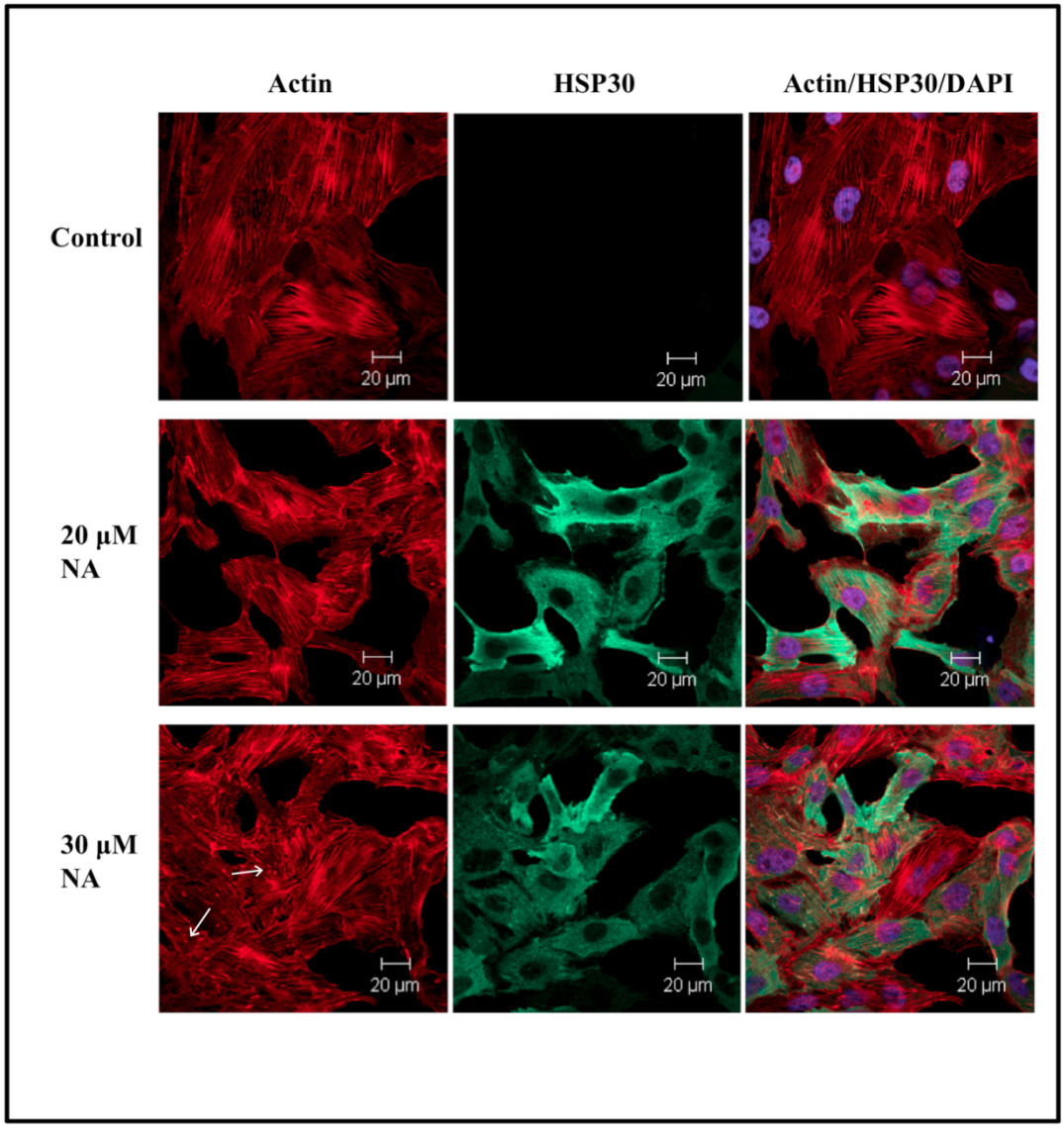

Actin
HSP30
Actin/HSP30/DAPI
Control
20 µM NA
30 µM NA
20 µm

Figure 7. Localization of HSP30 accumulation in A6 cells treated with $CdCl_2$. Cells were cultured on glass coverslips and maintained at 22 °C or incubated with 100 or 200 μM $CdCl_2$ for 18 h. HSP30 was indirectly detected with an anti-HSP30 antibody and a secondary antibody conjugated to Alexa-488 (green). Actin and nuclei were stained directly with phalloidin conjugated to TRITC (red) and DAPI (blue), respectively. The 20 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of three separate experiments.

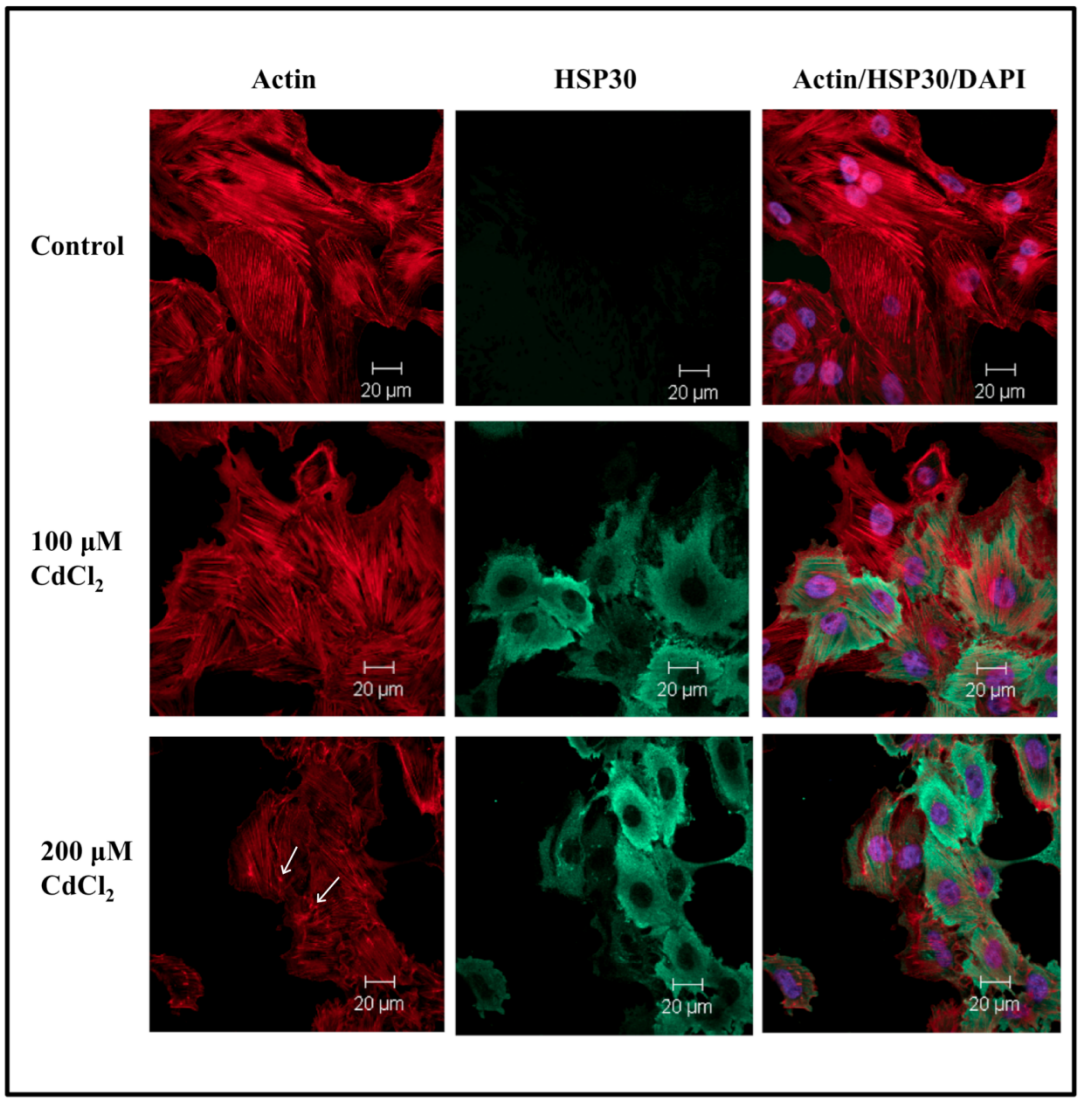
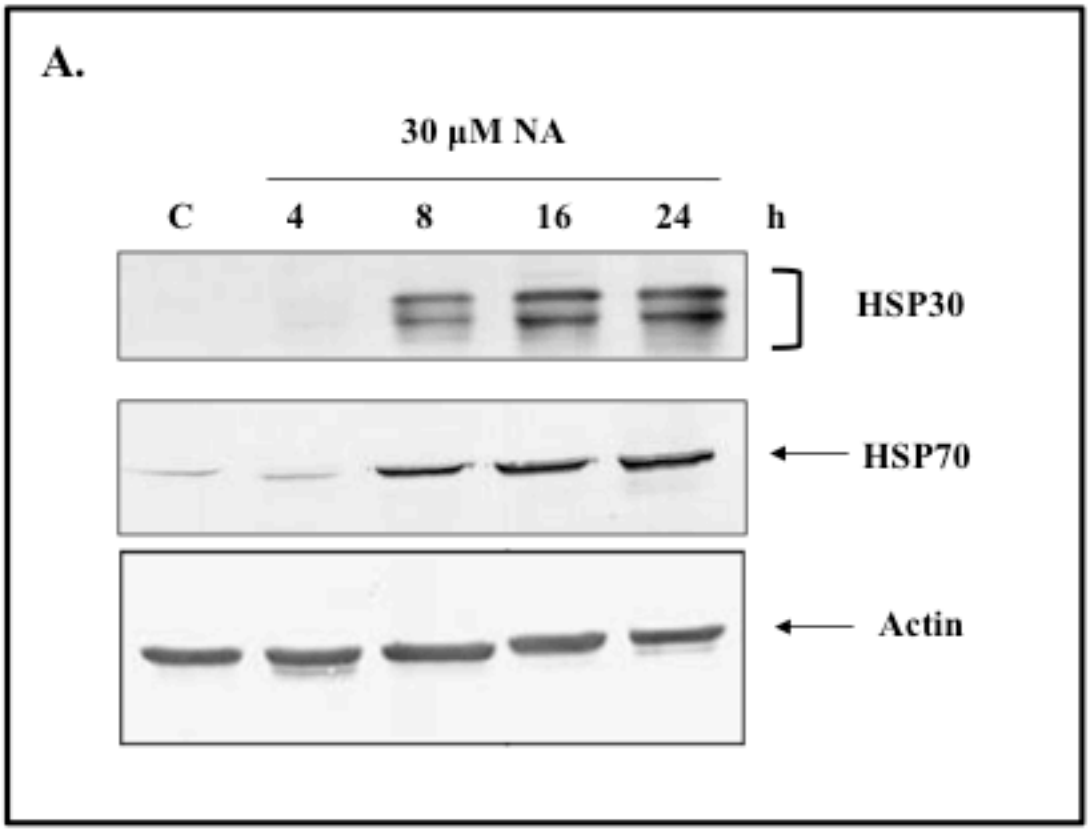

Actin
HSP30
Actin/HSP30/DAPI
Control
100 µM CdCl2
200 µM CdCl2
20 µm

Figure 8. A time-dependent increase in HSP30 and HSP70 in A6 cells following NA treatments. A) Cells were maintained at 22 °C (C) or treated with 30 μM NA for time periods ranging from 4 to 24 h. After treatment, cells were harvested and total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis of the signal intensity for HSP30 (black) and HSP70 (white) protein bands was performed using Image J software. The data are expressed as a percentage of the maximum band. Vertical bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control (C) cells and NA treated cells are indicated by * ($p<0.05$). These data are representative of three separate experiments.


A.
30 μM NA
C
4
8
16
24
h
HSP30
HSP70
Actin

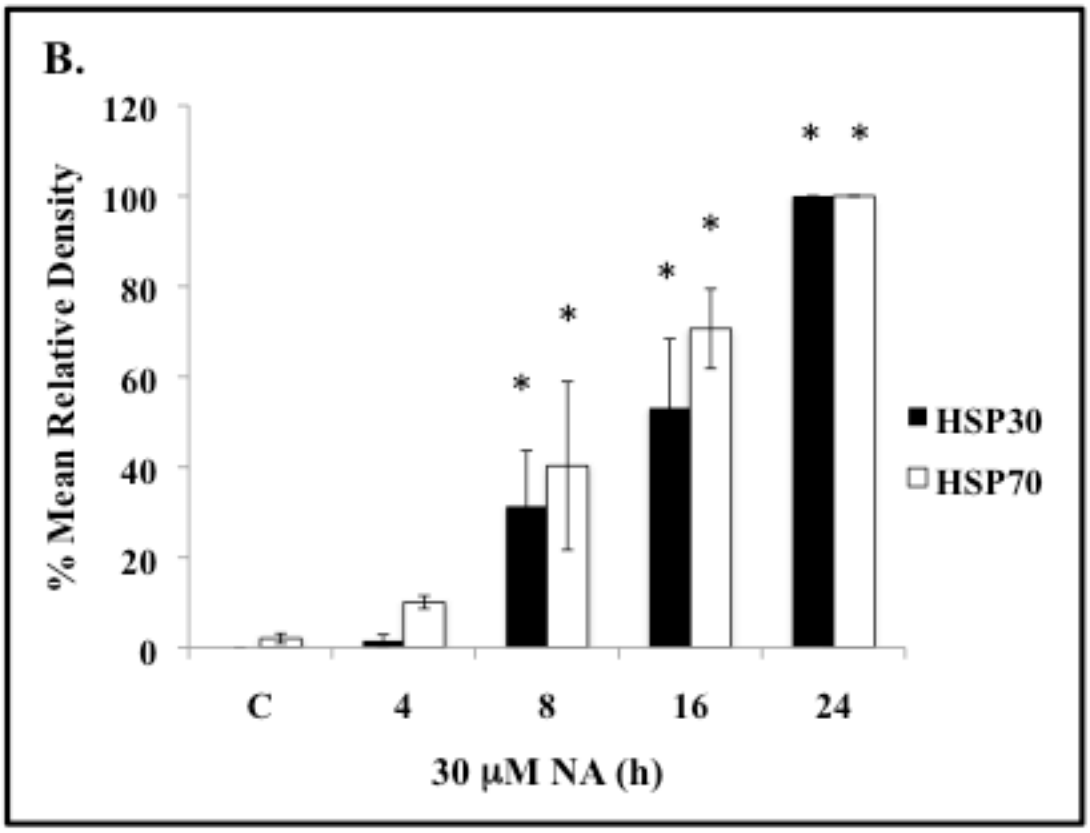
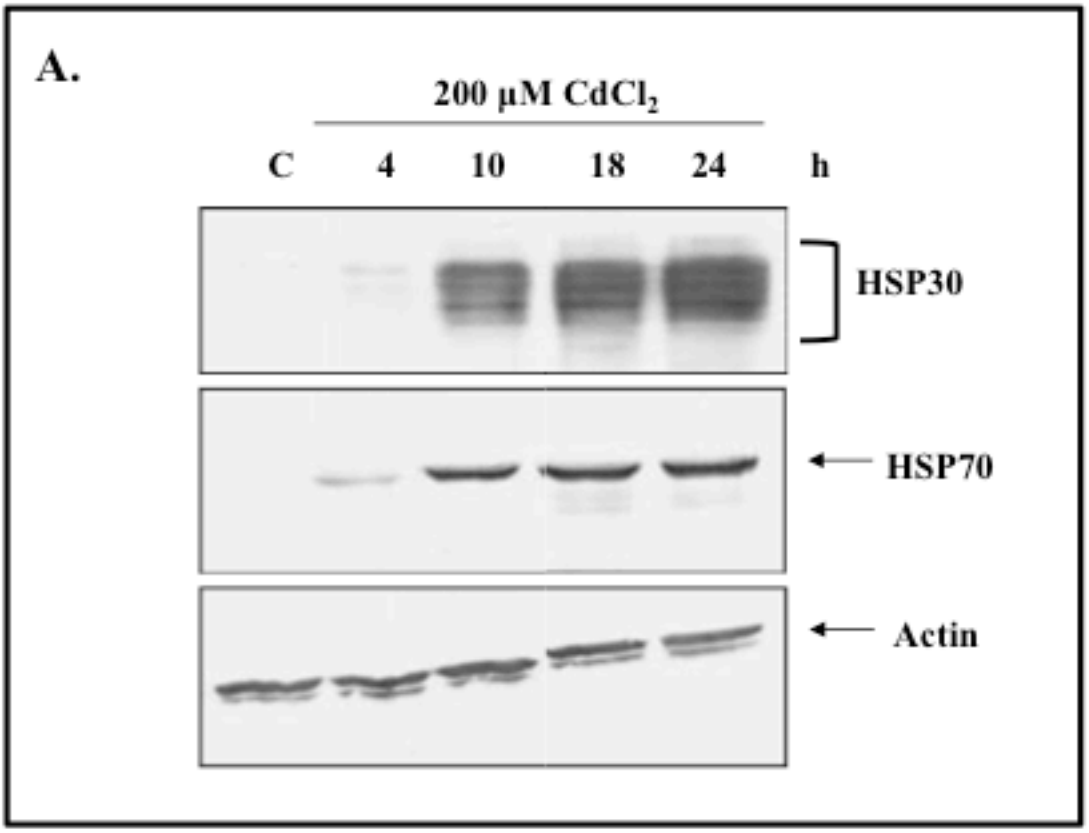
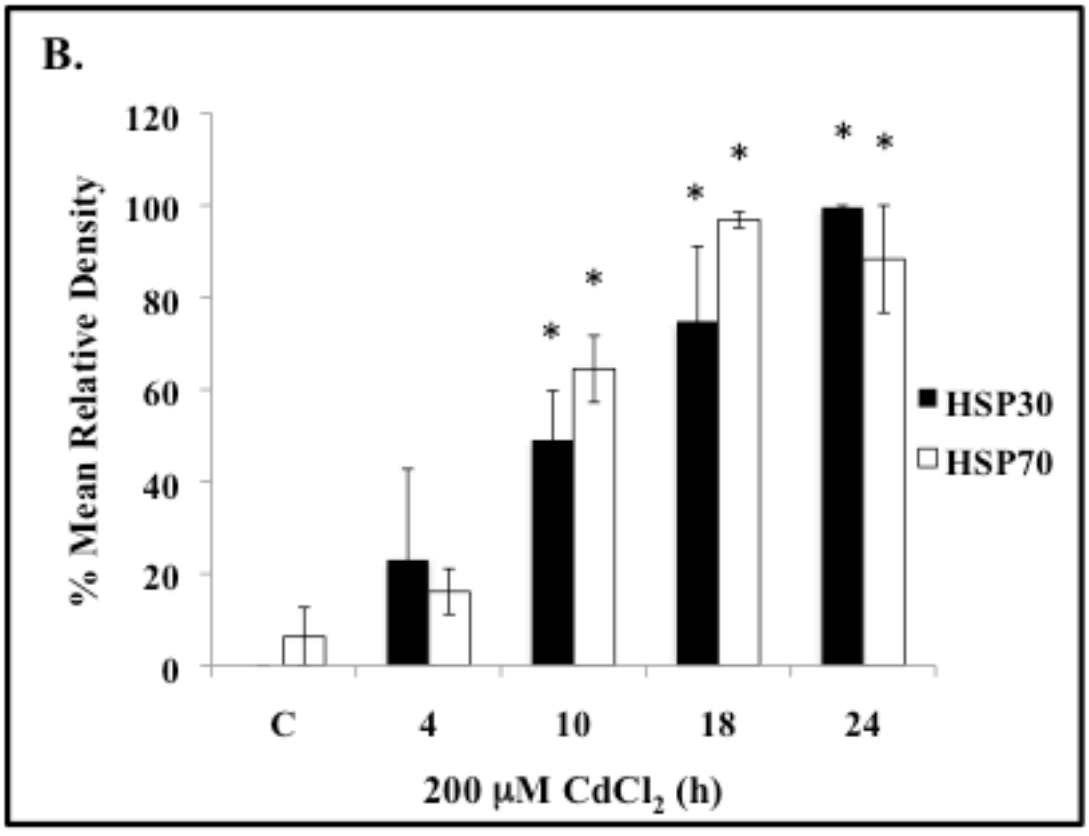

B.
% Mean Relative Density
120
100
80
60
40
20
0
C
4
8
16
24
30 μM NA (h)
HSP30
HSP70

Figure 9. Time course of $CdCl_2$–induced HSP30 and HSP70 accumulation. Cells were maintained at 22°C (C) or treated with 200 μM $CdCl_2$ for periods of time ranging from 4 to 24 hours. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis employing anti-*Xenopus* HSP30, anti-*Xenopus* HSP70 and anti-actin polyclonal antibodies. B) Image J software was used to carry out densitometric analysis of HSP30 (black bars) and HSP70 (white bars) bands in the western blot images. The percentage of the maximum signal acquired for each protein was used to express results and the standard error is represented by vertical bars. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control and cadmium chloride treatments are indicated as * ($p<0.05$). These results are representative of at least three different experiments.


A.
200 μM CdCl2
C
4
10
18
24
h
HSP30
HSP70
Actin

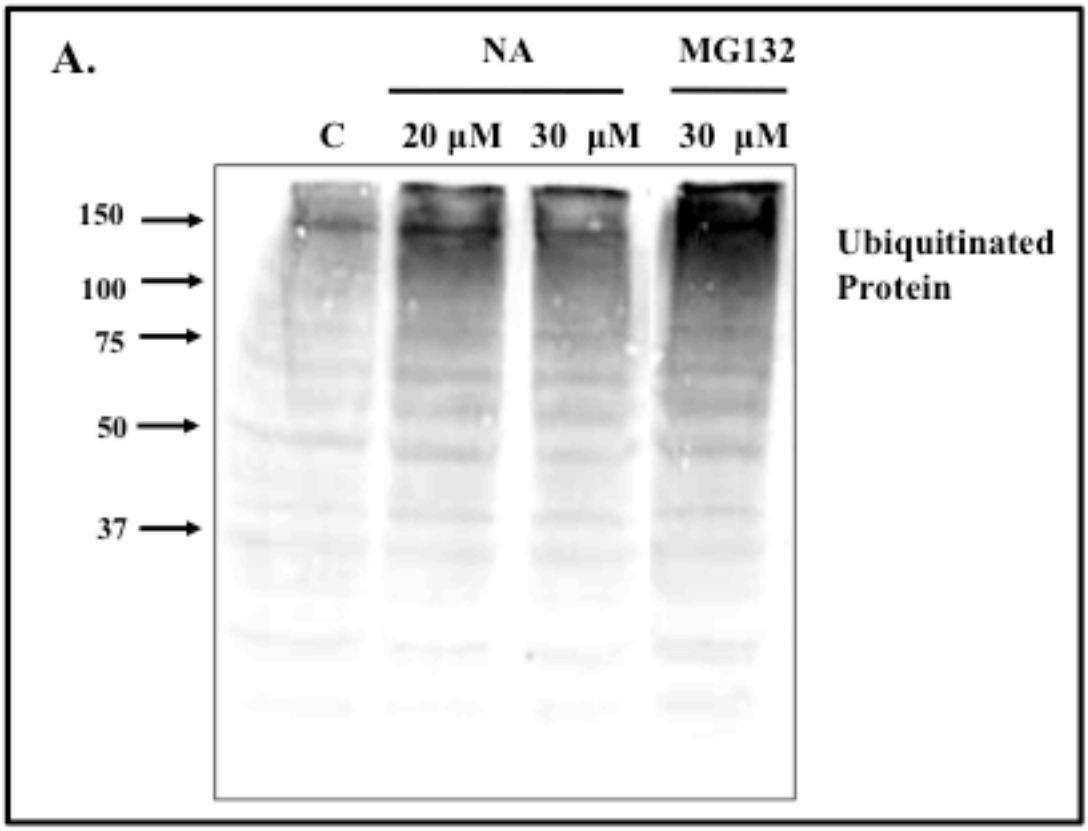

B.
% Mean Relative Density
120
100
80
60
40
20
0
C
4
10
18
24
200 μM CdCl2 (h)
HSP30
HSP70

exposure. Maximal HSP30 and HSP70 accumulation was noted after 24 h and 18 h incubation with $CdCl_2$, respectively.

3.4 Relative levels of ubiquitinated protein increase with NA and $CdCl_2$ treatment

The impact of NA and $CdCl_2$ on proteasome activity in *Xenopus laevis* A6 cells was evaluated initially by examining the relative levels of ubiquitinated protein. The relative levels of ubiquitinated protein in cells was determined by immunoblot analysis using an anti-ubiquitin antibody. An increase in the relative level of ubiquitinated protein was observed in cells treated for 16 h with 20 or 30 μM NA in comparison to control cells (Fig. 10). MG132, a known proteasomal inhibitor, was employed in this study as a positive control. Densitometric analysis revealed a 1.6- and 1.7-fold increase in cells treated with 20 and 30 μM NA, respectively, compared to control cells. Time course studies showed an increase in the relative levels of ubiquitinated protein in cells treated with 30 μM NA from 4 to 24 h in comparison to control cells (Fig. 11). A 2.5-fold increase in the relative levels of ubiquitinated protein was noted after 16 or 24 h NA treatment in comparison to control. Similarly, treatment of cells with $CdCl_2$ also increased the relative levels of ubiquitinated protein. After 18 h treatment with 100 or 200 μM $CdCl_2$ there was an increase in ubiquitinated protein compared to cells maintained at 22 °C. Treatment with 200 μM $CdCl_2$ resulted in a 2-fold increase in the level of ubiquitinated protein as determined by statistical analysis (Fig. 12). Time course studies of the relative levels of ubiquitinated protein following treatment with $CdCl_2$ showed a slight increase after 4 h treatment with a significant increase in accumulation after 10, 18 and 24 h treatment (Fig. 13).

Figure 10. NA increased the relative level of ubiquitinated protein in A6 cells. A) Cells were maintained at 22 °C, treated with 20 or 30 μM NA for 16 h or treated with 30 μM MG132 for 16 h. Total protein was isolated and subjected to immunoblot analysis employing a mouse anti-ubiquitin antibody as described in the Materials and methods. B) Densitometric analysis was performed on each lane using Image J software. Results are expressed as a percentage of the maximal signal acquired. The standard error is represented by vertical bars. One-way ANOVA with a Tukey's post-test was used to determine the level of significance of the differences between samples. The symbol * represents the significant difference ($p<0.05$) between control and cells treated with NA. These results are representative of at least three separate experiments.


A.
NA
MG132
C
20 µM
30 µM
30 µM
150
100
75
50
37
Ubiquitinated Protein

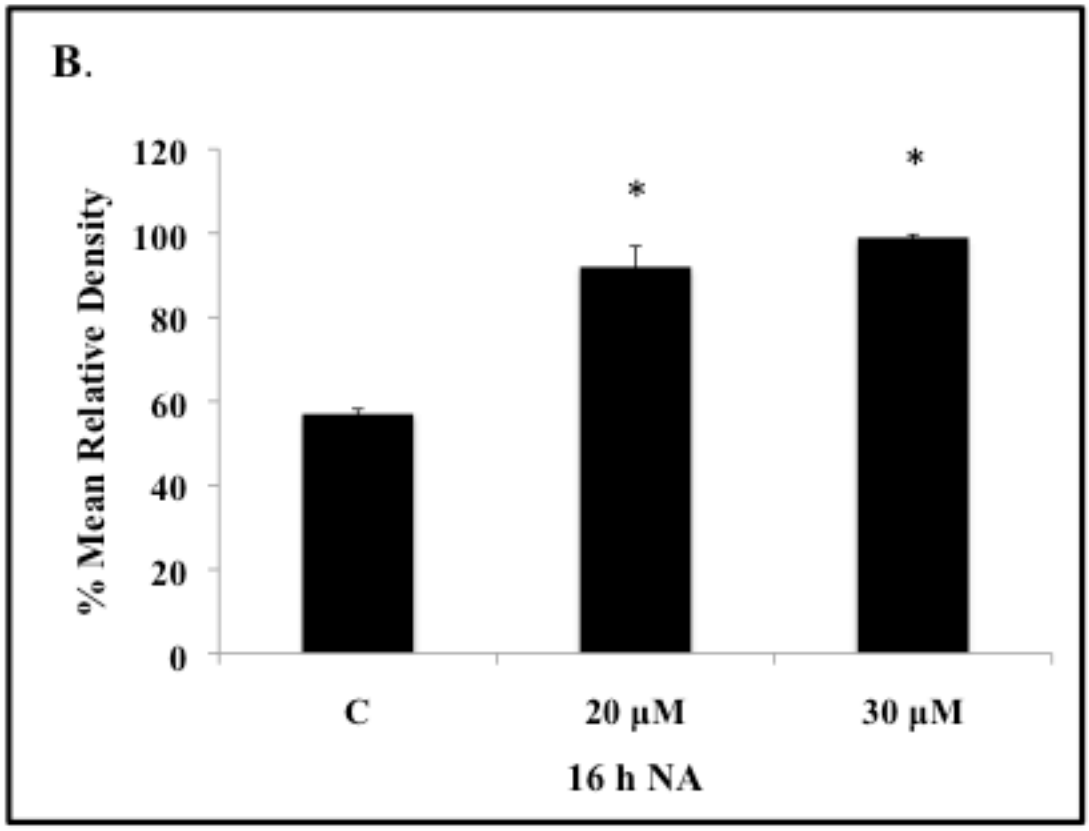
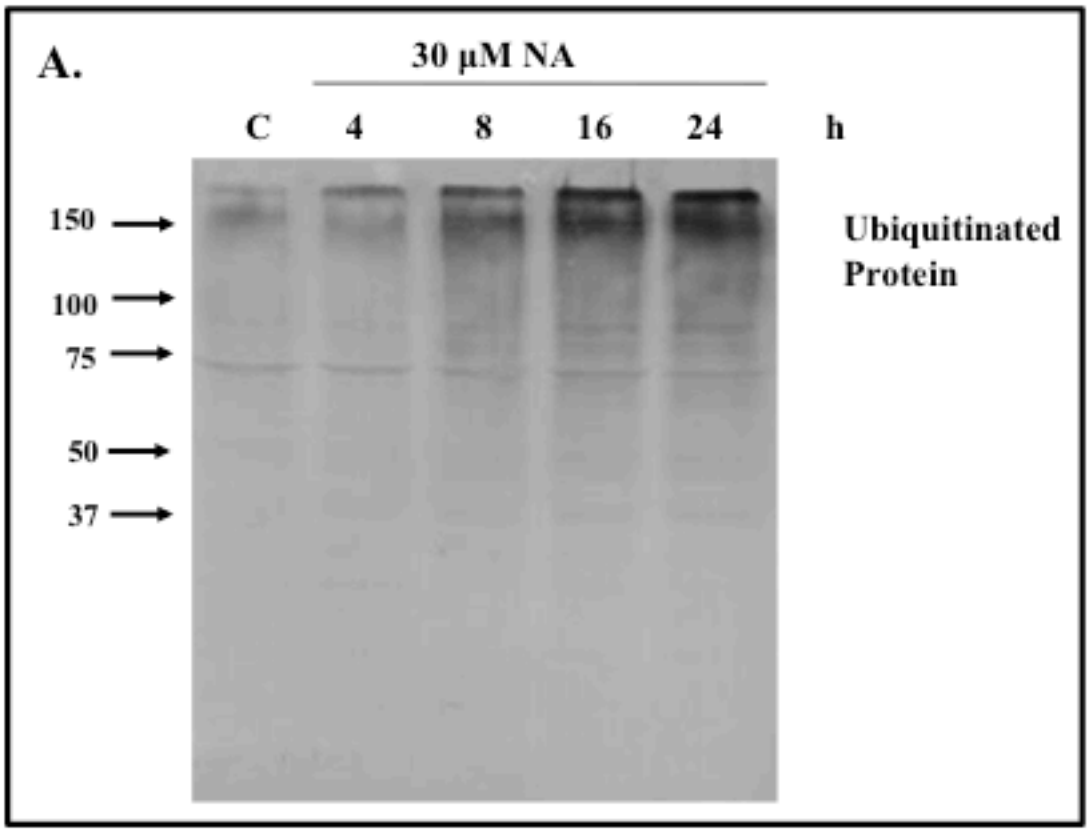

B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
C
20 µM
30 µM
16 h NA

Figure 11. Temporal pattern of ubiquitinated protein levels in A6 cells treated with NA. A) Cells were maintained at 22 °C or treated with 30 μM NA for 4, 8, 16 or 24 h. Total protein was isolated and subjected to immunoblot analysis. B) Image J software was used to perform densitometric analysis of the signal intensity for each lane of the immunoblots. The data are expressed as a percentage of the lane with the maximum signal while vertical bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control (C) and cells exposed to 30 μM NA are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A.
30 μM NA
C
4
8
16
24
h
150
100
75
50
37
Ubiquitinated Protein

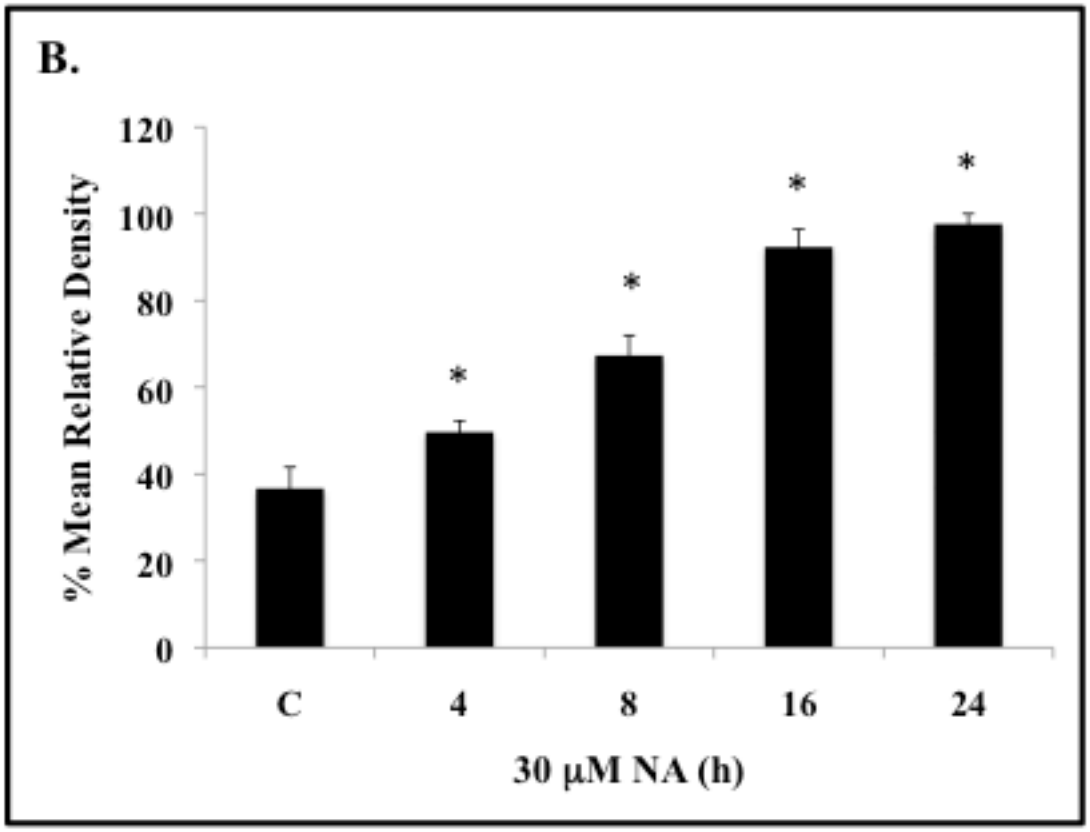
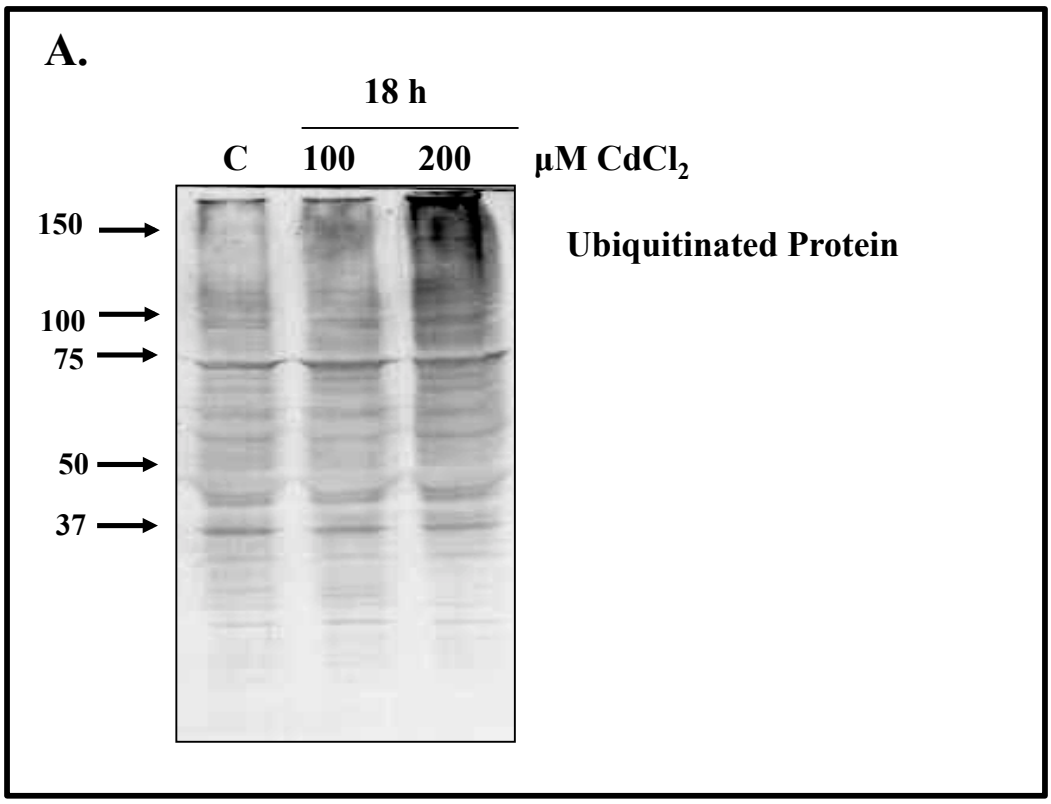

B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
*
*
C
4
8
16
24
30 μM NA (h)

Figure 12. Treatment with $CdCl_2$ increases the relative levels of ubiquitinated protein. A) Cells were maintained at 22 °C (C) or treated with 100 or 200 μM $CdCl_2$ for 18 h. Total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis of the signal intensity for the protein of the immunoblot was performed using Image J software. The data are expressed as a percentage of the lane with the maximum signal. Vertical error bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control and cells exposed to cadmium chloride are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A.
18 h
C
100
200
μM CdCl2
150
100
75
50
37
Ubiquitinated Protein

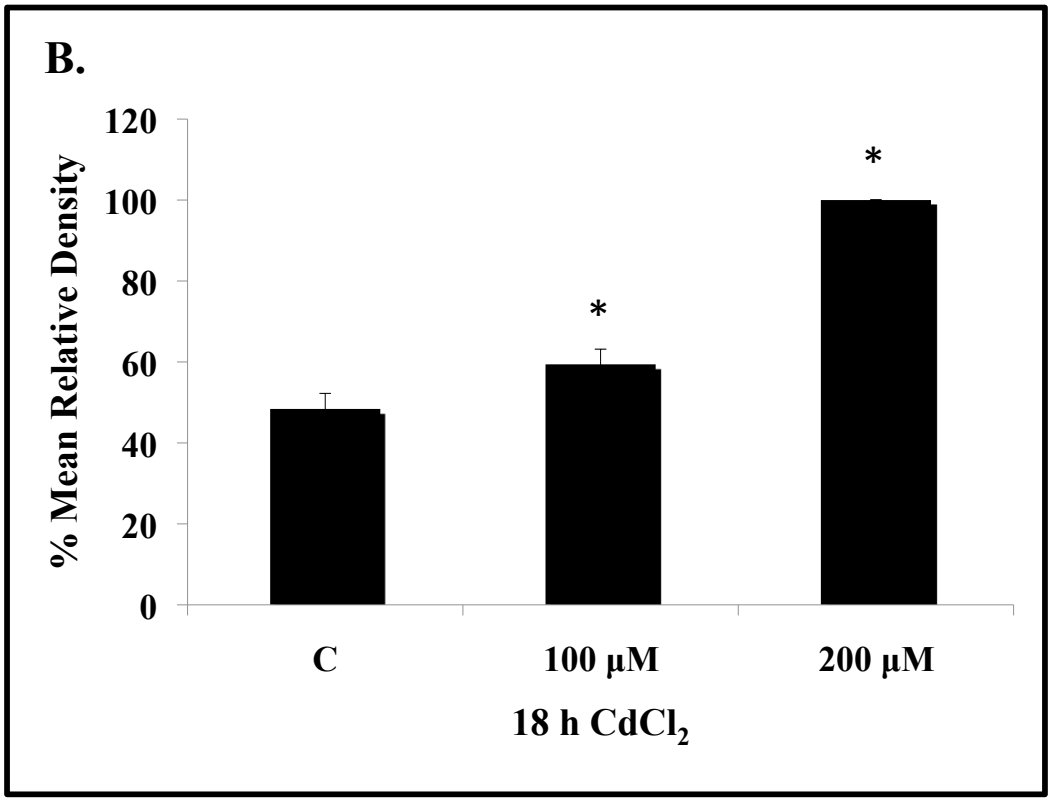
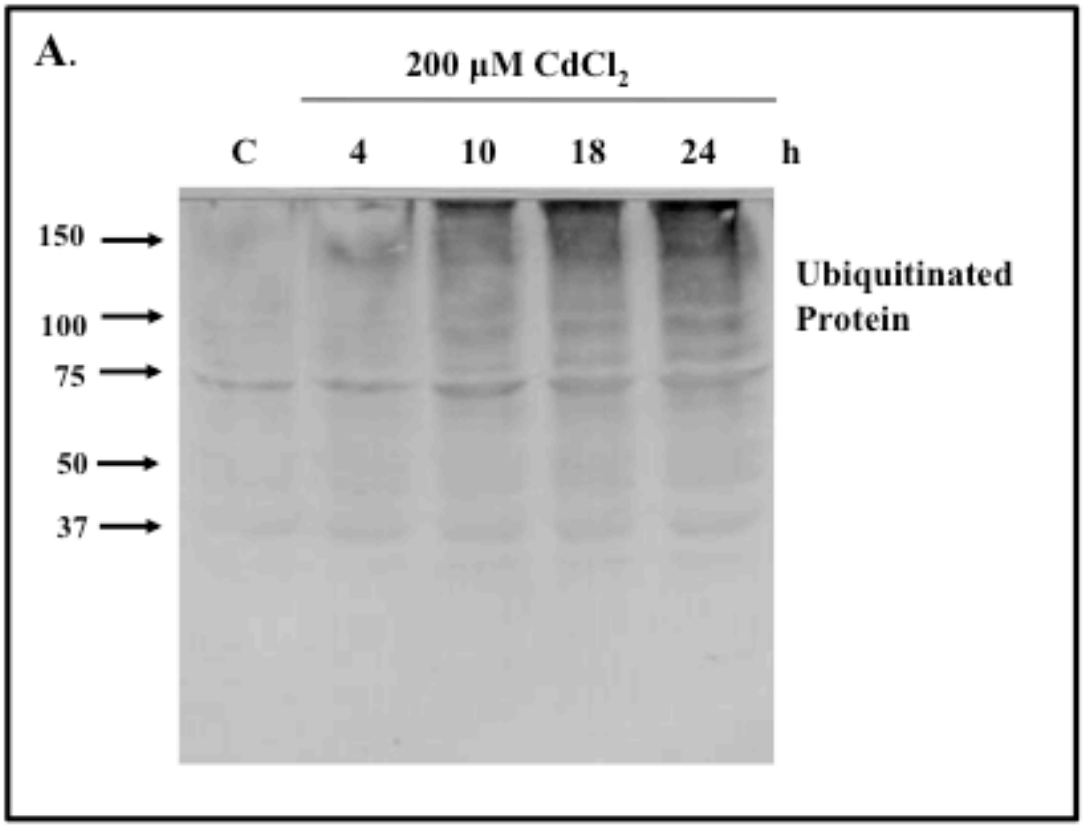
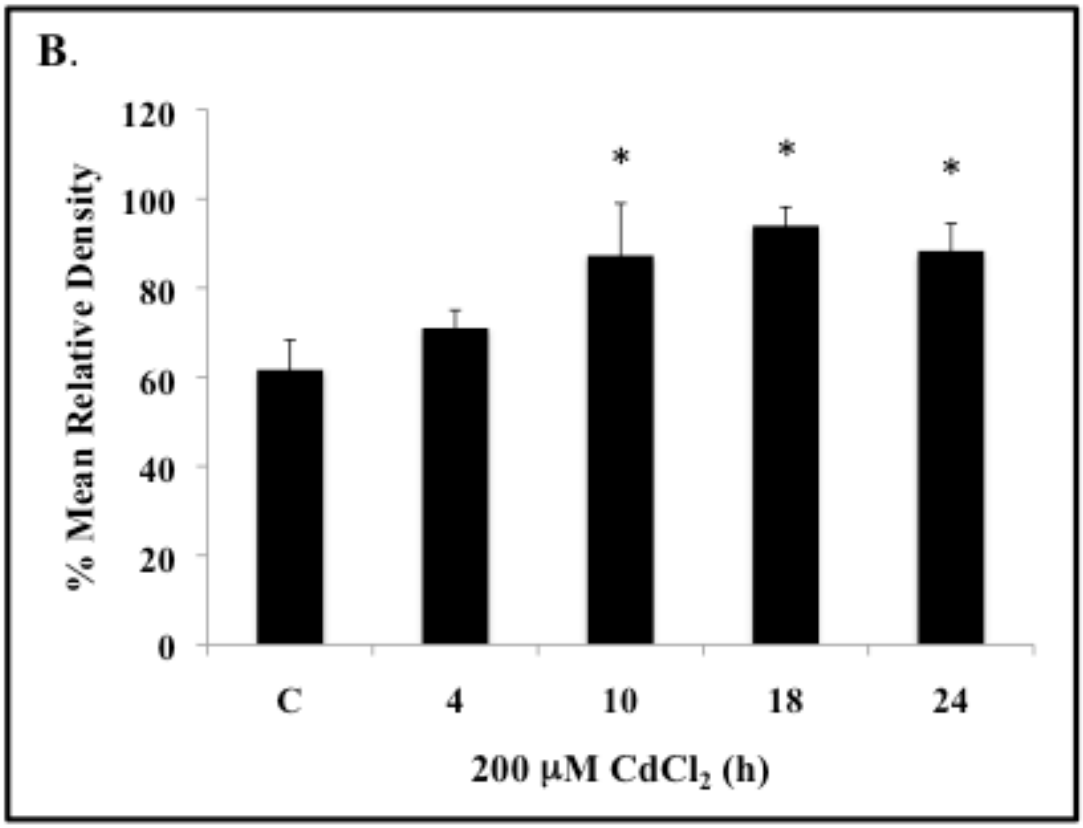

B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
C
100 μM
200 μM
18 h CdCl2

Figure 13. Temporal examination of ubiquitinated protein levels of A6 cells treated with $CdCl_2$. A) Cells were maintained at 22 °C or treated with 200 μM $CdCl_2$ for 4, 10, 18 or 24 h. After treatment, total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis was performed using Image J software to determine the signal intensity for each lane of the immunoblots. The data are expressed as a percentage of the lane with the maximum signal while vertical bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control (C) and cells exposed to 200 μM $CdCl_2$ are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A.
200 µM CdCl2
C
4
10
18
24
h
150
100
75
50
37
Ubiquitinated Protein


B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
*
C
4
10
18
24
200 µM CdCl2 (h)

3.5. NA and $CdCl_2$ decrease chymotrypsin(CT)-like activity in *Xenopus* A6 cells.

Another method to examine the impact of NA and $CdCl_2$ on proteasome activity in A6 cells involved a measurement of the relative levels of CT-like activity. CT-like activity is a component of the 26S proteasome. CT-like activity, as determined by means of a cell-based assay, decreased approximately 40% in A6 cells treated with 20 or 30 μM NA for 16 h when compared to control cells (Fig. 14). In cells treated with 100 or 200 μM $CdCl_2$ for 18 h resulted in 40% and 70% decrease, respectively, in CT-like activity compared to control (Fig. 15).

3.6 Involvement of HSF1 activation in NA- and $CdCl_2$-induced HSP and ubiquitinated protein accumulation

In order to determine any possible association between heat shock protein accumulation and proteasome activity a HSF1 inhibitor, KNK437, was employed. Previously, our laboratory demonstrated that KNK437 pretreatment inhibited both heat shock and chemical stress-induced *hsp* gene expression in A6 cells (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008; Young et al., 2009). As shown in Figures 16 and 17, when cells were pretreated with KNK437 prior to NA or $CdCl_2$, there was extensive inhibition of HSP30 and HSP70 accumulation. Subsequent analysis demonstrated that KNK437 caused 90% and 70% inhibition of 20 and 30 μM NA-induced and 100% inhibition of $CdCl_2$-induced HSP30 and HSP70 accumulation. Further examination of the relationship of HSP accumulation and proteasome activity looked at the relative levels of ubiquitinated protein after pretreatment with KNK437. Treatment with KNK437 alone had no significant effect of the relative levels of

Figure 14. Effect of NA on chymotrypsin-like activity of A6 cells. Cells were maintained at 22 °C (C) or treated with 20 or 30 μM NA for 16 h. After treatment cells were suspended in L-15 media and chymotrypsin-like activity was determined as described in the Materials & methods. The CT-like activity was measured and expressed as a percentage of control. The level of significance of the differences between samples was calculated by one-way ANOVA and a Tukey's post-test. Significant differences between control cells and cells treated with NA indicated as * ($p<0.05$). These data are representative of three separate experiments.

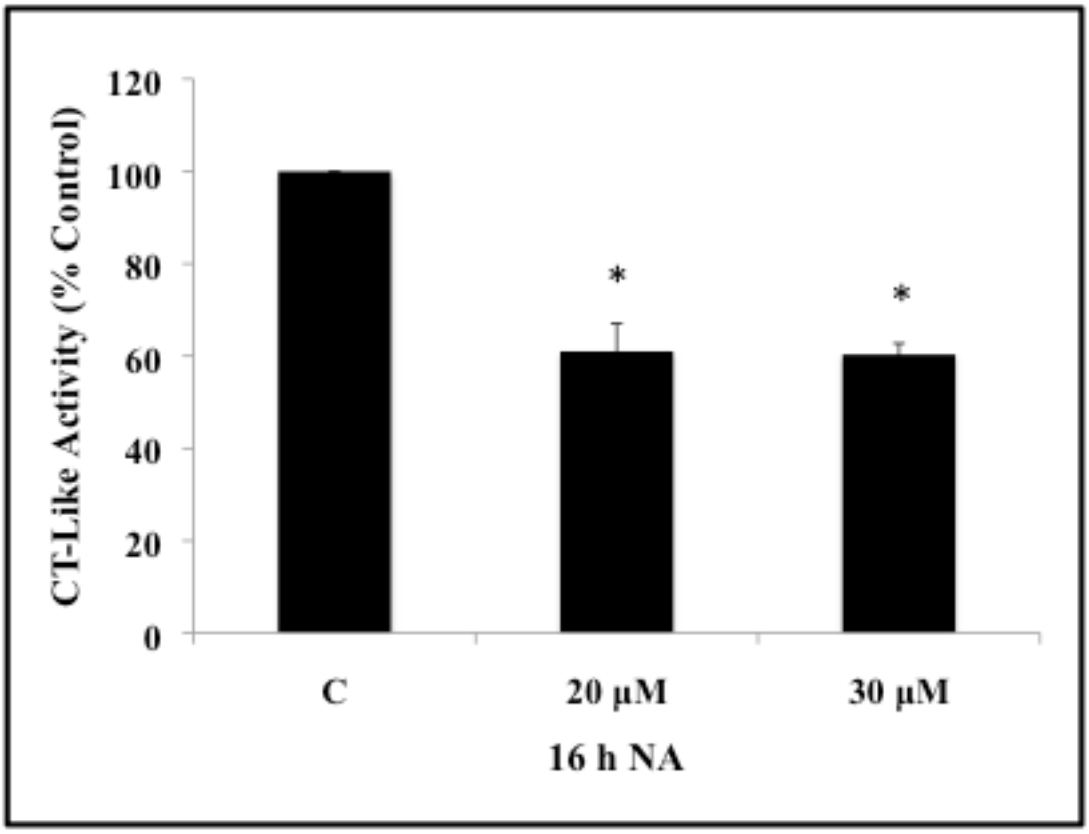

CT-Like Activity (% Control)
120
100
80
60
40
20
0
*
*
C
20 µM
30 µM
16 h NA

Figure 15. Inhibition of chymotrypsin-like activity in A6 cells treated with $CdCl_2$. Cells were maintained at 22 °C (C) or treated with 100 or 200 μM $CdCl_2$ for 18 h. The relative levels of chymotrypsin-like activity was determined and expressed as a percentage of control. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between control cells and A6 cells treated with $CdCl_2$ are indicated as * ($p<0.05$). These results were derived from three different experiments.

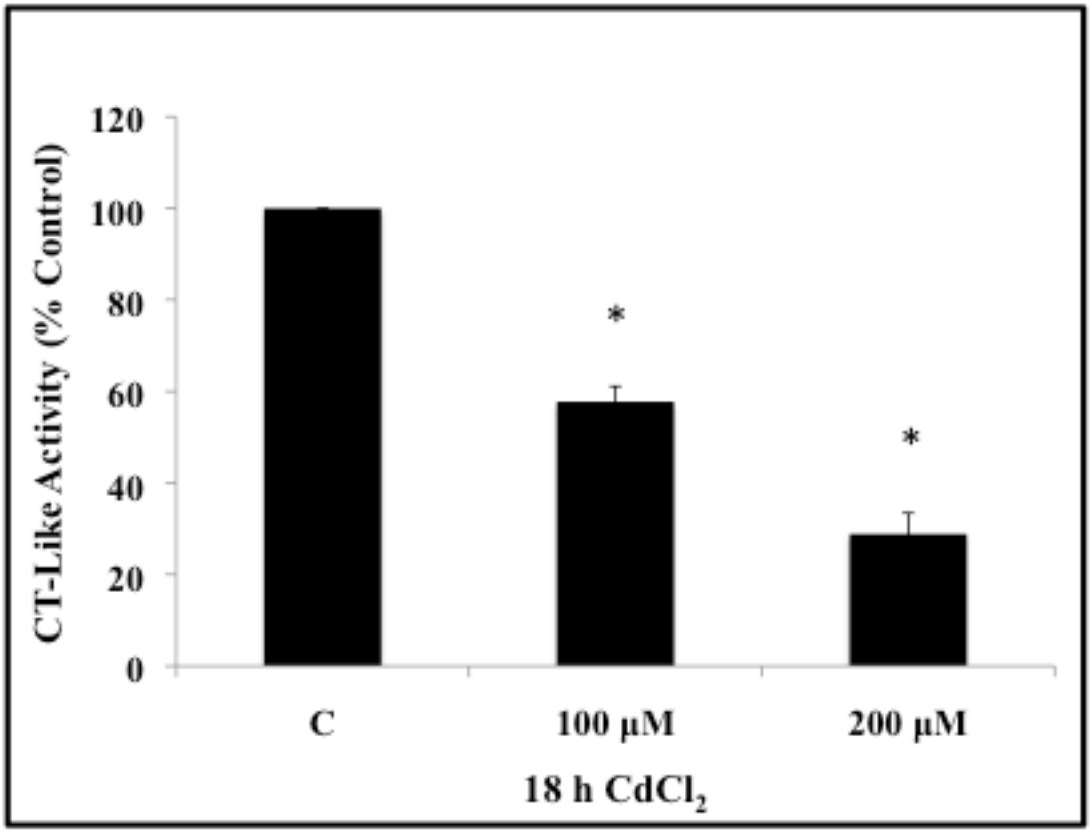
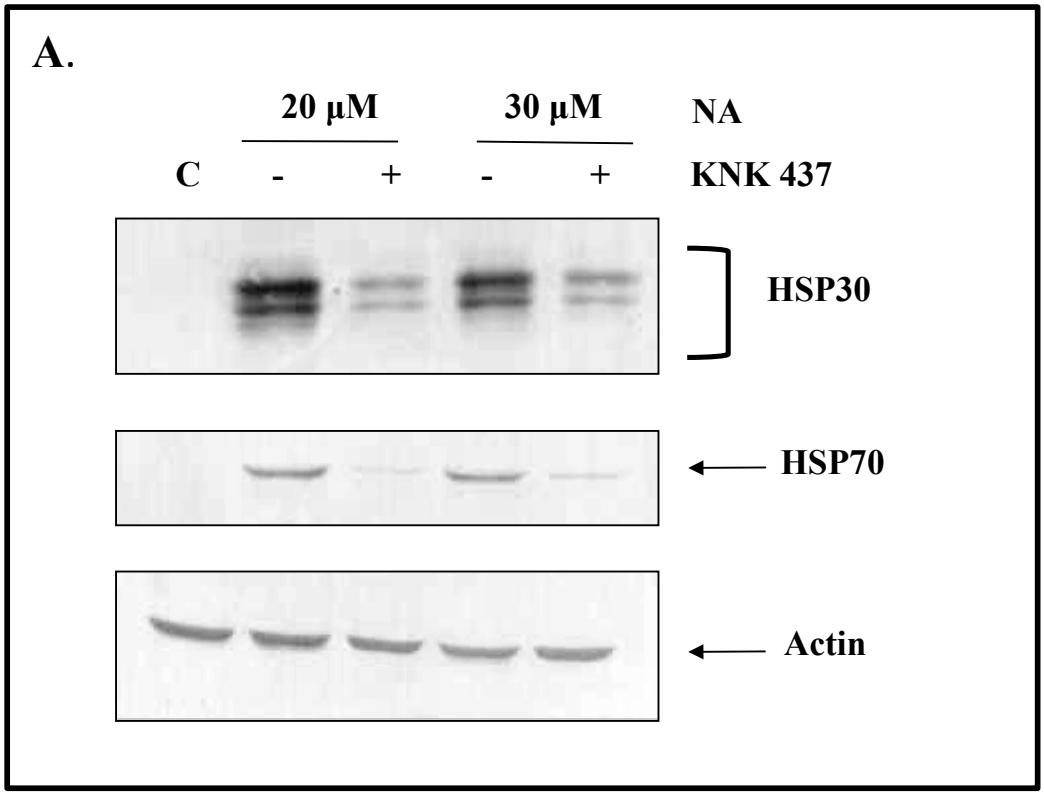

CT-Like Activity (% Control)
120
100
80
60
40
20
0
*
*
C
100 µM
200 µM
18 h CdCl2

Figure 16. Effect of KNK437 on NA-induced HSP30 and HSP70 accumulation. Cells were maintained at 22 °C (C) or treated with 20 or 30 μM NA with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis. B) Image J software was used to carry out densitometric analysis of HSP30 (black) and HSP70 (white) bands in the western blot images. Results are expressed as a percentage of the signal acquired without pretreatment with KNK437 for each protein. Vertical error bars represent the standard error. The level of significance of the differences between samples with and without pretreatment with KNK437 was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between cells with or without pretreatment with KNK437 are indicated as * ($p<0.05$). These results are representative of at least three different experiments.


A.
20 µM
30 µM
NA
C
-
+
-
+
KNK 437
HSP30
HSP70
Actin

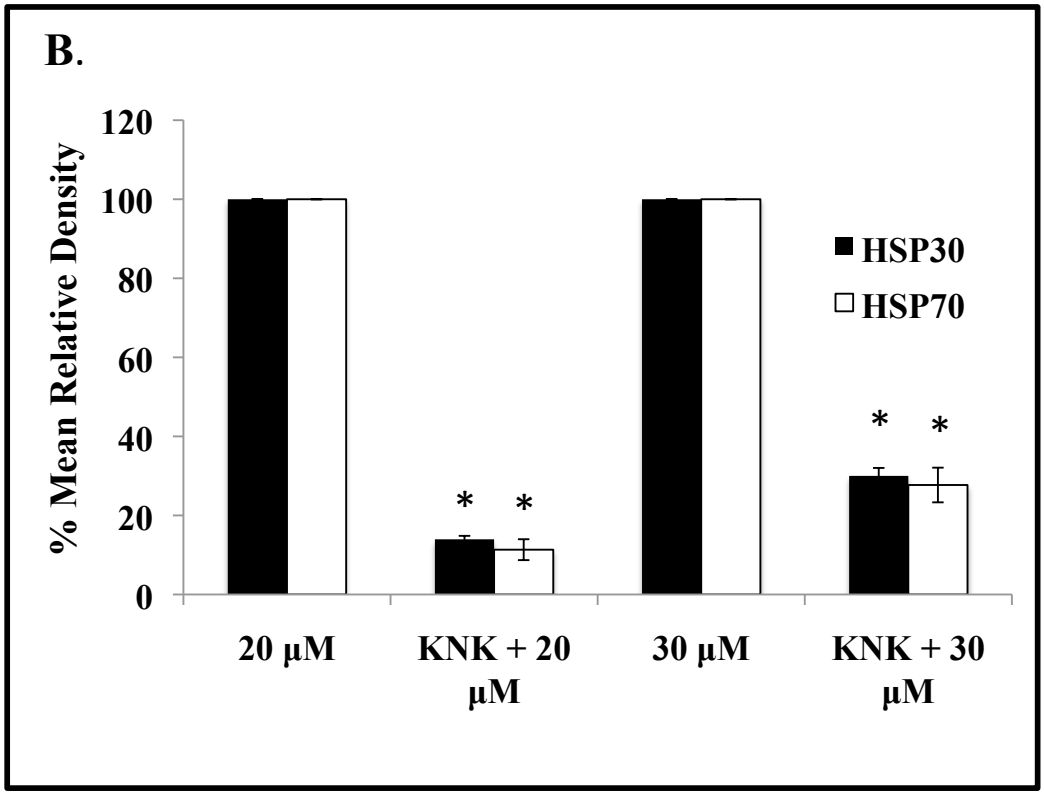
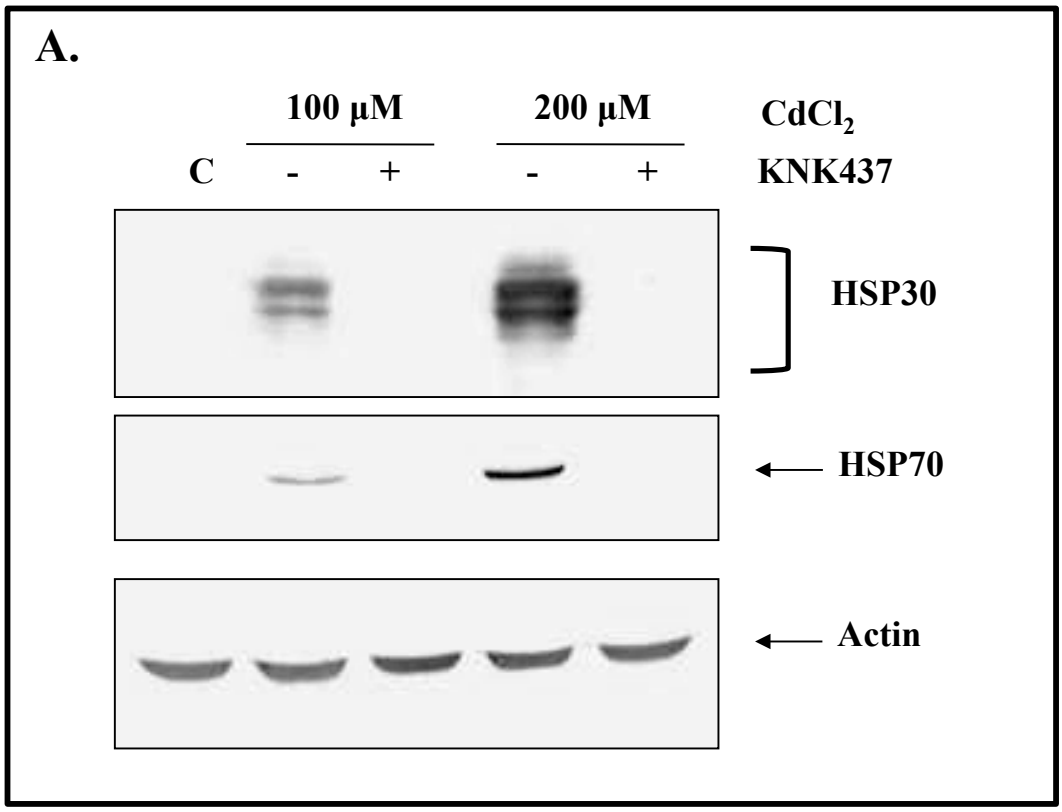
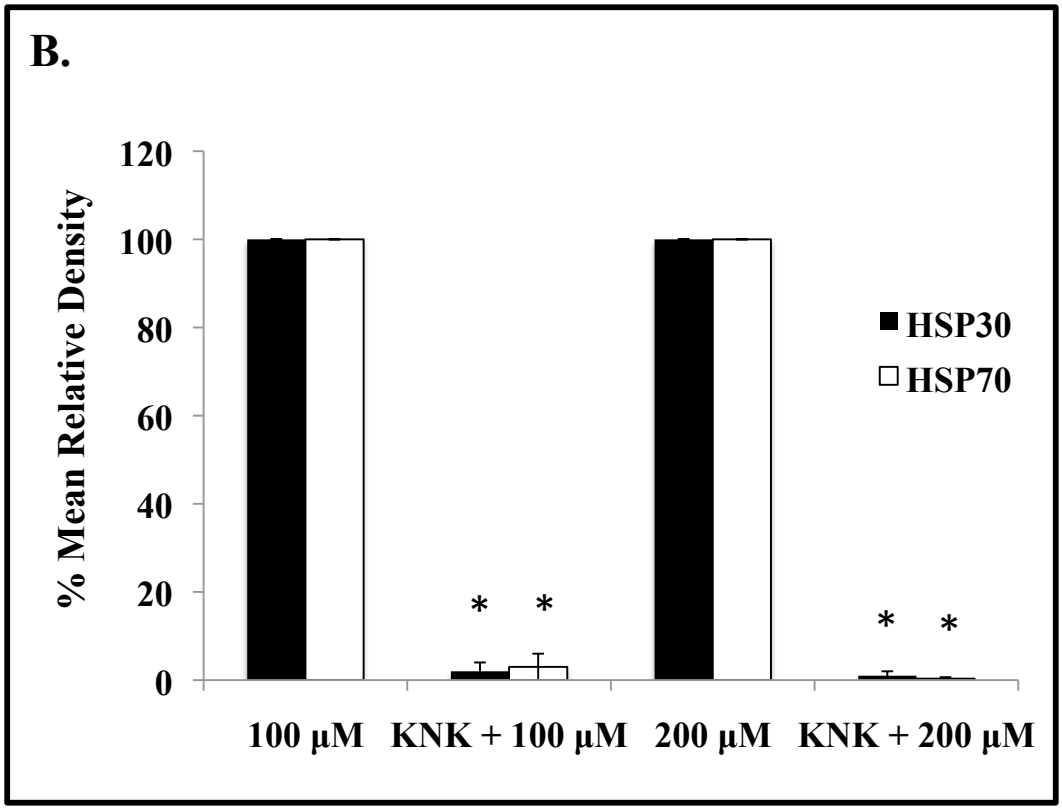

B.
% Mean Relative Density
120
100
80
60
40
20
0
HSP30
HSP70
*
*
*
*
20 µM
KNK + 20 µM
30 µM
KNK + 30 µM

Figure 17. Effect of KNK437 on $CdCl_2$-induced HSP30 and HSP70 accumulation. Cells were maintained at 22 °C or treated with 100 or 200 μM $CdCl_2$ with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437 at 22 °C. Total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis of HSP30 (black) and HSP70 (white) bands in the western blot images was carried out using Image J software. Results are expressed as a percentage of the signal acquired without pretreatment with KNK437 for each protein. Vertical error bars represent the standard error. The level of significance of the differences between samples with and without pretreatment with KNK437 was calculated by one-way ANOVA with a Tukey's post-test. Significant differences between cells with or without pretreatment with KNK437 are indicated as * ($p<0.05$). These results are representative of at least 3 different experiments.


A.
100 µM
200 µM
CdCl2
C
-
+
-
+
KNK437
HSP30
HSP70
Actin

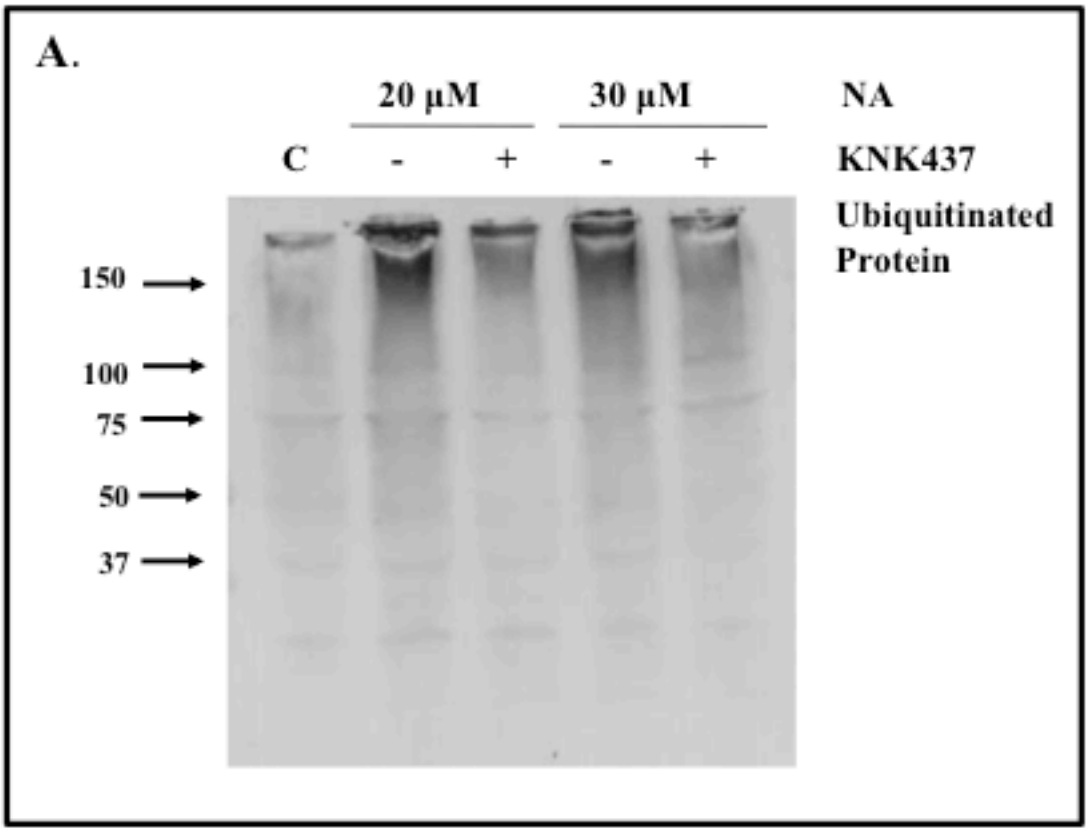

B.
% Mean Relative Density
120
100
80
60
40
20
0
HSP30
HSP70
*
*
*
*
100 µM
KNK + 100 µM
200 µM
KNK + 200 µM

ubiquitinated protein. A 2 h pretreatment with KNK437 followed by 16 h NA reduced the levels of ubquitinated protein. Densitometric analysis revealed that ubiquitinated protein levels decreased by 25% and 15% when pretreated with KNK437 followed by 20 and 30 μM NA, respectively, as shown in figure 18. Figure 19 shows the effect of pretreatment with KNK437 prior to incubation with $CdCl_2$ on the relative levels of ubiquitinated proteins. Significant decreases of 25% and 40% were seen in A6 cells pretreated by KNK437 followed by 100 or 200 μM $CdCl_2$, respectively, for 18 h. Pretreatment with KNK437 also had no effect on cellular morphology, which is in agreement with results found previously in our laboratory (Voyer and Heikkila, 2008).

3.7 KNK437 had no effect on NA- or $CdCl_2$-induced inhibition of CT-like activity.

In order to examine the effect of inhibiting HSP accumulation on proteasome activity, A6 cells were pretreated with KNK437 prior to NA or $CdCl_2$ and CT-like activity was measured. KNK437, alone, had no effect on CT-like activity compared to control cells. As previously mentioned treatment of A6 cells with NA decreased CT-like activity by approximately 40% compared to control cells. A 2 h pretreatment with KNK437 followed by 16 h NA resulted in no significant change between cells pretreated compared to cells without a pretreatment with KNK437 (Figure 20). In A6 cells treated with 100 μM or 200 μM $CdCl_2$ there was a decrease of approximately a 35% and 75%, respectively, compared to control cells. In cells pretreated with KNK437 prior to $CdCl_2$ there was no significant difference in the CT-like activity of cells compared to cells treated with $CdCl_2$ without pretreatment (Figure 21).

Figure 18. KNK437 decreases the relative level of ubiquitinated protein. A) Cells were maintained at 22 °C or treated with 20 or 30 μM NA with (+) or without (-) a 2 h pretreatment with 100 μM KNK437. After treatment cells were harvested and total protein was isolated and subjected to immunoblot analysis. B) Densitometric analysis of ubquitinated protein was performed on immunoblots using Image J software. Results are expressed as a percentage of NA treatment without KNK437 pretreatment. Vertical bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant difference between cells with or without pretreatment is represented by * ($p<0.05$) or ∇ ($p<0.15$). These results are representative of 3 separate experiments.


A.
20 µM
30 µM
NA
C
-
+
-
+
KNK437
Ubiquitinated Protein
150
100
75
50
37

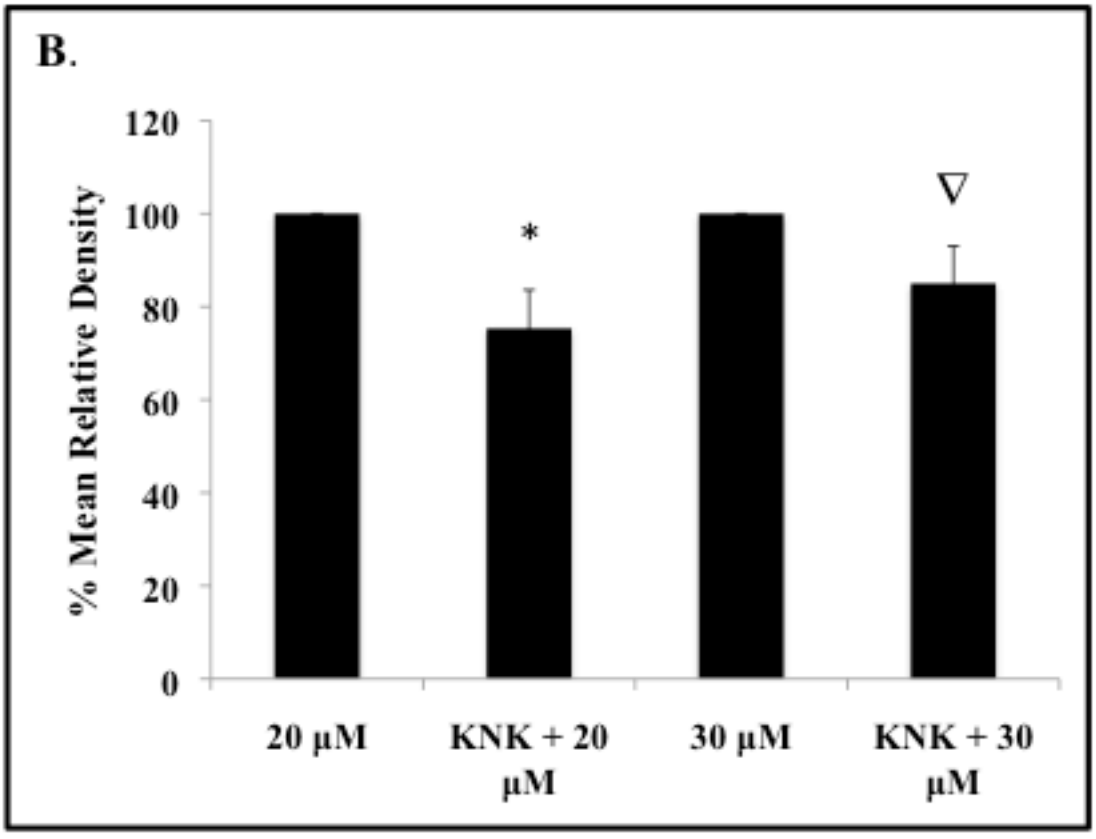
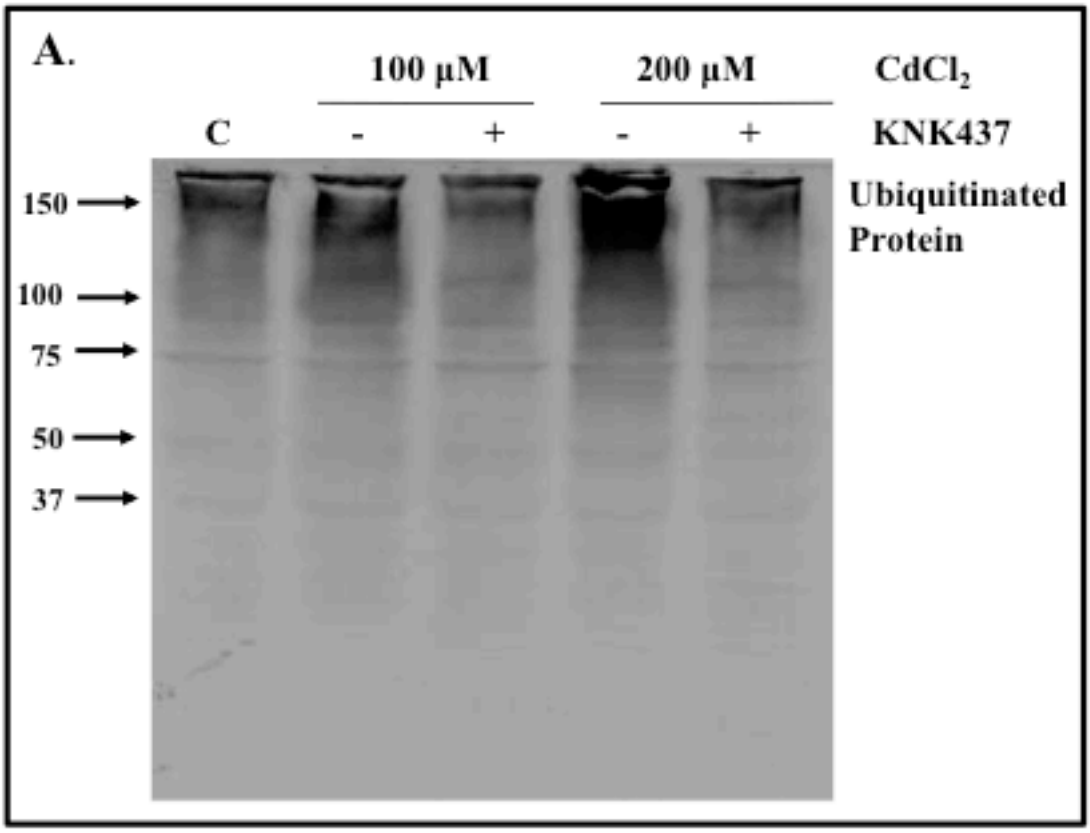

B.
% Mean Relative Density
120
100
80
60
40
20
0
*
∇
20 µM
KNK + 20 µM
30 µM
KNK + 30 µM

Figure 19. The relative levels of ubiquitinated protein in cells pretreated with KNK437. A) Cells were maintained at 22 °C or treated with 100 or 200 μM $CdCl_2$ with (+) or without (-) a 2 h pretreatment with 100 μM KNK437. Total protein was isolated and subjected to immunoblot analysis. B) Image J software was used to carry out densitometric analysis of ubiquitinated protein on immunoblots. Results are expressed as a percentage of $CdCl_2$ treatment without KNK437 pretreatment. Vertical bars represent the standard error. The level of significance of the differences between samples was calculated by one-way ANOVA with a Tukey's post-test. Significant difference between cells with or without pretreatment with KNK437 is represented by * ($p<0.05$). These results are representative of 3 separate experiments.


A.
100 µM
200 µM
CdCl2
C
-
+
-
+
KNK437
150
100
75
50
37
Ubiquitinated Protein

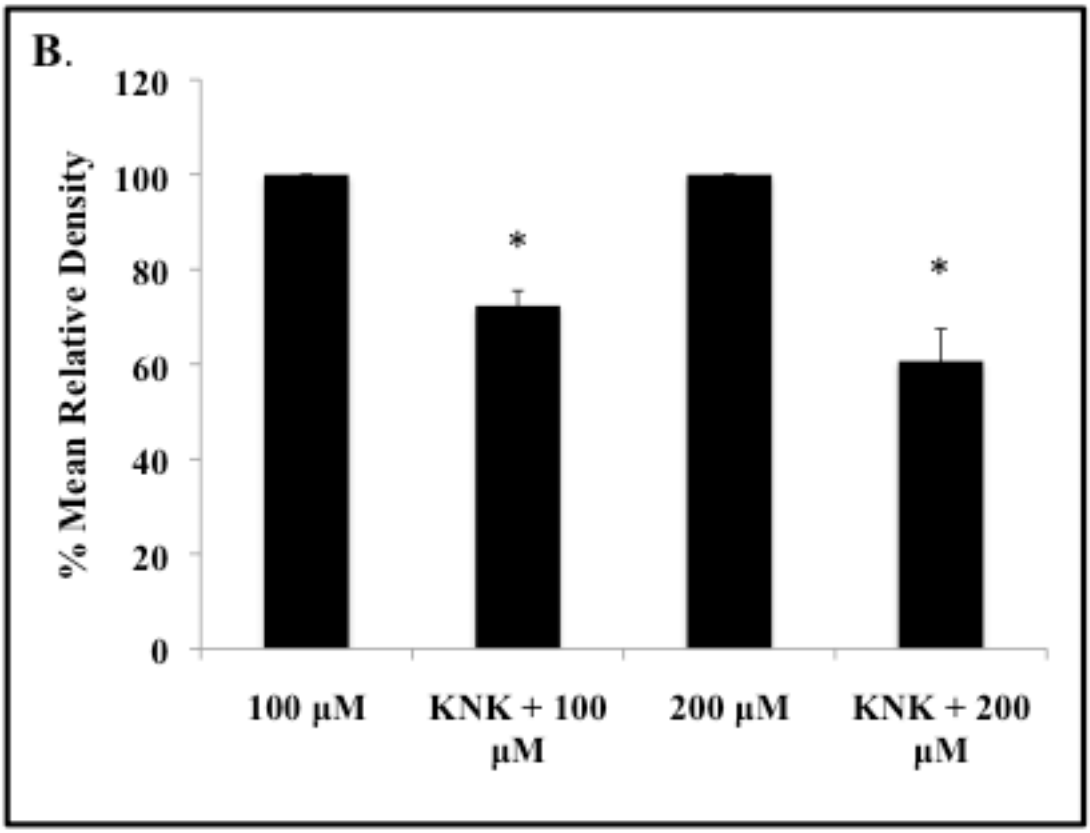

B.
% Mean Relative Density
120
100
80
60
40
20
0
*
*
100 µM
KNK + 100 µM
200 µM
KNK + 200 µM

Figure 20. Pretreatment with KNK437 had no effect on NA-induced changes in CT-like activity. Cells were maintained at 22 °C or treated with 20 or 30 μM NA with or without a 2 h pretreatment with 100 μM KNK437. The CT-like activity was measured and expressed as a percentage of control. The level of significance of the differences between samples with or without pretreatment with KNK437 was calculated by one-way ANOVA with a Tukey's post-test. No significant difference was noted between samples with and without pretreatment with KNK437. These results are representative of 3 separate experiments.

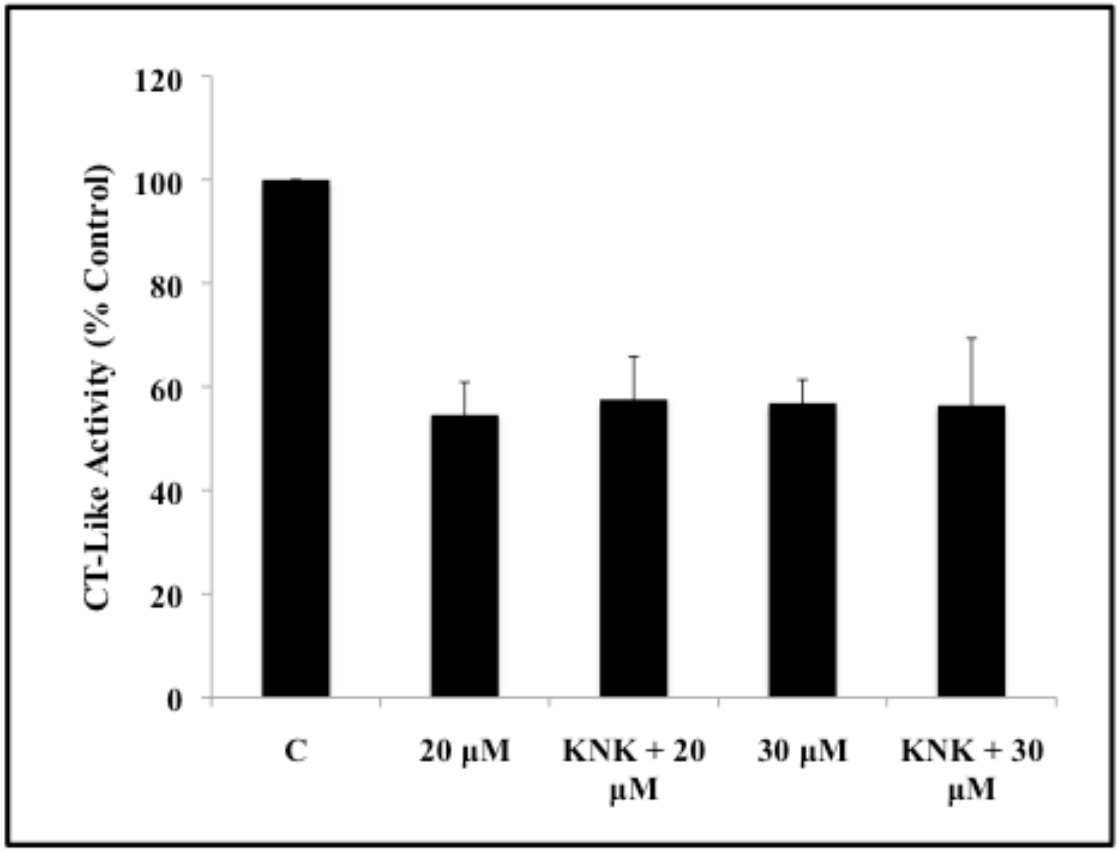
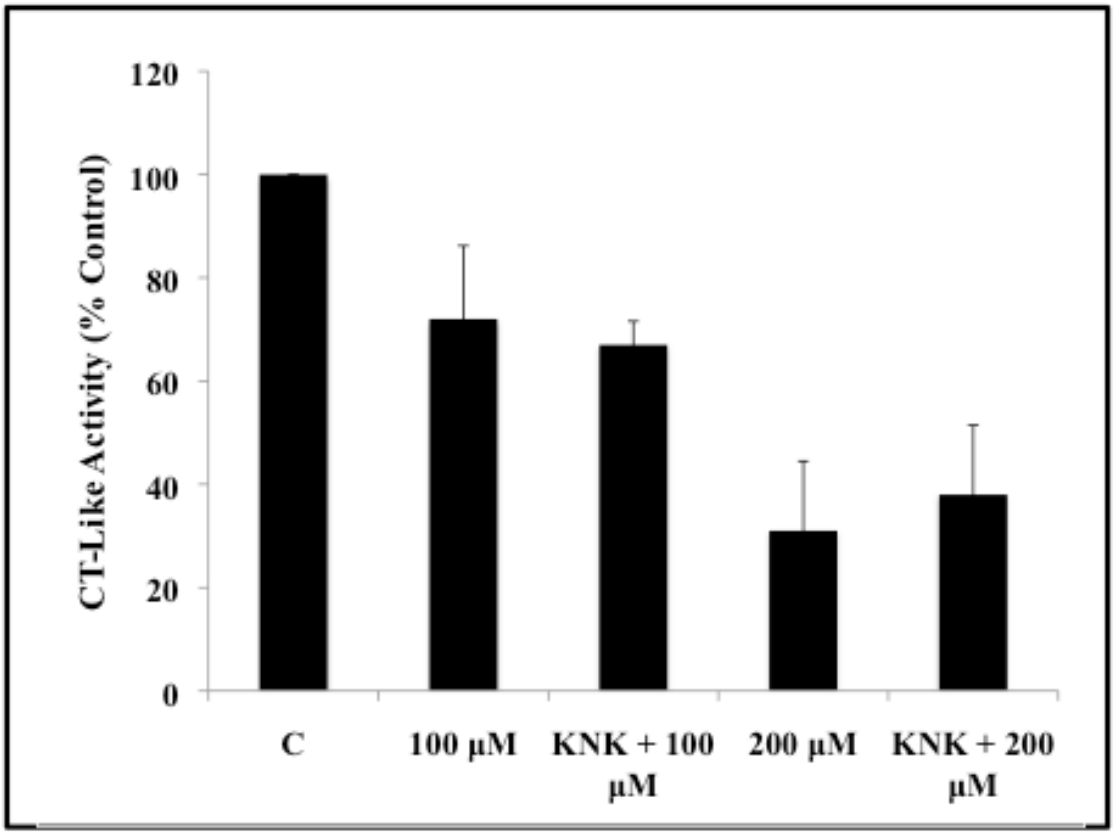

CT-Like Activity (% Control)
120
100
80
60
40
20
0
C
20 µM
KNK + 20 µM
30 µM
KNK + 30 µM

Figure 21. CT-like activity of A6 cells treated with $CdCl_2$ with or without pretreatment with KNK437. Cells were maintained at 22 °C or treated with 100 or 200 μM $CdCl_2$ with or without a 2 h pretreatment with 100 μM KNK437. The CT-like activity was measured and expressed as a percentage of control. The level of significance of the differences between samples with or without pretreatment with KNK437 was calculated by one-way ANOVA with a Tukey's post-test. No significant difference was observed between samples with and without pretreatment with KNK437. These results are representative of 3 separate experiments.


CT-Like Activity (% Control)
120
100
80
60
40
20
0
C
100 µM
KNK + 100 µM
200 µM
KNK + 200 µM

4. Discussion

The present study has shown that NA and $CdCl_2$ can induce HSP accumulation and inhibit proteasomal activity in *Xenopus laevis* A6 kidney epithelial cells. For example, treatment of A6 cells with 20 or 30 μM NA significantly increased HSP30 and HSP70 accumulation compared to control. Furthermore, the relative levels of HSP30 and HSP70 increased in a time-dependent manner from 8 to 24 h when treated with 30 μM NA or 200 μM $CdCl_2$. These findings are in agreement with earlier studies showing that treatment of A6 cells with NA or $CdCl_2$ induced HSP30 and HSP70 accumulation (Ohan et al., 1998; Phang et al., 1999; Fernando and Heikkila, 2000; Gellalchew and Heikkila, 2005; Young et al., 2009; Woolfson and Heikkila, 2009). In other systems, arsenite-treated rat astrocytes or human bladder urothelial cells showed significantly higher levels of HSPs compared to control cells (Fauconneau et al., 2002; Rossi et al., 2002). Also, in cadmium treated rat kidney NRK-52E cells and zebrafish embryos increases in HSP levels were reported (Madden et al., 2002; Hallare et al., 2005).

Immunocytochemistry and LSCM were employed to verify the immunoblot analyses and to examine the localization of HSP30 in A6 cells treated with NA or $CdCl_2$. Cells exposed to NA (20 and 30 μM) or $CdCl_2$ (100 and 200 μM) displayed enhanced accumulation of HSP30 primarily in the cytoplasm in a punctate pattern. The granular pattern of stress-induced HSP30 accumulation may be caused by the formation of multimeric structures which are required for sHSP function (Ohan et al., 1998; MacRae, 2000; Van Montfort et al., 2001). The actin cytoskeleton has been used an indicator of cellular viability and health (Wiegant et al., 1987; Ohtsuka et al., 1993). The current study showed that treatment of A6 cells with NA or $CdCl_2$ resulted in the presence of

actin aggregates which suggests disruption of the actin cytoskeleton. These results are comparable to those found previously in our laboratory (Gellalchew and Heikkila, 2005; Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009).

The exact mechanism of activation of *hsp* gene expression in response to NA or $CdCl_2$ in A6 cells is not known at this time. However, The regulation of NA- or $CdCl_2$-induced HSP accumulation in *Xenopus* occurs, at least in part, at the level of transcription since both of these stressors can induce HSF1-HSE binding as detected by gel mobility shift analysis (Gordon et al., 1997). Also, our laboratory recently determined in studies employing KNK437, an inhibitor of HSP accumulation, that *hsp* gene expression in *Xenopus* cells by NA or $CdCl_2$ is likely controlled, at least in part, by the activation of HSF1 (Voyer and Heikkila, 2008). Under stress conditions, unfolded or damaged protein is thought to be a signal for the activation of *hsp* gene expression (Voellmy et al., 2004). Increased concentrations of non-native proteins can result in aggregate formation, which is detrimental to cell function (Hartl 1996; Lee and Goldberg, 1998; Hartl and Hayer-Hartl, 2009). It has been reported that arsenite toxicity may be caused by a decrease in sulfhydryl groups and by generating reactive oxygen species which increase the levels of protein carbonyls (Chen et al., 1998; Liu et al., 2001; Del Razo et al., 2001; Samuel et al., 2005). Also, it was suggested that cadmium substitutes for zinc in proteins and reacts with thiol groups within cells to induce the accumulation of abnormal proteins (Waisberg et al., 2003; Galazyn-Sidorczuk et al., 2009). Therefore, treatment of cells with NA or $CdCl_2$ may lead to an increase in the intracellular level of damaged proteins which initiates the activation of HSF1 leading to *hsp* gene expression.

In *Xenopus laevis* A6 cells, like other eukaryotic systems, the ubiquitin-proteasome system (UPS) is the primary degradation pathway for misfolded or damaged proteins (Lee and Goldberg, 1998; Malik et al., 2001). Previous studies have shown that inhibiting the activity of the proteasome leads to the accumulation of damaged or unfolded proteins within cells which are targeted for degradation by the addition of polyubiquitin chains (Bush et al., 1997; Lee and Goldberg, 1998; Malik et al., 2001; Liao et al., 2006). The 26S proteasome is responsible for enzymatic degradation of ubiquitin tagged proteins. In other studies, an indicator of proteasome inhibition is an increase in the relative levels of ubiquitinated protein (Mimnaugh et al., 1997; Melikova et al., 2006). In *Xenopus* A6 cells incubation with 20 or 30 μM NA increased the relative levels of ubiquitinated protein by 1.6- and 1.7-fold, respectively. Also, treatment of A6 cells with 100 or 200 μM $CdCl_2$ increased the relative levels of ubiquitinated protein by 0.5- and 2-fold, respectively. In a survey of the scientific literature, the effect of NA on proteasome activity has been examined only in mammalian cells (Tsou et al., 2005; Medina-Diaz et al., 2009; Kirkpatrick, 2003). For example, an increase in ubiquitinated protein was shown in mouse CYP3A4 cells following treatment with NA or other arsenite metabolites (Medina-Diaz et al., 2009). Also, the relative levels of ubiquitinated protein in rabbit renal-cortical slices and human embryonic kidney cells (HEK293) increased following treatment with arsenite (Kirkpatick et al., 2003). Furthermore, Kirkpatrick et al. (2003) demonstrated that treatment with arsenite had no effect on the activity of the ubiquitin-conjugating enzymes suggesting that an increase in ubiquitinated protein results from impaired degradation of targeted proteins. Finally, $CdCl_2$-induced increases in ubiquitinated protein levels has been demonstrated only in mammalian cells with the

exception of studies employing two different marine bivalves, mussels and clams (Thevenod and Friedmann, 1999; Figueiredo-Pereira and Cohen, 1999; Othumpangat et al., 2005; McDonagh and Sheegan, 2006; Chora et al., 2008; Li et al., 2008; Yu et al., 2008; McDonagh, 2009; Yu et al., 2011).

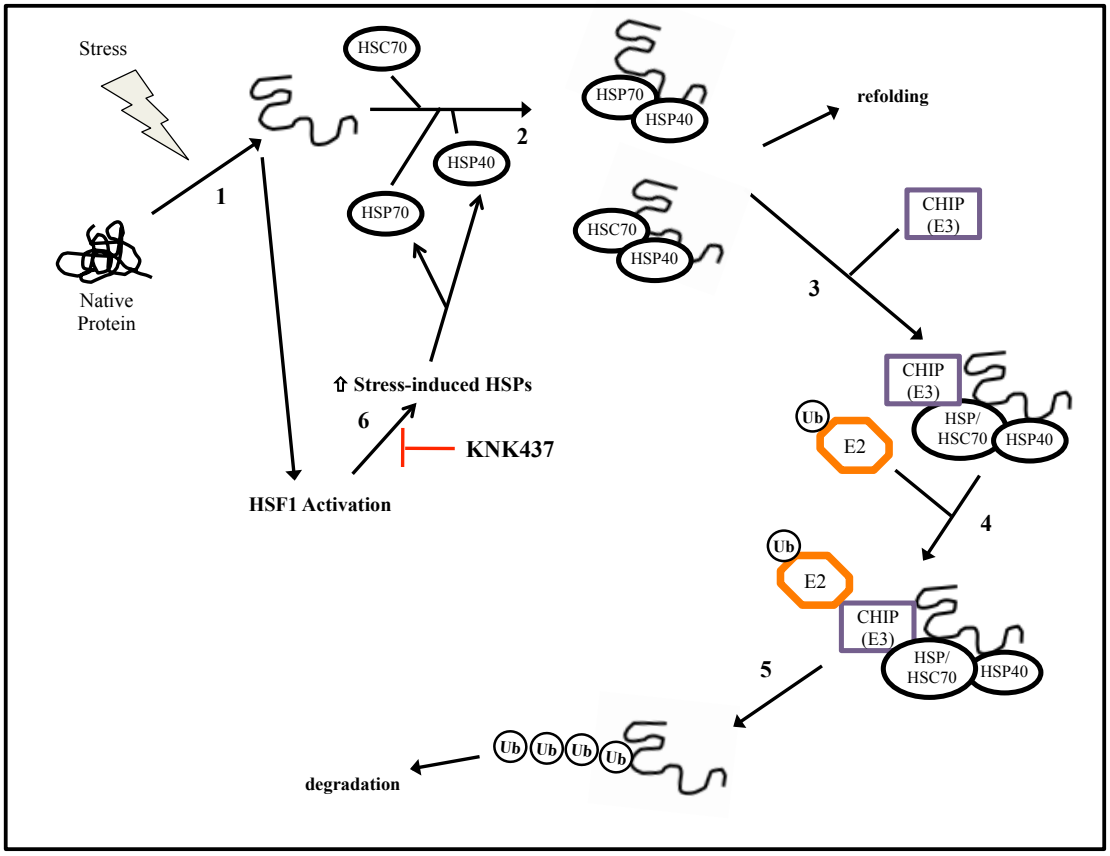
In order to further examine the effect of NA and $CdCl_2$ agents on proteasome activity in A6 cells, a cell-based assay was used to examine chymotrypsin (CT)-like activity, which is a component of the 26S proteasome. The present study revealed that treatment with 20 or 30 μM NA decreased CT-like activity in A6 cells by 40% compared to control cells. A6 cells treated with 100 or 200 μM $CdCl_2$ showed a decrease in CT-like activity of 40% and 70%, respectively. Taken together, the NA- or $CdCl_2$-induced accumulation of ubiquitinated protein and the decrease in chymotrypsin-like activity was suggestive of NA- and $CdCl_2$-induced proteasomal inhibition in *Xenopus laevis* A6 cells. In other studies which examined only mammalian systems, rabbit renal-cortical slices treated with 10 μM arsenite for 24 h displayed a 40% decrease in CT-like activity compared to control (Kirkpatrick et al., 2003). Also, in HEK293 cells a 15% decrease in CT-like activity was observed after 24 h treatment with 10 μM arsenite in comparison to control cells (Kirkpatrick et al., 2003). Finally, in rat sertoli cells, treatment with 10 or 20 μM cadmium resulted in no significant changes in CT-like activity compared to control (Yu et al., 2008). While the exact mechanism of NA or $CdCl_2$-induced proteasome inhibition in A6 cells is not known at this time, it is possible that these agents may damage key proteins involved in protein ubiquitination or the normal functioning of the proteasome complex (Rea et al., 2003; Su et al., 2006; Yu et al., 2010).

As previously mentioned it is likely that the induction of *hsp* gene expression in *Xenopus* by NA or $CdCl_2$ involves the activation of HSF1 and binding to the HSE (Ovsenek and Heikkila, 1990). To examine a possible association between NA- or $CdCl_2$-induced HSP accumulation and proteasomal activity an HSF1 inhibitor, KNK437, was employed. KNK437 is a benzylidene lactam compound which has been shown to inhibit HSF1 activation (Yokota et al., 2000). Treatment of human tumour cells with KNK437 inhibited the acquisition of thermotolerance and prevented their protection from heat-induced apoptosis (Yokota et al., 2000; Sahin et al., 2011). Previously, our laboratory demonstrated that KNK437 was capable of inhibiting heat-induced *hsp30*, *hsp47* and *hsp70* mRNA and HSP30 and HSP70 protein accumulation in A6 cells (Manwell & Heikkila, 2007; Voyer and Heikkila, 2008). The present study examined the effect of KNK437 pretreatment on NA- and $CdCl_2$-induced HSP accumulation and the inhibition of proteasomal activity. A 2 h pretreatment with KNK437 followed by incubation with NA or $CdCl_2$ significantly reduced both HSP30 and HSP70 accumulation in A6 cells which confirmed previous studies in our laboratory (Voyer and Heikkila, 2008). While KNK437, alone, had no significant effect on the levels of ubiquitinated protein, pretreatment with KNK437 induced a 25% and 20% reduction in the relative levels of ubiquitinated protein in cells treated with 20 and 30 μM NA, respectively, compared to A6 cells treated with NA and no pretreatment. In A6 cells treated with 100 and 200 μM $CdCl_2$, pretreatment with KNK437 reduced the ubiquitinated protein levels by 30% and 40%, respectively. However, KNK437 pretreatment had no effect on the NA- or $CdCl_2$-induced inhibition of CT-like activity. These results suggest that inhibiting

HSP accumulation by KNK437 inhibited the ubiquitination of damaged proteins induced by NA or $CdCl_2$.

Recent studies have shown that HSP70 can have an effect on client protein turnover by promoting protein ubiquitination (Pratt et al., 2010). Ubiquitination involves three sequential steps catalyzed by activating (E1), conjugating (E2) and ligase (E3) enzymes. In previous studies it was suggested that HSP70 is the main substrate recognition factor of the carboxyl terminus of Hsc70-interacting protein (CHIP), an E3 ubiquitin ligase (Esser et al., 2004). CHIP binds to HSC70 and HSP70 through its amino-terminal tetratricopeptide domain and binds to E2 conjugating enzymes by the carboxy-terminal U-box domain. Peng et al. (2004) reported that in the absence of chaperones there is a basal level of polyubiquitination, however the addition of purified HSP70 and HSP40 to the CHIP-containing reaction enhanced the ubiquitination of protein. Also, the cystic fibrosis transmembrane conductance receptor undergoes degradation that is both CHIP and HSP70 dependent (Meacham et al., 2001). Finally, luciferase undergoes CHIP-dependent ubiquitination when it is misfolded and bound to HSP70 (Murata et al., 2001). These studies indicate that CHIP preferentially ubiquitinates HSP70-bound substrates. However, it is also possible that inhibiting HSP70 accumulation (and possibly other HSPs) could have a negative effect on the mechanism by which ubiquitination occurs. A simplified model of the possible interaction of HSP70 and CHIP during ubiquitination is shown in Figure 20. Stressful stimuli cause native proteins in the cell to unfold, which recruits molecular chaperones such as HSP70 to prevent aggregation of unfolded proteins. CHIP targets HSP70 bound substrates for ubiquitination and binds to HSP70 and E2 conjugating enzymes which

Figure 22. Proposed model of HSP70-dependent ubiqutination by CHIP. (1) Stressful stimuli cause native proteins in the cell to unfold which activates HSF1. (2) This recruits HSP70 and other molecular chaperones to prevent aggregation of unfolded proteins. (3) CHIP, an E3 ubiquitin ligase, targets HSP70 bound substrates for ubiquitination and binds to HSP70 and E2 conjugating enzymes (4). This results in polyubiquitinated proteins that are destined for degradation (5). KNK437 inhibits HSF1 activation which decreases the amount of stress-inducible HSPs and the number of HSP70 bound substrates that can undergo ubiquitination (6).

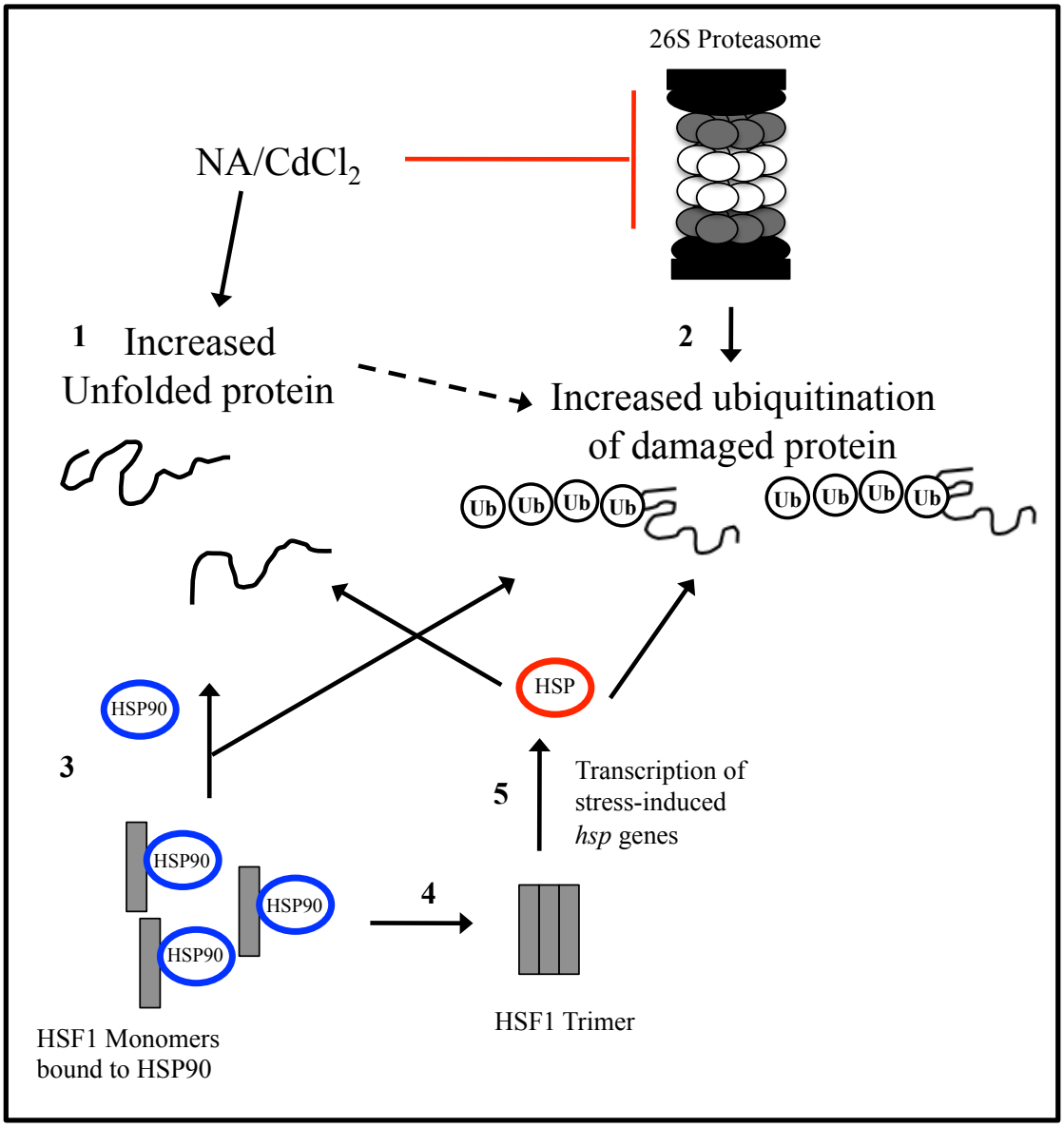

Stress
Native Protein
1
HSC70
HSP40
HSP70
2
HSP70
HSP40
refolding
HSC70
HSP40
CHIP (E3)
3
⇧ Stress-induced HSPs
6
KNK437
HSF1 Activation
CHIP (E3)
Ub
E2
HSP/ HSC70
HSP40
4
Ub
E2
CHIP (E3)
HSP/ HSC70
HSP40
5
Ub Ub Ub Ub
degradation

results in polyubiquitinated proteins destined for degradation. A sHSP, HSP27, was shown to interact with polyubiquitin chains and suggested to be an activator of the ubiquitin-proteasome system. Parcellier et al. (2003) demonstrated that HSP27 may be involved in the ubiquitination of several cellular proteins, but the mechanism of how these proteins are selected for HSP27-mediated ubiquitination remains to be determined. Given this latter study, it is possible that the *Xenopus* sHSP, HSP30, may play a similar role in protein ubiquitination.

An overview of how NA and $CdCl_2$ could induce *hsp* gene expression through increased levels of unfolded protein and/or ubiquitinated damaged protein is shown in Figure 21. Exposure of cells to NA or $CdCl_2$ causes an increase in unfolded protein and inhibits proteasome activity which results in an increase in ubiquitinated damaged protein. These events result in the recruitment of HSPs which are bound to HSF1 to bind to unfolded protein or damaged protein destined for ubiquitination. This results in HSF1 activation and transcription of stress-inducible *hsps*. Newly synthesized stress-inducible HSPs bind to the unfolded protein in the cell to inhibit aggregation as well as to aid in CHIP-dependent ubiquitination of damaged protein.

In summary, this study has shown that NA and $CdCl_2$ induce HSP30 and HSP70 accumulation in *Xenopus* cells. In addition, this study has demonstrated for the first time, in poikilothermic vertebrates, that NA and $CdCl_2$ increased the accumulation of ubiquitinated protein and inhibited chymotrypsin-like activity. Also, inhibiting stress-induced HSP accumulation using KNK437 decreased the relative levels of ubiquitinated protein resulting from NA or $CdCl_2$ treatment. Furthermore, this study showed that the inhibition of chymotrypsin-like activity by treatment with NA or $CdCl_2$ was not affected

Figure 23. Overview of the effect of NA and $CdCl_2$ on proteasome activity and *hsp* gene expression. NA and $CdCl_2$ increase unfolded protein (1) and inhibit proteasomal activity which results in a build up of ubiquitinated protein (2). This recruits HSP90 to unfolded protein or for CHIP-dependent ubiquitination of damaged protein leaving HSF1 as a free monomer (3). HSF1 trimerizes (4) and translocates to the nucleus binding to the HSE of *hsp* genes initiating transcription of stress-inducible *hsps* (5). Newly synthesized HSPs bind to the unfolded protein in the cell or associate with CHIP to ubiquitinate damaged protein destined for degradation.


26S Proteasome
NA/CdCl2
1 Increased
Unfolded protein
2
Increased ubiquitination
of damaged protein
Ub
Ub
Ub
Ub
Ub
Ub
Ub
Ub
HSP
HSP90
3
5
Transcription of
stress-induced
hsp genes
HSP90
HSP90
HSP90
4
HSF1 Monomers
bound to HSP90
HSF1 Trimer

by KNK437 pretreatment. The finding that both NA and $CdCl_2$ can induce proteasome inhibition in *Xenopus* A6 cells as indicated by enhanced protein ubiquitination and inhibition of CT-like activity is of importance given that both of these agents are widespread environmental pollutants. Also, NA and $CdCl_2$ can have teratogenic and carcinogenic effects in humans and other organisms (Del Razo et al., 2001; Waisberg et al., 2003). Given that $CdCl_2$ and NA are often found as water contaminants, aquatic organisms, such as amphibians and fish, are very susceptible to their deleterious effects. In the future it will be of interest to determine if other aquatic species undergo proteasomal inhibition after exposure to NA or $CdCl_2$ and whether proteasome activity can be used as a molecular biomarker for environmental contamination. Additionally, understanding the effect of various stressors on proteasome function and *hsp* gene expression is of importance due to the potential therapeutic role of HSPs in various human diseases. Proteasome dysfunction and *hsp* gene expression have been implicated in numerous disease states including Alzheimer's disease (Morimoto, 2008).

Given the findings of this study, further analysis of the relationship between NA- and $CdCl_2$-induced proteasome inhibition and the accumulation of HSPs is required. Therefore, future studies could look at the specific-involvement of HSP70 and HSP30 in ubiquitination. KNK437 is a broad inhibitor of *hsp* gene expression. Studies involving siRNA can elicit highly sequence-specific gene silencing. Transfection with siRNA could inhibit specific expression of HSPs to further examine the role of HSP70 and HSP30 in ubiquitination. In pancreatic cancer cells, HSP70 downregulation by siRNA resulted in the induction of apoptosis (Dudeja et al., 2009). In rat brain astrocytes treated with MG132, downregulation of HSP25 by siRNA caused cells to be more sensitive to

stress induced by proteasomal inhibition, impaired microfilament organization and resulted in cell death (Goldbaum et al., 2009). Other studies in *Xenopus* A6 cells could employ co-immunoprecipitation assays in order to examine the relationship between CHIP, ubiquitinated protein, HSP70 and HSP30. For example, in HEK293 cells CHIP and HSP70 were shown to bind heat-stressed luciferase, as determined by co-immunoprecipitation (Rosser et al., 2007).

References

Abdulle, R., Mohindra, A., Fernando, P., Heikkila, J.J. 2002. *Xenopus* small heat shock proteins, Hsp30C and Hsp30D, maintain heat- and chemically denatured luciferase in a folding-competent state. Cell Stress Chaperones 7, 6-16.

Ali, A., Salter-Cid, L., Flajnik, M.F., Heikkila, J. J. 1996a. Isolation and characterization of a cDNA encoding a *Xenopus* 70-kDa heat shock cognate protein, Hsc70.I. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 113, 681-687.

Ali, A., Salter-Cid, L., Flajnik, M.J., Heikkila, J.J. 1996b. Molecular cloning of a cDNA encoding a *Xenopus laevis* 70-kDa heat shock cognate protein, hsc70.II. Biochem. Biophys. Acta. 1309, 174-178.

Amin, J., Ananthan, J., Voellmy, R. 1988. Key features of heat shock regulatory elements. Mol. Cell Biol. 8, 3761-3769.

Arrigo, A-P., Landry, J. 1994. Expression and function of the low-molecular weight heat shock proteins. In: The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Awasthi, N., Wagner, B.J. 2005. Upregulation of heat shock protein expression by proteasome inhibition: an antiapoptotic mechanism in the lens. Invest. Ophthalmol. Vis. Sci. 46, 2082-2091.

Barbier, O., Jacquillet, G., Tauc, M., Poujeol, P., Cougnon, M. 2004. Acute study of interaction among cadmium, calcium, and zinc transport along the rat nephron in vivo. Am. J. Physiol. Renal Physiol. 287, 1067-1075.

Bienz, M. 1984. *Xenopus hsp70* genes are constitutively expressed in injected oocytes. EMBO J. 3, 2477-2483.

Bjerregaard, H. 2007. Effects of cadmium on differentiation and cell cycle progression in cultured *Xenopus* kidney epithelial (A6) cells. Altern. Lab Anim. 35, 343-348.

Blechinger, S.R., Kusch, R.C., Haugo, K., Matz, C., Chivers, D.P., Krone, P.H., 2007. Brief embryonic cadmium exposure induces a stress response and cell death in the developing olfactory system followed by long-term olfactory deficits in juvenile zebrafish. Toxicol. Appl. Pharmacol. 224, 72-80.

Bode, A.M., Dong, Z. 2002. The paradox of arsenic: molecular mechanisms of cell transformation and chemotherapeutic effects. Crit. Rev. Oncol. Hematol. 42. 5-24.

Bogyo, M., Gaczynska, M., Ploegh H.L. 1997. Proteasome inhibitors and antigen presentation. Biopolymers 43, 269-280.

Boorstein, W.R., Ziegelhoffer, T., Craig, E.A. 1994. Molecular evolution of the HSP70 multigene family. J. Mol. Evol. 38, 1-17.

Briant, D., Ohan, N., Heikkila, J.J. 1997. Effect of herbimycin A on *hsp30* and *hsp70* heat shock protein gene expression in *Xenopus* cultured cells. Biochem. Cell Biol. 75, 777-782.

Bush, K.T., Goldberg, A.L., Nigam, S.K. 1997. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J. Biol. Chem. 272, 9086-9092.

Chen, Y.C., Lin-Shiau, S.Y., Lin, J.K. 1998. Involvement of reactive oxygen species and caspase 3 activation in arsenite-induced apoptosis. J. Cell Physiol. 177, 324-333.

Chou, I.N. 1989. Distinct cytoskeletal injuries induced by As, Cd, Co, Cr and Ni compounds. Biomed. Environ. Sci. 2, 358-365.

Chora, S., McDonagh B., Sheehan, D., Starita-Geribaldi, M., Romeo, M., Bebianno, M.J. 2008. Ubiquitination and carbonylation as markers of oxidative-stress in *Ruditapes decussates*. Mar. Environ. Res. 66, 95-97.

Darasch, S., Mosser, D.D., Bols, N.C., Heikkila, J.J. 1988. Heat shock gene expression in *Xenopus laevis* A6 cells in response to heat shock and sodium arsenite treatments. Biochem. Cell Biol. 66, 862-870.

Daugaard, M., Rohde, M., Jäättelä, M. 2007. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett. 581, 3702-3710.

Del Razo, L.M., Quintanilla-Vega, B., Brambila-Colombres, E., Calderon-Aranda, E.S., Manno, M., Albores, A. 2001. Stress proteins induced by arsenic. Toxicol. Appl. Pharmacol. 177, 132-148.

Dudeja, V., Mujumdar, N., Phillips, P., Chugh, R., Borja-Cacho, D., Dawra, R.K. Vickers, S.M., Saluja, A.K. 2009. Heat shock protein 70 inhibits apoptosis in cancer cells through simultaneous and independent mechanisms. Gastroenterology 136, 1772-1782.

Esser, C., Alberti, S., Hohfeld, J. 2004. Cooperation of molecular chaperones with the ubiquitin/proteasome system. Biochim. Biophys. Acta 1695, 171-188.

Fauconneau, B., Petegnief, V., Sanfeliu, C., Piriou, A., Planas, A.M. 2002. Induction of heat shock proteins (HSPs) by sodium arsenite in cultured astrocytes and reduction of hydrogen peroxide-induced cell death. J. Neurochem. 83; 1338-1348.

Feige, U., Morimoto, R.I., Yahara, I., Polla, B.S. 1996. "Stress-inducible cellular responses," Birkhauser Verlag., Basel: Switzerland.

Fernando, P., Heikkila, J.J. 2000. Functional characterization of *Xenopus* small heat shock protein, Hsp30C: The carboxyl end is required for stability and chaperone activity. Cell Stress Chaperones 5, 148-159.

Fernando, P. Megeney, L.A., Heikkila, J.J. 2003. Phosphorylation-dependent structural alterations in the small hsp30 chaperone are associated with cellular recovery. Exp. Cell Res. 286, 175-185.

Figueiredo-Pereira, M.E., Cohen, G. 1999. The ubiquitin/proteasome pathway: friend or foe in zinc-, cadmium-, and H_2O_2-induced neuronal oxidative stress. Mol. Biol. Rep. 26, 65-69.

Franck, E., Madsen, O., van Rheede, T., Ricard, G., Huynen, M.A., de Jong, W.W. 2004. Evolutionary diversity of vertebrate small heat shock proteins. J. Mol. Evol. 59, 792- 805.

Galazyn-Sidorczuk, M., Brzoska, M.M., Jurczuk, M., Moniuszko-Jakoniuk, J. 2009. Oxidative damage to proteins and DNA in rats exposed to cadmium and/or ethanol. Chem. Biol. Interac. 180, 31-38.

Gauley, J., Heikkila, J.J. 2006. Examination of the expression of the heat shock protein gene, *hsp110*, in *Xenopus laevis* cultured cells and embryos. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 145, 225-234.

Gellalchew, M., Heikkila, J.J. 2005. Intracellular localization of *Xenopus* small heat shock protein, hsp30, in A6 kidney epithelial cells. Cell Biol. Int. 29, 221-227.

Goldbaum, O., Riedel, M., Stahnke, T., Richter-Landsberg, C. 2009. The small heat shock protein HSP25 protects astrocytes against stress induced by proteasomal inhibition. Glia 57, 1566-1577.

Gordon, S., Bharadwaj, S., Hnatov, A., Ali, A., Ovsenek, N. 1997. Distinct stress-inducible and developmentally regulated heat shock transcription factors in *Xenopus* oocytes. Dev. Biol. 181, 47-63.

Guerra, L., Favia, M., Fanelli, T., Calamita, G., Svetlo, M., Bagorda, A., Jacobson, K.A., Reshkin, S. J., Casavola, V. 2004. Stimulation of *Xenopus* P2Y1 receptor activates CFTR in A6 cells. Pflugers Arch. 449, 66-75.

Hallare, A.V., Pagulayan, R., Lacdan, N., Kohler, H.R., Triebskorn, R. 2005. Assessing water quality in a tropical lake using biomarkers in zebrafish embryos: developmental toxicity and stress protein responses. Environ. Monit. Assess. 104, 171-187.

Hartl, F.U. 1996. Molecular chaperones in cellular protein folding. Nature 381, 571-579.

Hartl, F.U, Hayer-Hartl, M. 2009. Converging concepts of protein folding in vitro and in vivo. Nat Struct. Mol. Biol. 16, 574-581.

Heikkila, J.J. 2003. Expression and function of small heat shock protein genes during *Xenopus* development. Semin. Cell Dev. Biol. 14, 259-266.

Heikkila, J.J. 2004. Regulation and function of small heat shock protein genes during amphibian development. J. Cell Biochem. 93, 672-680.

Heikkila, J.J. 2010. Heat shock protein gene expression and function in amphibian model systems. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156, 19-33.

Hohfeld, J., Jentsch, S. 1997. GrpE-like regulation of the hsc70 chaperone by the anti-apoptotic protein BAG-1. EMBO Journal 16, 6209-6216.

Katschinski, D. M. 2004. On heat and cells and proteins. News Physiol. Sci. 19, 11-15.

Khan, S., Heikkila, J.J. 2011. Curcumin-induced inhibition of proteasomal activity, enhanced HSP accumulation and the acquisition of thermotolerance in *Xenopus laevis* A6 ells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 158, 566-576.

Kim, K.K., Kim, R., Kim, S. 1998. Crystal structure of a small heat-shock protein. Nature 394, 595-599.

Kirkpatrick, D.S., Dale, K.V., Catania, J.M., Gandolfi, A.J., 2003. Low-level arsenite causes accumulation of ubiquitinated proteins in rabbit renal cortical slices and HEK293 cells. Toxicol. Appl. Pharmacol. 186, 101-109.

Krone, P.H., Snow, A., Ali, A., Pasternak, J.J., Heikkila, J.J., 1992. Comparison of the regulatory and structural regions of the *Xenopus laevis* small heat-shock protein-encoding gene family. Gene 110, 159-166.

Lee D.H., Goldberg A.L. 1998. Proteasome inhibitors: valuable new tools for cell biologists. Trends Cell Biol. 8, 397–403.

Le Goff, P., Le Drean, Y., Le Person, C., Le Jossic-Corcos, C., Ainouche, A., Michel, D. 2004. Intracellular trafficking of heat shock factor 2. Exp. Cell Res. 294, 480-493.

Lehman, N.L. 2009. The ubiquitin proteasome system in neuropathology. Acta Neuropathol. 118, 329-347.

Li, L., Yang, H., Chen, D., Cui, C., Dou, Q.P. 2008. Disulfiram promotes the conversion of carcinogenic cadmium to a proteasome inhibitor with pro-apoptotic activity in human cancer cells. Toxicol. Appl. Pharmacol. 229, 206-214.

Liao, W., Li, X., Mancini, M., Chan, L. 2006. Proteasome inhibition induces differential heat shock protein response but not unfolded protein response in HepG2 cells. J. Cell Biochem. 99, 1085-95.

Liu, J., Kadiiska, M.B., Liu, Y., Lu, T., Qu, W., Waalkes, M.P. 2001. Stress-related gene expression in mice treated with inorganic arsenicals. Toxicol. Sci. 61, 314-320.

Lundgren, J., Masson, P., Mirzaei, Z., Young, P. 2005. Identification and characterization of a *Drosophila* proteasome regulatory network. Mol. Cell. Biol. 25, 4662-4675.

MacRae, T.H. 2000. Structure and function of small heat shock/alpha-crystallin proteins: established concepts and emerging ideas. Cell Mol. Life Sci. 57, 899-913.

Madden, E.F., Akkerman, M., Fowler, B.A. 2002. A comparison of 60, 70 and 90 kDa stress protein expression in normal rat NRK-52 and human HK-2 kidney cell lines following in vitro exposure to arsenite and cadmium alone or in combination. J. Biochem. Mol. Toxicol. 16, 24-32.

Malik, B., Schlanger, L., Al-Khalili, O., Bao, H.F., Yue, G., Price, S.R., Mitch, W.E., Eaton, D.C. 2001. Enac degradation in A6 cells by the ubiquitin-proteosome proteolytic pathway. J. Biol. Chem. 276, 12903-12910.

Manwell, L.A., Heikkila, J.J. 2007. Examination of KNK437- and quercetin-mediated inhibition of heat shock-induced heat shock protein gene expression in *Xenopus laevis* cultured cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148, 521-530.

Masliah E., Rockenstein E., Veinbergs I., Mallory M., Hashimoto M., Takeda A., Sagara Y., Sisk A., Mucke L. 2000. Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 287, 1265–1269.

Matsumoto, P.S., Ohara, A., Duchatelle, P., Eaton, D.C. 1993. Tyrosine kinase regulates epithelial sodium transport in A6 cells. Am. J. Physiol. 264, 246-250.

McDonagh, B., Sheehan, D. 2006. Redox proteomics in the blue mussel *Mytilus edulis*: Carbonylation is not a pre-requisite for ubiquitination in acute free radical-mediated oxidative stress. Aquat. Toxicol. 79, 325-333.

McDonagh, B. 2009. Detection of ubiquitination in 2DE. Methods Mol. Biol. 519, 377-381.

Meacham, G.C., Patterson, C., Zhang, W., Younger, J.M., Cyr, D.M. 2001. The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat. Cell Biol. 3, 100-105.

Medina-Diaz, I.M., Estrada-Muniz, E., Reyes-Hernandez, O.D., Ramirez, P., Vega, L., Elizondo, G. 2009. Arsenite and its metabolites, MMA^{III} and DMA^{III}, modify CYP3A4, PXR and RXR alpha expression in the small intestine of CYP3A4 transgenic mice. Toxicol. Appl. Pharmacol. 239, 162-168.

Melikova, M.S., Kondratov, K.A., Kornilova, E.S. 2006. Two different stages of epidermal growth factor (EGF) receptor endocytosis are sensitive to free ubiquitin depletion produced by proteasome inhibitor MG132. Cell Biol. Int. 30, 31-43.

Mendez-Armenta, M., Rios, C. 2007. Cadmium neurotoxicity. Environ. Toxicol. Pharmacol. 23, 350-358.

Mimnaugh, E.G., Chen, H.Y., Davie, J.R., Celis, J.E., Neckers, L. 1997. Rapid deubiquitination of nucleosomal histones in hyman tumour cells caused by proteasome inhibitors and stress response inducers: effects on replication, transcription, translation, and the cellular stress response. Biochemistry 36, 14418-14429.

Miskovic D., Salter-Cid, L., Ohan, N., Flajnik, M., Heikkila, J.J. 1997. Isolation and characterization of a cDNA encoding a *Xenopus* immunoglobulin binding protein, BiP (grp78). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 116, 227-34.

Morimoto, R.I. 1998. Regulation of the heat shock transcriptional response: Cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. 12, 3788-3796.

Morimoto, R.I. 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes Dev. 22, 1427-1438.

Mosser, D.D., Kotzbauer, P.T., Sarge, K.D., Morimoto, R.I. 1990. In vitro activation of heat shock transcription factor DNA-binding by calcium and biochemical conditions that affect protein conformation. Proc. Natl. Acad. Sci. 87, 3748-3752.

Mosser, D.D., Caron, A.W., Bourget, L., Meriin, A.B., Sherman, M.Y., Morimoto, R.I., Massie, B. 2000. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol. Cell Biol. 20, 7146-7159.

Mouchet, F., Baudrimont, M., Gonzalez, P., Cuenot, Y., Bourdineaud, J.P., Boudou, A., Gauthier, L. 2006. Genotoxic and stress inductive potential of cadmium in *Xenopus laevis* larvae. Aquat. Toxicol. 78, 157-166.

Mouchet, F., Gauthier, L., Baudrimont, M., Gonzalez, P., Mailhes, C., Ferrier, V., Devaux, A. 2007. Comparative evaluation of the toxicity and genotoxicity of cadmium in amphibian larvae (*Xenopus laevis* and *Pleurodeles waltl*) using the comet assay and the micronucleus test. Environ. Toxicol. 22, 422-435.

Muller, M., Gauley, J., Heikkila, J.J. 2004. Hydrogen peroxide induces heat shock protein and proto-oncogene mRNA accumulation in *Xenopus laevis* A6 kidney epithelial cells. Can. J. Physiol. Pharmacol. 82, 523-529.

Murata, S., Minami, Y., Minami, M., Chiba, T., Tanaka, K. 2001. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. EMBO Rep. 2, 1133-1138.

Nakamoto, H., Vigh, L. 2007. The small heat shock proteins and their clients. Cell. Mol Life Sci. 64, 294-306.

Noonan, E.J., Place, R.F., Giardina, C., Hightower, L.E. 2007. Hsp70B' regulation and function. Cell Stress Chaperones 12, 242-253.

Ohan, N.W., Tam, Y., Fernando, P., Heikkila, J.J. 1998. Characterization of a novel group of basic small heat shock proteins in *Xenopus laevis* A6 kidney epithelial cells. Biochem. Cell Biol. 76, 665-671.

Ohnishi, K., Takahashi, A., Yokota, S., Ohnishi, T. 2004. Effects of a heat shock protein inhibitor KNK437 on heat sensitivity and heat tolerance in human squamous cell carcinoma cell lines differing in p53 status. Int. J. Radiat. Biol. 80, 607-614.

Ohtsuka, K., Liu, Y.C., Kaneda, T. 1993. Cytoskeletal thermotolerance in NRK cells. Int. J. Hyperthermia 9, 115-124.

Othumpangat, S., Kashon, M., Joseph, P. 2005. Eukaryotic translation initiation factor 4E is a cellular target for toxicity and death due to exposure to cadmium chloride. J. Biol. Chem. 280, 25162-25169.

Ovsenek, N., Heikkila, J.J. 1990. DNA sequence-specific binding activity of the heat shock transcription factor is heat-inducible before midblastula transition of early *Xenopus* development. Development 110, 427-433.

Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantome, A., Plenchette, S., Khochbin, S., Solary, E., Garrido, C. 2003. HSP27 is a ubiquitin-binding protein involved in I-kBa proteasomal degradation. Mol. Cell Biol. 23, 5790-5802.

Peng, H.M., Morishima, Y., Jenkins, G.J., Dunbar, A.Y., Lau, M., Patterson, C., Pratt, W.B., Osawa, Y. 2004. Ubiquitination of neuronal nitric-oxide synthase by CHIP, a chaperone-dependent E3 ligase. J. Biol. Chem. 279, 52970-52977.

Perisic, O., Xiao, H., Lis, J.T. 1989. Stable binding of *Drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell 59, 797-806.

Phang, D., Joyce, E.M., Heikkila J.J. 1999. Heat shock-induced acquisition of thermotolerance at the levels of cell survival and translation in *Xenopus* A6 kidney epithelial cells. Biochem. Cell Biol. 77, 141-151.

Pirkkala, L., Nykanen, P., Sistonen, L. 2001. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. FASEB J. 15, 1118-1131.

Pratt, W.B., Morishima, Y., Peng, H.M., Osawa, Y. 2010. Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergoes oxidative and toxic damage. Exp. Biol. Med. (Maywood) 235, 278-289.

Pritts, T.A., Hungness, E.S., Hershko, D.D., Robb, B.W., Sun, X., Luo, G.J., Fischer, J.E., Wong, H.R., Hasselgren, P.O. 2002. Proteasome inhibitors induce heat shock response and increase IL-6 expression in human intestinal epithelial cells. Am. J. Physiol. Regul. Integr. Comp. Physiol. 282, 1016-1026.

Rabindran, S.K., Haroun, R.I, Clos, J., Wisniewski, J., Wu, C. 1993. Regulation of heat shock factor trimer formation: role of a conserved leucine zipper. Science 259, 230- 234.

Rafferty, K.A. 1969. Mass culture of amphibian cells: Methods and observations concerning stability of cell type. In M. Mizell, *Biology of amphibian tumors* pp. 58-81. Berlin: Springer Verlag.

Rea, M.A., Gregg, J.P., Quin, Q., Phillips, M.A., Rice, R.H. 2003. Global alteration of gene expression human keratinocytes by inorganic arsenic. Carcinogenesis 24, 747-756.

Ritossa, F. 1962. A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia 18, 571-573

Ross, C.A., Pickart, C.M. 2004. The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative disease. Trends Cell Biol. 14, 703-711.

Rossi, M.R., Somji, S., Garrett, S.H., Sens, M.A., Nath, J., Sens D.A. 2002. Expression of hsp 27, hsp 60, hsc 70 and hsp 70 stress response genes in cultured human urothelial cells (UROtsa) exposed to lethal and sublethal concentrations of sodium arsenite. Environ. Health Perspect. 110, 1225-1232.

Rosser, M.F.N., Washburn, E., Muchowski, P.J., Patterson, C., Cyr, D.M. 2007. Chaperone functions of the E3 ubiquitin ligase CHIP. J. Biol. Chem. 282, 22267-22277.

Sahin, E., Sahin, M., Sanlioglu, A.D., Gumuslu, S. 2011. KNK437, a benzylidene lactam compound, sensitizes prostate cancer cells to the apoptotic effect of hyperthermia. Int. J. Hyperthermia 27, 63-73.

Samuel, S., Kathirvel, R., Jayavelu, T., Chinnakkannu, P. 2005. Protein oxidative damage in arsenic induced rat brain: influence of DL-a-lipoic acid. Toxicol. Lett. 155, 27-34.

Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F.U., Moarafei, I. 2000. Structure of the TPR domain-peptide complexes: critical elements in the assembly of the HSP70-HSP90 multichaperone machine. Cell 101, 199-210.

Schuetz, T.J., Gallo, G.J., Sheldon, L., Tempst, P., Kingston, R.E. 1991. Isolation of a cDNA for HSF2: Evidence for two heat shock factor genes in humans. Proc. Natl. Acad. Sci. 88, 6911-6915.

Shamovsky, I., Nudler, E. 2008. New insights into the mechanisms of heat shock response activation. Cell Mol. Life Sci. 65, 855-861.

Song, X., Chen, Z., Wu, C., Zhao, S. 2010. Abrogating HSP response augments cell death induced by As2O3 in glioma cell lines. Can. J. Neurol. Sci. 37, 504-511.

Stangl, K., Gunther, C., Frank, T., Lorenz, M., Meiners, S., Ropke, T., Stelter, L., Moobed, M., Baumann, G., Kloetzel, P.M., Stangl, V. 2002. Inhibition of the ubiquitin-proteasome pathway induces differential heat-shock protein response in cardiomyocytes and renders early cardiac protection. Biochem. Biophys. Res. Commun. 291, 542-549.

Stankiewicz, M., Nikolay, R., Rybin, V., Mayer M.P. 2010. CHIP participates in protein triage decisions by preferentially ubiquitinating HSP70-bound substrates. FEBS J. 277, 3353-3367.

Stromer, T., Ehrnsperger, M., Gaestel, M., Buchner, J. 2003. Analysis of the interaction of small heat shock proteins with unfolding proteins. J. Biol. Chem. 278, 18015-18021.

Su, P-F., Hu, Y-J., Ho, I-C., Cheng, Y-M., Lee, T.C. 2006. Distinct gene expression profiles in immortalized human urothelial cells exposed to inorganic arsenite and its methylated trivalent metabolites. Environ. Health Perspect. 114, 394-403.

Tanaka, M., Asashima, M., Atomi, Y. 2003. Proliferation and differentiation of *Xenopus* A6 cells under hypergravity as revealed by time-lapse imaging. In Vitro Cell Dev. Biol. Anim. 39, 71-79.

Taylor J.P., Hardy J., Fischbeck K.H. 2002. Toxic proteins in neurodegenerative disease. Science 296, 1991–1995.

Thevenod, F., Friedmann, J.M. 1999. Cadmium-mediated oxidative stress in kidney proximal tubule cells induces degradation of Na^+/K^+-ATPase through proteasomal and endo-/lysosomal proteolytic pathways. FASEB J. 13, 1751-1761.

Tsou, T.C., Tsai, F.Y., Hseih, Y.W., Li, L.A., Yeh, S.C., Chang, L.W. 2005. Arsenite induces endothelial cytotoxicity by down-regulation of vascular endothelial nitric oxide synthase. Toxicol. Appl. Pharmacol. 208, 277-284.

Van Montfort, R., Slingsby, C., Vierling, E. 2001. Structure and function of the small heat shock protein/alpha-crystallin family of molecular chaperones. Adv. Protein Chem. 59, 105-156.

Voellmy, R. 2004. On mechanisms that control heat shock transcription factor activity in metazoan cells. Cell Stress Chaperones 9, 122-133.

Voyer, J., Heikkila, J.J. 2008. Comparison of the effect of heat shock factor inhibitor, KNK437, on heat shock- and chemical stress-induced *hsp30* gene expression in *Xenopus laevis* A6 cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 151, 253-261.

Waisberg, M., Pius, J., Hale, B., Beyersmann, D. 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192, 95-117.

Walcott, S.E., Heikkila, J.J. 2010. Celastrol can inhibit proteasome activity and upregulate the expression of heat shock protein genes, *hsp30* and *hsp70*, in *Xenopus laevis* A6 cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156, 285-293.

Wiegant, F.A., van Bergen en Henegouwen, P.M., van Dongen, G. and Linnemans, W.A. 1987. Stress-induced thermotolerance of the cytoskeleton of mouse neuroblastoma N2A cells and rat Reuber H35 hepatoma cells. Cancer Res. 47, 1674-1680.

Woolfson, J.P., Heikkila, J.J. 2009. Examination of cadmium-induced expression of the small heat shock protein gene, *hsp30*, in *Xenopus laevis* A6 kidney epithelial cells. Comp. Biochem. Physiol. A Mol. Integr. Physiol., 152, 91-99.

Xiao, H., Lis, J.T. 1988. Germline transformation used to define key features of heat-shock response elements. Science 239, 1139-1142.

Yang, H., Landis-Piwowar, K.R., Chen, D., Milacic, V., Dou, Q.P. 2008. Natural compounds with proteasome inhibitory activity for cancer prevention and treatment. Curr. Protein Pept. Sci. 9, 227 - 239.

Yokota, S., Kitahara, M., Nagata, K. 2000. Benzylidene lactam compound, KNK437, a novel inhibitor of acquitision of thermotolerance and heat shock protein induction in human colon carcinoma cells. Cancer Res. 60, 2942-2948.

Young, J.T., Gauley, J., Heikkila, J.J. 2009. 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. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 153, 417-424.

Young, J.T., Heikkila, J.J. 2010. Proteasome inhibition induces *hsp30* and *hsp70* gene expression as well as the acquisition of thermotolerance in *Xenopus laevis* A6 cells. Cell Stress Chaperones 15, 323-334.

Yu, X., Hong, S., Faustman, E.M. 2008. Cadmium-induced activation of stress signaling pathways, disruption of ubiquitin-dependent protein degradation and apoptosis in primary rat sertoli cell-gonocyte cocultures. Toxicol. Sci. 104, 385-396.

Yu, X., Robinson, J.F., Sidhu, J.S., Hong, S., Faustman, E.M. 2010. A system-based comparison of gene expression reveals alterations in oxidative stress, disruption of ubiquitin-proteasome system and altered cell cycle regulation after exposure to cadmium and methylmercury in mouse embryonic fibroblast. Toxicol. Sci. 114, 356-377.

Yu, X., Sidhu, J.S., Hong, S., Robinson, J.F., Ponce, R.A., Faustman, E.M. 2011. Cadmium induced p53-dependent activation of stress signaling, accumulation of ubiquitinated proteins, and apoptosis in mouse embryonic fibroblast cells. Toxicol. Sci. 120, 403-412.