Examination of the effect of the natural plant extract, withaferin A, on heat shock protein gene expression in *Xenopus laevis* A6 cells

by

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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.

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Abstract

In eukaryotes, the ubiquitin-proteasome system (UPS) degrades most cellular protein. Inhibition of the UPS has been associated with different disease states and can affect various intracellular processes including the activation of heat shock protein (*hsp*) gene expression. During cellular stress, HSPs act as molecular chaperones by inhibiting protein aggregation and assisting in their refolding once normal conditions are re-established. In the present study, Withaferin A (WA), a steroidal lactone with possible anti-inflammatory and antitumor properties, was found to inhibit proteasome activity and induce the expression of *hsp* genes in the amphibian model system, *Xenopus laevis*. Treatment of *Xenopus* kidney epithelial A6 cells with WA produced an increase in the accumulation of ubiquitinated protein and a significant decrease in chymotrypsin-like activity. Furthermore, immunoblot analysis revealed that WA induced HSP30 and HSP70 accumulation. For example, cells treated with 5 μM WA for 18 h resulted in the optimal accumulation of HSP30 and HSP70. Northern blot analysis revealed that exposure of cells to 5 μM WA induced *hsp30* and *hsp70* mRNA accumulation in a time-dependent manner up to 12 h. The activation of heat shock factor 1 (HSF1) DNA-binding may be involved in WA-induced *hsp* gene expression in A6 cells, since pretreatment with the HSF1 inhibitor, KNK437, reduced the accumulation of HSP30 and HSP70. Also, WA acted synergistically with mild heat shock to enhance HSP accumulation to a greater extent than the sum of both stressors individually. In cells recovering from WA, the relative levels of HSP30 and HSP70 accumulation remained elevated from 6 to 12 h after removal of WA. Immuocytochemical analysis and laser scanning confocal microscopy revealed that WA-induced HSP30 accumulation occurred

primarily in the cytoplasm with some staining in the nucleus in a granular or punctate pattern. Prolonged exposure to WA resulted in some disorganization of the actin cytoskeleton as well as large cytoplasmic HSP30 staining structures in some cells. Prior exposure of cells to WA treatment conferred thermotolerance since it protected them against a subsequent thermal challenge at 37 °C. In conclusion, this study has shown that WA can induce an inhibition of proteasome activity and an increase *hsp* gene expression. Activating the heat shock response is a potential avenue for novel drug therapies, which can confer cytoprotection in disease states involving cytotoxic protein aggregation.

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List of Abbreviations

ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APS	ammonium persulfate
ATCC	American type culture collection
BCA	bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BiP	immunoglobulin-binding protein
BSA	bovine serum albumin protein standard
BSA fraction V	bovine serum albumin fraction V
C	control
CT	chymotrypsin
DAPI	4,6-diamidino-2-phenylindole
DEPC	diethylpolycarbonate
DIG	digoxigenin
DMSO	dimethylslphoxide
DTT	dithiothreitol
ECL	enzymatic chemilumniscence
EDTA	ethylene-diamine-tetraacetic acid
e-EF1α	elongation factor 1-α
EEVD	Glu-Glu-Val-Asp
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxythyl)-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
HSR-1	heat shock RNA-1
KNK-437	N-formyl-3, 4-methylenedioxy-benzylidene-γ-butryolactam
L-15	Leibovitz -15 media
LB	luria-bertani
LSCM	laser scanning confocal microscopy
MAPKAPK-2	mitogen activated protein kinase activating protein kinase -2
MG132	carbobenzyxy-L-leucyl-L-leucyl-L-leucinal
MOPS	3-(N-morpholino)propanesulfonic acid
NF-κB	nuclear factor kappa B
NBT	4-nitro blue tetrazolium
PBS	phosphate buffered saline
RBC	red blood cells
ROS	reactive oxygen species
SDS	sodium dodecylslfate
SHSP	small heat shock protein
PAGE	polyacrylamide gel electrophoresis
SSC	saline-sodium citrate
TBS-T	tris buffered saline – tween 20
TEMED	tetra-methyl-ethylene-diamine
Tris	tris(hydroxymethyl)aminomethane
TRITC	rhodamine-tetramethylrhodamine-5-isothiocynate phalloidin
WA	withaferin A
UB	ubiquitinated protein
UPS	Ubiquitinp roteasome system

1 Introduction

Protein homeostasis, encompassing RNA metabolism and processing, protein synthesis, folding, translocation, assembly and disassembly is regulated by a complex network of components in the cell (Morimoto, 2008). These processes, accomplished in the crowded cellular environment, are influenced by physical, metabolic and environmental pressures, such as oxidation by reactive oxygen species (ROS), temperature and changes in small molecule metabolite concentrations (Powers, et al., 2009). Deregulation of proteastasis can result in loss of function, gain of toxic function and aggregation of proteins. Chaperones, such as heat shock proteins (HSPs), help circumvent this problem by holding protein in a folding competent state, preventing aggregation of unfolded polypeptides and refolding unfolded protein to their native conformation or promoting protein degradation when necessary (Powers, et al., 2009). Increased HSP accumulation protects against stress-induced damage with a direct correlation between the level of stress and the amount of HSP synthesized (Gupta et al., 2010).

1.1 Heat Shock Proteins

Heat shock proteins were first observed by Ritossa (1962) in the salivary glands of *Drosophila melanogaster* larvae, in response to elevated temperature. HSPs were initially named because of their induction by heat shock. However, they are expressed in response to many factors including cell growth and development, environmental influences, pathophysiological stress and protein conformation diseases (Morimoto, 2008; Gupta et al.,

2010). Synthesis of inducible HSPs, via the heat shock response (HSR), occurs as a result of cellular stress such as exposure to heat, alcohol, hypoxia, heavy metals, hydrogen peroxide and the accumulation of abnormal proteins (Shamovsky and Nudler, 2008). Constitutively expressed HSPs were later found in addition to the stress inducible forms. Under normal conditions, the constitutively expressed HSPs perform maintenance duties including folding of nascent chains, regulation of protein quality control, and assisting in apoptotic regulation by interacting with members of the death cascade (Morimoto, 2008; Kalmar and Greensmith, 2009). A defining characteristic of HSPs is their ability to prevent misfolding, and potential aggregation with other unfolding intermediates, as well as to hold proteins in a folding competent state so as to allow their refolding (Morimoto, 2008). As molecular chaperones, HSPs work to capture non-native intermediates and nascent chains by recognizing exposed hydrophobic stretches, normally hidden inside the native conformation (Morimoto and Santoro, 1998).

HSPs have been found in all organisms, from bacteria to mammals, and are classified into groups according to their size (Shamovsky and Nudler, 2008). In vertebrates, the six families identified thus far are HSP100 (including HSP110), HSP90 HSP70, HSP60, HSP40 and the small heat shock proteins (sHSPs) that range from 10 – 42 kDa. This introduction will focus on the HSP70 family and the sHSPs.

1.1.1 HSP70

The 70 kDa heat shock protein (HSP70) is involved in a wide array of folding processes including, folding of newly synthesized proteins, transport of proteins across membranes and refolding of misfolded proteins (Morimoto, 2008). HSP70 promotes folding

of nascent chains on ribosomes under normal conditions, and holds unfolded intermediates in a folding competent state to prevent aggregation by interacting with hydrophobic surfaces exposed during cell stress (Katschinski, 2004). Folding of proteins into their native conformation is restricted to HSP70, while its ability to hold proteins in a folding competent state is common among many HSPs (Morimoto, 2008). Biological activity of regulatory proteins is controlled through transient interactions of HSP70 with nuclear receptors, kinases and transcription factors (Mayer and Bukau, 2005). HSP70 also promotes degradation of proteins, by the proteasome or lysosome, when they are no longer needed (Kalmar and Greensmith, 2009). Interaction of HSP70 with members of the caspase-independent and caspase-dependent apoptosis pathways is necessary for inhibition of apoptosis and cell survival (Beere et al., 2000; Mayer and Bukau, 2005). HSP70 regulates this broad and complex network of cellular functions through diversification in *hsp70* evolution, which generated specialized HSP70 chaperones, substrate specificity via co-chaperones and cooperation with other chaperone systems (Mayer and Bukau, 2005).

As a collective, the HSP70 molecular chaperones are the most abundant family of HSPs as they are found in every organism in multiple cellular compartments. Members of the HSP70 family include the stress inducible HSP70 and constitutive heat shock cognate (HSC70), both relegated to the cytoplasm, as well as a mitochondrial HSP70 (mitHSP70) and immunoglobulin binding protein (BiP) also known as glucoses-regulated protein 78 (GRP78), which is located in the endoplasmic reticulum (Meimaridou et al., 2009). The HSP70s are a highly conserved family of proteins, having 74% identity between the amino acid sequence of *Drosophila* and *Xenopus* HSP70 while the heat shock cognate, HSC70, has 92% identity between *Xenopus*, mouse, rat and bovine (Heikkila et al., 1997).

The multi-domain organization of HSP70 includes a 45 kDa amino-terminal ATPase domain, an 18 kDa peptide binding domain near the carboxyl end and a G/C rich domain containing a 7 kDa EEVD regulatory motif at the extreme C-terminal (Freeman et al., 1995; Morimoto and Santoro 1998; Daugaard et al., 2007; Shamovsky and Nudler, 2008). In addition, HSP70 relies on an ATPase cycle, which consists of alternation between an ADP bound state with high substrate affinity and an ATP bound state with low substrate affinity (Mayer and Bukau, 2005). ATP binding induces conformational changes in the substrate-binding domain opening a pocket and helical lid for binding of HSP70 client proteins (Bukau et al., 2006).

HSP70 binds a wide variety of proteins to accomplish its diverse cellular processes. Co-chaperones like the DNAJ family, Hip, Hop and Bag influence these interactions by conferring client protein specificity (Mayer and Bukau, 2005; Morimoto, 2008; Kalmar and Greensmith, 2009). Protein misfolding is detected by HSP70, which then forms complexes with HSP90 and co-chaperones. The J domain family of proteins (DNAJ) mediate ATP hydrolysis dependent locking of substrates in the binding cavity of HSP70. Chaperone activity is dependent on the J domain of these co-chaperones interacting with the ATPase domain of HSP70. Association of the HSP70 complex with HSP70-HSP90 organizing protein (HOP) or HSP-interacting protein (HIP) increase ATPase activity and thus refolding. If the HSP70-HSP90 complex interacts with other co-chaperones such as CHIP or BAG this will result in decreased HSP70-ATPase activity. The BAG family of proteins, particularly BAG-1, may antagonize the function of HIP by competing for the ATPase domain of HSP70. Co-chaperone assisted repression of HSP70 folding activity results in ubiquitination of the client protein and subsequent degradation.

1.1.2 Small HSPs

The small HSP superfamily is a ubiquitous group of proteins within the cell and is involved in numerous cellular processes such as actin capping and decapping, cellular differentiation, prevention of apoptosis and acquisition of thermotolerance (MacRae, 2000; Mounier and Arrigo, 2002; Heikkila, 2004). sHSPs also interact with unfolding proteins, holding them in a folding competent state. SHSPs are the most diverse family of stress proteins in structure and function, yet they are found in nearly every cellular compartment in almost every organism (Franck et al., 2004; Nakamoto and Vigh, 2007). Many organisms express multiple members of the sHSP family often with several members in one cellular compartment (Franck et al., 2004).

SHSPs have a varied domain structure (Kim et al., 1998; Franck et al., 2004; Sun and MacRae, 2005a; Nakamoto and Vigh, 2007). The only conserved region, named for the protein in which it was first discovered, is a C-terminal α-crystallin domain, comprised of 80-100 amino acids. This region is folded into a β-sandwich conformation, which consists of two anti-parallel β-sheets and intersubunit binding of these sheets facilitates dimer formation, the building blocks for oligomeric complexes. Flanking the α-crystallin domain are a non-conserved amino terminal domain and a flexible carboxyl terminal extension, required for stability and chaperone activity. The varied size of sHSPs, from 10-42 kDa, is primarily due to the variable length of the N-terminal region and presence or absence of the C-terminal extension.

Almost all sHSPs assemble into large oligomeric complexes of 9 to over 30 subunits and up to 2 MDa in size (Haley et al., 1998; Kim et al., 1998; Nakamoto and Vigh, 2007).

The oligomeric crystal structure was defined in several organisms as a hollow globular sphere and consisted of multiple different sHSPs in one complex. SHSP quaternary structure was found to be dynamic and variable with subunits that freely and rapidly exchanged between oligomers (Haley et al., 1998; van Montfort et al., 2002; Stromer et al., 2003). Formation of oligomers and their reversible dissociation into smaller subunits was required for in vitro chaperone activity (van Montfort et al., 2001; Stromer et al, 2003; Nakamoto and Vigh, 2007) and involved temperature-regulated exposure of their hydrophobic surfaces, making them available for substrate binding. Substrate-bound sHSPs may rejoin formed multimers as several substrate proteins were found bound to each assembly, up to one client per sHSP dimer (van den Oetelaar et al., 1990; Bova et al., 1997; Stromer et al., 2003; Haslbeck et al., 2005). SHSP complexes suppress the aggregation of denaturing proteins by holding them in a folding competent state such that the client proteins can be folded by other chaperones, such as HSP70 and HSP90, upon stress attenuation (Arrigo, 1998; Nakamoto and Vigh, 2007). Phosphorylation of sHSPs resulted in the formation of smaller multimeric units and severely inhibited chaperone activity (Lavoie et al., 1995; Fernando et al., 2003; Shashidharamurthy et al., 2005). The phosphorylation-induced changes attenuated sHSP-substrate binding and may enable ATP-dependent HSPs access to client proteins for refolding. Client protein specificity has not been detected thus far which may account for the wide variety of sHSP-associated processes (Stromer et al., 2003).

Numerous sHSPs have been identified in almost every organism, the most widely studied being HSP27, αA-crystallin and αB-crystallin. These three sHSPs were found in all vertebrates studied to date (Miron et al., 1991; Sawada et al., 1992; Lu et al., 1995; Behrens et al., 1998; Kawazoe et al., 1999; Posner et al., 1999; Norris and Hightower, 2002;

Panasenko et al., 2002; Franck et al., 2004; Heikkila, 2010). The α-crystallins are major constituents of the human eye lens and as such prevent proteins from forming light scattering aggregates. αA-crystallin is confined to the lenticular compartment, however αB-crystallin is involved in cytoprotective and regulatory processes in many other tissues (Franck et al., 2004; Haslbeck et al., 2005). HSP27, also known as HSP25 in mice or HSPB1, has protein holding chaperone capabilities and its expression is elevated during heat shock (Brown et al., 2007).

1.1.2.1 HSP30

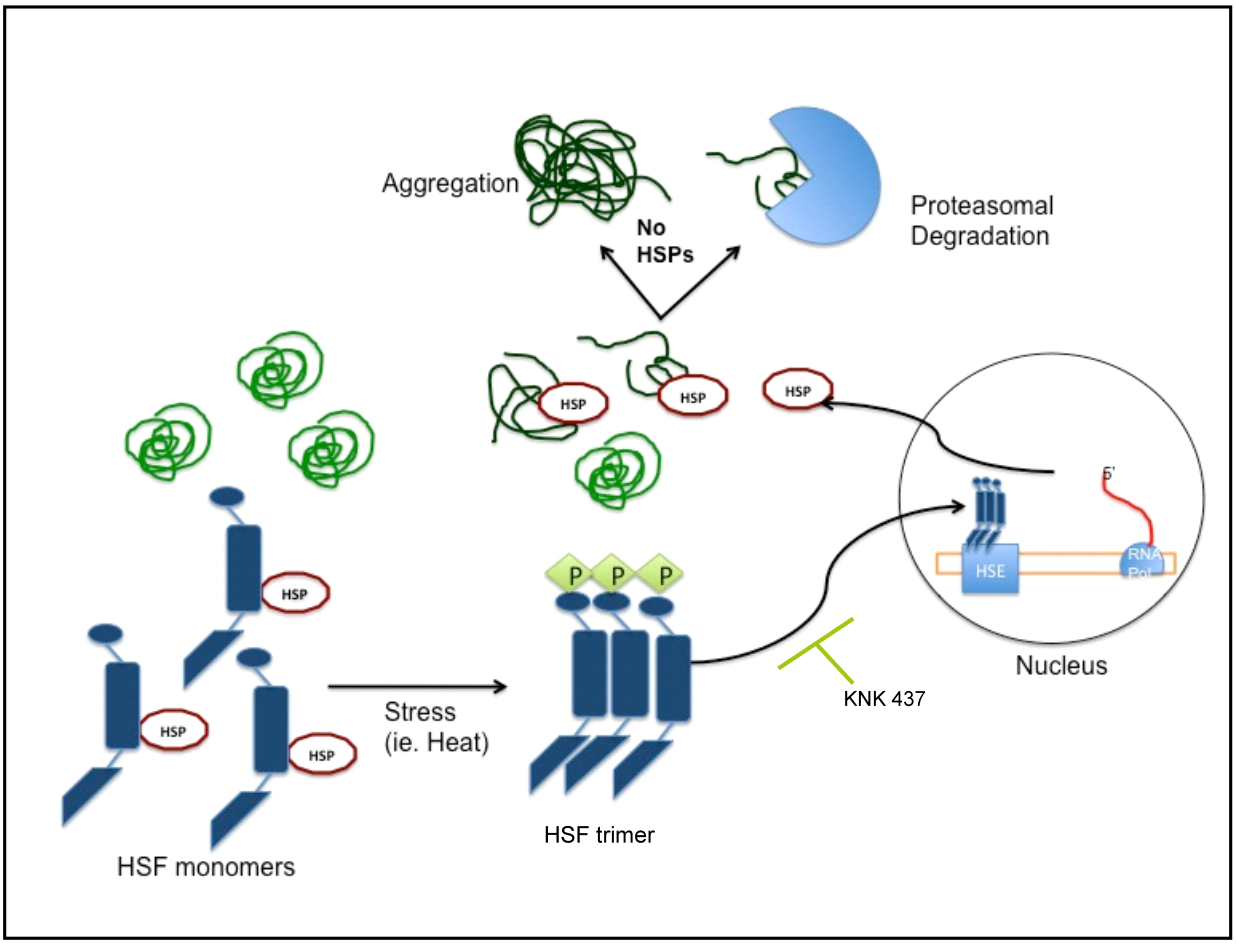
An additional group of sHSPs, parologous to those in humans, is the HSP30 family. First discovered in amphibian systems, it was thought to be the counterpart to HSP25/27. However, it is now known that HSP30 is a distinct family of HSPs with homologs found in avian, amphibian and fish classes (Krone et al., 1992; Tam and Heikkila, 1995; Helbing et al., 1996; Norris et al., 1997; Norris and Hightower, 2002; Katoh et al., 2004; Kondo et al., 2004; Wang et al., 2007). HSP30 has properties very similar to those of HSP27 and other sHSPs including chaperone activity, stress inducibility, phosphorylation-induced structural alterations and muscle localization.

1.2 Heat Shock Factor 1 and the Heat Shock Response

Heat shock factor 1 (HSF1) is responsible for stress-induced *hsp* gene expression in eukaryotes (Shamovsky and Nudler, 2008). Rapid HSF1 activation, upon exposure to cellular stress, involves trimerization of pre-existing HSF1 monomers, translocation to the nucleus and subsequent DNA binding (Fig. 1) (Morimoto, 2008). HSFs are poorly conserved among

different species, apart from the organization of their protein domains (Shamovsky and Nudler, 2008). The most highly conserved domain, an N-terminal helix-turn-helix DNA binding region, is the point of interaction between HSF1 and the heat shock element (HSE) which is located in the upstream promoter region of *hsp* genes (Akerfelt et al., 2007; Morimoto, 2008). DNA binding requires at least two copies of HSF1, however the active trimer has much greater affinity for the HSE than either the HSF1 monomer or dimer (Rabindran et al., 1993; Shamovsky and Nudler, 2008). Trimerization occurs via three separate leucine zipper domains: hydrophobic repeat-A (HR-A) and HR-B near the N-terminus, and HR-C located closer to the C-terminus. The N-terminal repeats of adjacent transcription factors are required for trimer formation, while the HR-C maintains HSF1 in its monomeric state. Under non-stressed conditions, intramolecular coiled-coil interactions, between the N-terminal and C-terminal leucine zippers, presumably masks the hydrophobic domains from interacting with other HSF1 monomers. Heat shock, as well as other cellular stress, induces a conformational change that disrupts the intramolecular interactions and

Figure 1. Model of the heat shock response. During cellular stress, HSP has greater affinity for unfolded protein than for HSF1 monomers. HSF is then phosphorylated and trimerizes into an active state whereupon it translocates to the nucleus to bind HSE. Transcription and translation follows to increase HSP accumulation in the cytoplasm. Adapted from Riordan et al. (2006).


Aggregation
No
HSPs
Proteasomal
Degradation
HSP
HSP
HSP
HSP
HSP
HSP
HSP
Stress
(ie. Heat)
P
P
P
HSF monomers
HSF trimer
KNK 437
5'
HSE
RNA
Pol
Nucleus

consequently HSF1 trimer repression. A C-terminal activation domain and a regulatory domain located within the central region are also necessary for HSF1 activity (Shamovsky and Nudler, 2008; Pirkkala et al., 2001). Control of the activation domain is through the regulatory region, which contains sites for constitutive and inducible serine pohosphorylation. For example, phosphorylation at serine 303, 307 and 363 are key for the negative regulation of HSF1 activity at normal temperatures, while phosphorylation at serine 230 is enhanced upon heat shock, and has a positive effect on the transcription factor's activity. Sumoylation, the addition of ubiquitin-like peptides to specific lysine residues in the regulatory domain, may also modulate DNA binding and transcriptional activity of HSF1 (Hong et al., 2001).

HSF1 is the primary regulator of the stress-induced increase in *hsp* gene expression that protects against proteotoxic stress (Akerfelt et al., 2007). *hsp* gene expression is also regulated at the levels of mRNA stability, translation, activity and the subcellular localization of chaperones (Katschinski, 2004). In addition, post-translational modifications, such as phosphorylation, can alter the structure and function of some HSPs (Fernando et al., 2003; Papp et al., 2003; Nakamoto and Vigh, 2007). The fine balance of proteostasis maintained in the cell, demands a fast and efficient response to proteotoxic stress. Once there is an excess of HSPs available to process the unfolded protein, HSF1 re-associates with HSPs in its inactive monomeric form (Morimoto, 1998). *Hsp* genes are only transcribed when HSF1 is bound to the HSE therefore cessation of the HSR occurs almost immediately after stress alleviates.

The mechanism of HSF1 activation is still largely unknown, however, formation of multichaperone complexes including HSP90, HSP70/HSP40, HIP, HOP and p23 were shown to inhibit HSF1 activation (Shamovsky and Nudler, 2008). An increase in protein intermediates appears during stress, shifting chaperone equilibrium away from HSF monomers alleviating its repression (Morimoto, 2008). Unfolded protein has been proposed as the shared mechanism behind the various stressors that induce the HSR and HSF1 activation. However, HSF1 activity may also be regulated by the tandem action of the translational elongation factor, eEF1α, and constitutively expressed heat shock RNA-1 (HSR1) (Shamovsky et al., 2006). Both eEF1α and HSR1 were required for HSF1 activation in in vitro and in vivo studies employing antisense oligonucleotides or short interfering RNA against HSR1 which depleted HSR1. eEF1α is a key component regulating the actin cytoskeleton and mRNA translation; HSR-induced protein synthesis inhibition and increased cytoskeletal collapse, may free eEF1α for interaction with HSF1 (Gross and Kinzy, 2005). Direct recognition of cell stress by HSR1, without unfolded protein, was also seen in vitro with purified HSF1 whose transcriptional activity increased in response to oxidative stress and low pH. Gross and Kinsey (2005) postulated that HSR1 may act as a the thermosensor of the cell, directly recognizing cell stress. In addition, HSF1 DNA binding activity was inhibited by a nuclear localized hydrophobic repeat containing protein, HSF binding protein (Morimoto, 1998). This indicates that the passive removal of HSPs may not act alone to activate HSF1 upon cell stress.

HSF1 is the master regulator of heat-inducible *hsp* gene expression in vertebrates, but other HSFs have been identified as well. HSF2, which is found in all vertebrates, is regulated by developmental conditions (Pirkkala et al., 2001) and although not activated in response to

classical stress, was shown to act cooperatively with HSF1 during stress (Ostling et al., 2007). In avian species an additional transcription factor, HSF3, was stress responsive and directly affected the activity and trimerization of HSF1 (Pirkkala et al., 2001). HSF3 was activated in response to severe heat shock and, unlike HSF1, the transcription of this factor was also induced with elevated temperature (Pirkkala et al., 2001). In mammals, HSF4, an as yet uncharacterized transcription factor, was preferentially expressed in the human heart, brain, skeletal muscle and pancreas. Lacking both the C-terminal hydrophobic repeat and transcriptional activation capability, it was suggested that HSF4 functions as a repressor of *hsp* gene expression (Nakai et al., 1997). In support of this, HSF4 specifically interacted with αB-crystallin promoter in post-natal development, possibly out-competing HSF1 and HSF2 for DNA binding (Somasundaram and Bhat, 2004).

1.3 Heat shock proteins and disease

Protein homeostasis is a dynamic relationship between relative protein levels and their function within the cell. Chaperone and co-chaperone levels, for example, can alter networks of proteins therefore enhancing or dampening some signaling pathways, with long-term imbalances likely having dramatic consequences on diverse cellular processes (Quinlan and van den Ijssel, 1999; Heikkila, 2003; Sun and MacRae, 2005b). SHSP synthesis and mutation have been linked to a number of diseases, including cataracts, ischemia during heart attack and stroke, Parkinson's, Alzheimer's and neuromuscular disorders. For example, a missense mutation in the gene that codes for αB-crystallin has been found to cause desmin-related myopathy with the formation of protein inclusions comprising the intermediate filaments of the desmin class (Quinlan and van den Ijssel, 1999). In addition, a frameshift

mutation in the same crystallin gene produced an aberrant protein and was shown to be associated with congenital cataracts (Vicart et al., 1998; Perng et al., Perng et al., 1999; Berry et al., 2001). HSPs have also been implicated in many cardiovascular diseases (Snoeckx et al., 2001; Gupta et al., 2004). Elevated levels of HSPs were found in patients with systemic hypertension, coronary artery disease, carotid atherosclerosis, and myocardial infarction and ischemia. There are many human diseases that have been linked to chaperone and proteasome dysfunction that are pathologically defined by the abnormal deposition of misfolded polypeptides. Among these are severe neuropathologies like prion diseases, Alzheimer's and Huntington's, in which misfolded proteins aggregate in fibrillar structures, known as amyloids (Taylor et al., 2002; Muchowski and Wacker, 2005). In addition, the neurodegenerative Parkinson's disease may involve abnormal accumulation of the protein α-synuclein bound to ubiquitin in the damaged cells. The α-synuclein-ubiquitin complex cannot be directed and degraded by the proteasome and forms cytotoxic Lewy bodies, proteinaceous cytoplasmic inclusions (Masliah et al., 2000; Muchowski and Wacker, 2005). Finding ways to alleviate proteotoxicity by enhancing molecular chaperones has shown promise as a potential therapeutic (Morimoto, 2008).

1.4 *Xenopus laevis*

Xenopus laevis is an excellent model organism useful for studying development, and its cell and molecular biology have also been extensively examined. It possesses physiological traits common to most other vertebrates and the cellular and molecular data collected using amphibian systems are, to a great extent applicable to mammals (Burggren and Warburton, 2007). As one of many species of the genus *Xenopus*, *X. laevis* is a South

African clawed frog native to Southern and Western Africa. This animal is entirely aquatic, preferring stagnat pools of water and spends little if any time on land (Deuchar, 1975). In such a habitat, *Xenopus* would encounter dynamic environmental changes and is therefore an interesting organism for stress response research. *Xenopus laevis* is also a desirable organism for experimental embryology and developmental analyses as the female frogs can be hormonally stimulated to lay eggs, the oocytes are fertilized in vitro and the embryos grow rapidly, reaching tadpole stage in a matter of days (Sive et al., 2000; Brown, 2004; Heikkila et al., 2007). The large egg size, approximately 1.1 mm in diameter, allows visual observation of developmental progression and facilitates microinjection as well as provides a large amount of protein and nucleic acids for molecular analysis.

Additionally, *Xenopus* continuous cell lines are useful tools for in vitro molecular analyses. The *Xenopus laevis* A6 epithelial cell line, which was used in this study, was initially established by Rafferty (1969) from normal kidney cells of the renal uriniferous tubule of the adult male frog. The A6 cell line is easy and inexpensive to maintain and has a rapid doubling time of approximately 24 h making it an excellent tool for cellular and molecular biology research. A6 cells have been utilized as an experimental system in many areas of research including, proliferation and differentiation, genetic profiling under zero gravity, the function of Cystic Fibrosis transmembrane conductance regulator channels, and the role of renal epithelial sodium channels in hypertension (Guerra, 2004; Bjerregaard, 2007; Ikuzawa et al., 2007; Wang et al., 2008). A6 cells have also been used to characterize the expression of numerous *Xenopus hsp* genes including *hsp110*, *hsp90*, *hsp70*, *BiP*, *hsp47* and *hsp30* (Darasch et al., 1988; Tam and Heikkila, 1995; Ali et al., 1996a; Ali et al., 1996b; Briant et al., 1997; Miskovic et al., 1997; Gauley and Heikkila, 2006; Hamilton and Heikkila,

2006). A6 cells have also been utilized to study *hsp* gene expression in response to various stressors including heat shock, sodium arsenite, herbimycin A, hydrogen peroxide, cadmium chloride, MG132 and celastrol (Darasch et al., 1988; Briant et al., 1997; Muller et al., 2004; Gauley and Heikkila, 2006; Woolfson and Heikkila, 2009; Young et al., 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). A6 cells have also been used to study functional characteristics of HSPs. For example, experiments in A6 cells determined that HSP30 assembles into high molecular weight aggregates in order to function as a molecular chaperone (Ohan et al., 1998).

1.4.1 *hsp30* and *hsp70* genes in *Xenopus laevis*

1.4.1.1 hsp30

The *hsp30* gene family is part of the s*hsp* gene superfamily and 2 gene clusters (*hsp30A/B* and *hsp30C/D/E*) have been isolated so far in *Xenopus laevis* (Heikkila et al., 1997; Heikkila, 2010). While the first to be identified were *hsp30A* and *hsp30B* (Bienz, 1984), it was later discovered that *hsp30A* had an insertional mutation in its coding region and resulted in a truncated protein, while *hsp30B* was a pseudogene. Subsequently, *hsp30C* and *hsp30D* were isolated and encoded functional 24 kDa proteins (Krone et al., 1992; Heikkila, 2003). The *hsp30E* gene, for which a partial gene fragment is available, has yet to be characterized (Krone et al., 1992; Heikkila et al., 1997). Analysis of *hsp30C* revealed the presence of multiple cis-acting elements: 2 TATA boxes, 3 HSEs and 1 CCAAT box at the same relative location. Furthermore, the *hsp30C* gene lacked introns but the AT rich 3' end contained a polyadenylation sequence and mRNA instability sequence (Heikkila, 2010).

1.4.1.2 *hsp70*

Members of the *hsp70* gene family were first isolated in *Xenopus* by Bienz (1984). Coding regions of the stress inducible *hsp70* gene and 2 constitutive heat shock cognate genes *hsc70.I* and *hsc70.II*, all of which are cytoplasmic, have been isolated or characterized in our laboratory (Bienz, 1984; Ali et al., 1996a; Ali et al., 1996b). To date, four-70 kDa genes have been isolated in *Xenopus*, *hsp70A-D*, although only the full gene of *hsp70A* was analyzed. As well as being intronless, the 5' region contained cis-acting elements including a TATA and CCAAT box and 3 HSEs (Heikkila, 2010).

1.4.2 Expression and function of HSP30 in *Xenopus laevis* A6 cells

Multiple HSP30 isoforms were detected using 2-D SDS PAGE and immunoblot analysis which suggested a relatively large *hsp30* gene family (Tam and Heikkila, 1995). Further analysis of the HSP30C and HSP30D protein structure revealed the presence of an α-crystallin domain, conserved in all eukaryotic sHSPs, (Krone et al., 1992) and a highly polar carboxyl-terminal end required for solubility of itself its client proteins (Fernando and Heikkila, 2000; Fernando et al., 2002). Additionally, HSP30C and HSP30D were capable of molecular chaperone activity, in vitro and in vivo, by inhibiting heat-induced client protein aggregation and maintaining heat or chemically denatured luciferase in a folding competent state (Fernando and Heikkila, 2000; Abdulle et al., 2002; Fernando et al., 2002).

SHSPs are some of the most strongly induced HSPs (Arrigo and Landry, 1994; Haslbeck, 2002). In A6 cells, *hsp30* gene expression was induced by heat shock, MG132, sodium arsenite, herbimycin A, hydrogen peroxide, celastrol, and cadmium treatment (Briant et al., 1997; Fernando et al., 2003; Muller et al., 2004; Woolfson and Heikkila, 2009; Walcott

and Heikkila, 2010; Young and Heikkila, 2010). Combined treatments with multiple stressors were found to act synergistically on the accumulation of HSP30 in A6 cells (Heikkila et al., 1987; Young et al., 2009). For example, cells treated with concurrent mild heat shock and sodium arsenite exhibited an elevated level of HSP30 accumulation that was greater than the sum of each stress individually (Young et al., 2009).

Although *hsp* gene expression is primarily regulated at the transcriptional level by HSF1, post-translational modifications may affect the structure and function of HSP30. Phosphorylation of serine residues, by mitogen-activated protein kinase activated protein kinase-2 (MAPKAPK-2), resulted in alterations of HSP30's secondary structure and the formation of smaller complexes, rather than the large, oligomeric structures required for its chaperone activity (Fernando et al., 2003). Fernando et al., (2003) proposed that during cellular recovery from stress in *Xenopus*, the role of phosphorylation was to accelerate the release of tightly bound HSP30 particles from its targets by inducing a change in the oligomeric assembly of HSP30. Thus, chaperones such as HSP70 are able to access the target proteins in order to assist in their refolding and reactivation.

1.4.3 Localization of HSP30 in A6 cells

Previous studies, using immunocytochemistry and laser scaning confocal microscopy (LSCM), reported that heat shock-induced HSP30 accumulation was observed primarily in the cytoplasmic region, in a punctate pattern, likely corresponding to high molecular weight complexes of sHSP associated with unfolded protein (Gellalchew and Heikkila, 2005). Similar findings with respect to HSP30 localization were observed in cadmium, MG132, sodium arsenite or celastrol-treated A6 cells (Voyer and Heikkila, 2008; Woolfson and

Heikkila, 2009; Walcott and Heikkila; 2010; Young and Heikkila, 2010). Previous studies have also shown co-localization of *Xenopus* HSP30 with F-actin cytoskeletal disorganization at higher heat shock temperatures, which suggests that HSP30 may be involved in protecting cytoskeletal proteins and other cytoplasmic proteins (Gellalchew and Heikkila, 2005; Manwell and Heikkila, 2007). Acquisition of stress-tolerance, determined by monitoring cellular morphology and appearance of cytoskeletal actin filaments, was conferred in A6 cells by heat shock or combinations of MG132, arsenite and heat shock. This was mediated by HSPs, such as HSP30, since HSF1 inhibition repressed tolerance (Young et al., 2009; Young and Heikkila, 2010). Additionally, in A6 cells treated with higher concentrations of sodium arsenite, accumulation of HSP30 was observed in the cytoplasm as well as a small amount in the nucleus, but not in the nucleolus (Gellalchew and Heikkila, 2005).

1.4.4 Expression and function of HSP70 in *Xenopus laevis* A6 cells

Several members of the *Xenopus* HSP70 family have been investigated including the stress-inducible HSP70, constitutively expressed HSC70 and BiP (Ali et al., 1997; Briant et al., 1997; Miskovic and Heikkila, 1999; Ali and Heikkila, 2002). HSC70 and HSP70 are cytosolic with functional nuclear localization signals while BiP is confined to the endoplasmic reticulum (Goldfarb et al., 2006; Heikkila et al., 2007). HSP70 and HSC70 are both involved in appropriate folding and trafficking of newly synthesized proteins in the cell, however, recent studies on surface proteins in oocytes suggest these HSPs have differential and antagonistic effects (Goldfarb et al., 2006). Previously in our laboratory, it was shown that *hsp70* gene expression was induced by a variety of stressors including heat shock, sodium arsenite, celastrol and cadmium (Heikkila et al., 1997; Young et al., 2009; Woolfson

and Heikkila, 2009; Walcott and Heikkila, 2010). In A6 cells, *hsp70* mRNA was not detected constitutively at 22 ºC however, heat shock-induced *hsp70* accumulation was detected at 30 ºC with peak levels at 35-37 ºC (Gauley and Heikkila, 2006). The stress-induced accumulation of *hsp70* mRNA and protein were also observed in A6 cells treated with various stressors such as herbimycin A, hydrogen peroxide, ethanol, celastrol and cadmium (Darasch et al., 1988; Briant et al., 1997; Muller et al., 2004; Gauley and Heikkila, 2006; Woolfson and Heikkila, 2009; Walcott and Heikkila, 2010).

1.5 Ubiquitin proteasome system

Protein homeostasis is dependent on the cooperation between protein synthesis, folding and degradation. Misfolded, aged or damaged proteins are targeted for degradation by the ubiquitin proteasome system (UPS). Ubiquitin molecules are reversibly added in long chains to target proteins by a cascade of ubiquitin activating, ubiquitin conjugating and ubiquitin ligating enzymes (Tanaka, 2009). Ubiquitin ligases provide the specificity to the ubiquitin pathway since they recognize and bind to specific substrate sequences. Downstream, ubiquitinated proteins are processed and degraded by the proteasome. The 26S proteasome complex is comprised of a 20S core particle and 2 capping 19S regulatory particles. The 19S cap particles are responsible for recognition and processing of ubiquitinated proteins before degradation by the 20S particle. The 20S core is a hollow cylinder of heptameric stacked rings with individual subunits of either α or β in an αββα conformation (Yang et al., 2007). The α subunits control substrate translocation into the catalytic core whereas the β subunits contain the catalytic sites with chymotrypsin-like (β5), trypsin-like (β2) and peptidyl glutamyl peptide hydrolyzing (β1) activity (Tanaka, 2009).The

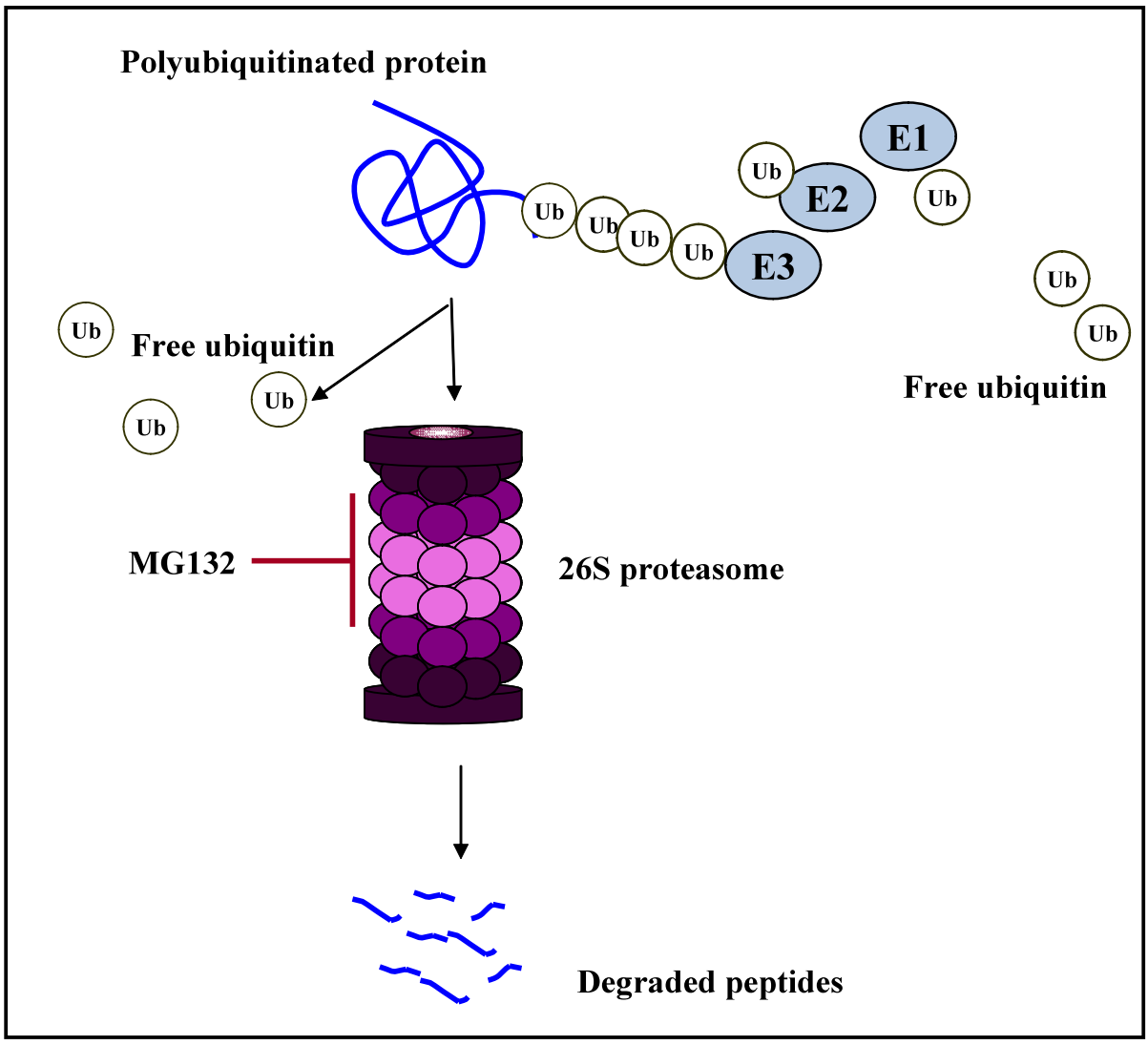
ubiquitinated substrate is then hydrolysed via an ATP-dependent mechanism and ubiquitin molecules are removed and recycled by deubiquitination enzymes present on the 19S particle. Figure 2 shows a simplified schematic of the UPS. As the primary proteolytic complex in the cell, the proteasome is tightly linked to cellular protein homeostasis. Control of protein degradation and turnover require the cooperative action of recognition by molecular chaperones, and recycling of peptides for the synthesis of new proteins. Inhibition of proteasomal activity results in a build up of polyubiquitinated protein which can act as a trigger for *hsp* gene expression.

1.6 Withaferin A

For centuries, traditional Indian medicine has utilized plants and their derivatives to treat ailments of the endocrine, cardiopulmonary, and central nervous systems (Mishra et al., 2000; Mohan et al., 2004). Known for its anti-inflammatory properties, Ashwaganda

Figure 2. A model of the UPS. Polypeptides are marked for proteasomal degradation by the addition of chains of ubiquitin molecules by ubiquitinating enzymes (E1, E2 and E3). Ubiquitin moieties are recognized by the 19S particle and the protein is subsequently

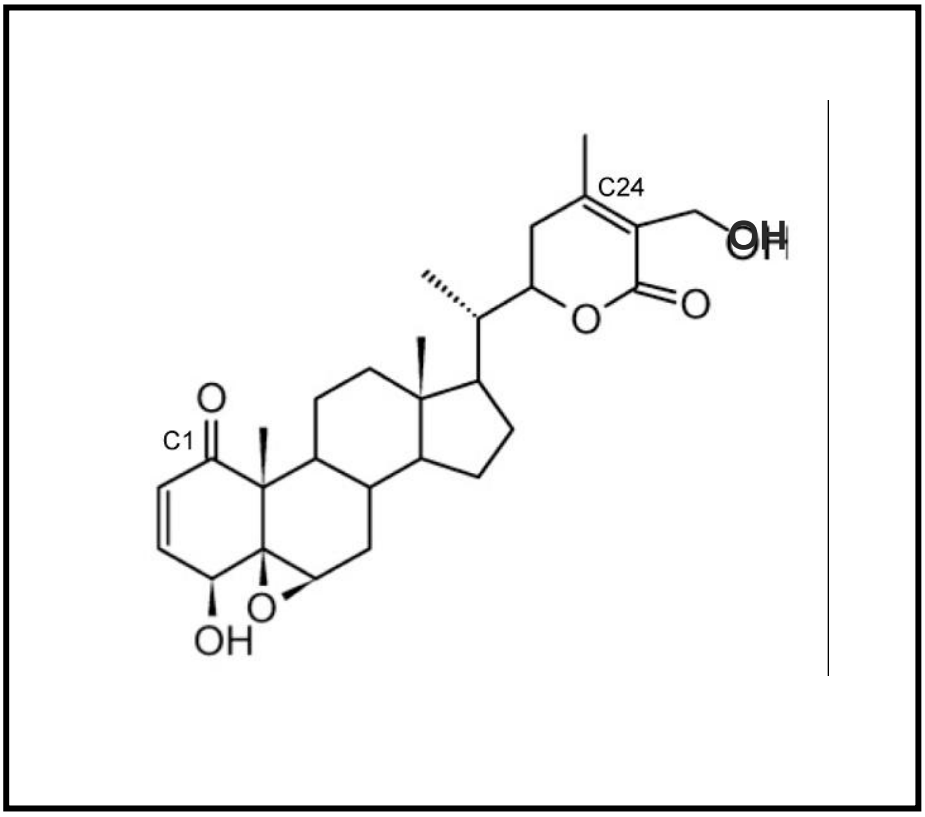
degraded within the 26S proteasome. Small peptide chains and free ubiquitin molecules are released for recycling by the cell. MG132 inhibits proteasomal activity (Adapted from Lee and Goldberg, 1998).


Polyubiquitinated protein
E1
E2
E3
Ub
Free ubiquitin
Free ubiquitin
MG132
26S proteasome
Degraded peptides

(*Withania somnifera*) has been gaining more attention lately with its acceptance as a dietary supplement in North America (Yang et al., 2007). Of the 40 compounds extracted from the leaves and roots of Ashwaganda, withaferin A (WA), a steroidal lactone (Fig. 3), is thought to be the active component responsible for its therapeutic properties (Mandal et al., 2008). Additional research has shown that WA inhibited angiogenesis by prevention of nuclear factor-κB (NFκB) activation, and had anti-carcinogenic, anti-tumor and radiosensitizing activity (Devi et al., 1995; Mohan et al., 2004; Malik et al., 2007; Yang et al., 2007; Mandal et al., 2008). For example WA was capable of inducing apoptosis in leukemic cells with no toxicity to normal human progenitor cells (Mandal, 2008). Furthermore, in Swiss albino mice, WA inhibited tumor growth and increased tumor free survival in a dose-dependent manner (Devi et al., 1995). Malik et al. (2007) determined that WA-induced apoptosis was mitochondrial-mediated and initiated by the generation of reactive oxygen species. Oxidative metabolites produce post-translational modifications often resulting in abhorrent or damaged proteins that may not fold properly. Unfolded protein is known to be an activator of the HSR and *hsp* gene expression.

Recently, WA was shown to potently inhibit the chymotrypsin-like activity of a purified rabbit 20S proteasome and 26S proteasome in human prostate cancer cells. Inhibition of prostate tumor cellular proteasome activity in culture and tissue by WA resulted in accumulation of ubiquitinated protein that was accompanied by apoptosis (Yang et al., 2007). It was also suggested that the conjugated ketone carbons at positions 1 and 24 of WA facilitated WA-induced proteasome inhibition by directly interacting with the catalytically active N-terminal threonine of the β5 subunit (Malik et al., 2007). Previously in our

Figure 3. Schematic of withaferin A molecular structure. Carbons 1 and 24 are marked (Adapted from Falsey et al., 2005).


C24
OH
O
O
O
C1
O
OH

laboratory, MG132 and lactacystin, both potent proteasomal inhibitors, were shown to induce expression of *hsp30* and *hsp70* in *Xenopus laevis* A6 cells (Young and Heikkila, 2010). Proteasome inhibition has great potential for use in cancer treatment and prevention (An et al., 1998; Orlowski, 1999; Drexler et al., 2000; Li et al., 2000; Soligo et al., 2001). Additionally, a recent study by Yu et al. (2010) on inhibition of HSP90 activity also determined that HSP70 accumulation increased in response to WA. Since the majority of studies performed to date have been on tumor cell lines, it would be interesting to study WA-treated non-cancerous epithelial cells. In hamsters, for example, WA elicited a protective effect on normal RBCs, yet, induced apoptosis in cancerous cells (Manoharan et al., 2009). In order to realize the therapeutic potential of this plant extract, its effect on proteostasis including *hsp* gene expression, needs to be understood more clearly.

1.7 Objectives

Xenopus laevis is an excellent vertebrate model system from which developmental and molecular results are readily transferable. However, the experimental parameters need to be determined in a continuous cell line prior to utilization of *Xenopus* embryos. The primary goal of this thesis was to determine the effect of WA on proteasome activity and *hsp* gene expression in *Xenopus laevis* A6 kidney epithelial cells. The objectives for this study are as follows:

- To analyze the accumulation of cellular protein conjugated to ubiquitin in cells treated with WA
- To examine chymotrypsin-like activity of the 26S proteasome in WA-treated cells
- To investigate the effect of WA on the expression of *hsp30* and *hsp70* genes expression
- To determine whether HSF1 activation was involved in WA-induced accumulation of HSP30 and HSP70
- To examine the pattern of HSP30 and HSP70 accumulation in cells simultaneously exposed to both WA and a mild heat shock
- To analyze the WA-induced localization of HSP30 accumulation in A6 cells
- To examine the effects of WA on cytoskeleton organization
- Lastly, to explore whether WA treatment could confer an acquired state of thermotolerance

2 Materials and methods

2.1 Culturing of *Xenopus laevis* A6 kidney epithelial cells

The *Xenopus laevis* A6 kidney epithelial cell line (CCL-102; American Type Culture Collection (ATCC), Rockville, MD) were maintained as described in Phang et al. (1999). Cells were cultured at 22 °C in 55% Leibovitz (L)-15 media (Sigma-Aldrich, Oakville, ON) supplemented with 10% (v/v) fetal bovine serum (Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) in T75 cm2 BD Falcon tissue culture flasks (VWR International, Mississauga, ON). When cells reached confluence, old media was removed by aspiration, 2 ml of Versene [0.02% (w/v) KCl, (0.8% (w/v) NaCl, 0.02% (w/v) KH2PO4, 0.115% (w/v) Na2HPO4, 0.02% (w/v) Na2EDTA] was added to the flasks for 2 min and then aspirated off. Cells were lifted off the surface of the flask using 1 ml of 1 X trypsin (Sigma) in 100% Hank's balanced salt solution (HBSS; Sigma) for approximately 30 sec and then removed by aspiration. Free cells were resuspended in fresh media and then distributed evenly into additional tissue culture flasks. Cell treatments on the A6 cell line were performed with cells that reached 90-100% confluence.

2.2 Treatment of *Xenopus* A6 kidney epithelial cells

Withaferin A (≥ 99% purity; Enzo Life Sciences, Plymouth Meeting, PA) was dissolved in dimethylsulphoxide (DMSO; Sigma) to make a stock solution of 2.21 mM which was aliquoted and stored at -20 °C. This working stock was used to prepare 2, 5, or 10 μM concentrations of withaferin A used for subsequent experiments. MG132 (Sigma) and

KNK437 (N-formyl-3, 4-methylenedioxy-benzylidene-γ-butyrolactam; Calbiochem, Gibbstown, NJ) were also dissolved in DMSO to prepare 5 mg/mL stock solutions. Experiments with MG132 used 30 μM concentrations while all treatments with KNK437 used 100 μM concentrations. Appropriate volumes of each chemical were added to fresh L-15 media followed by gentle shaking to ensure even distribution prior to adding each treatment to tissue culture flasks containing A6 cells. Cells treated with 5 μM WA for 2 h were allowed to recover for 2 h in withaferin A-free L-15 media. Treatments with 100 μM KNK437 were performed 6 h prior to treatment with 5 μM withaferin A. KNK437 at a concentration of 100 μM was employed in a number of previous studies to suppress *hsp* gene expression by inhibiting HSF1–HSE binding activity in mouse, human and *Xenopus* A6 cells with no detectable effect on cell viability (Ohnishi et al., 2004; Manwell and Heikkila, 2007; Takahashi et al., 2008; Voyer and Heikkila, 2008; Young and Heikkila, 2010). Some flasks of A6 cells were heat shocked for 4, 8 or 12 h in a water bath set at 30 °C. Heat shock treatments at 33 °C were for 2 h in a water bath set to the appropriate temperature, followed by a 2 h recovery period at 22 °C.

2.3 Harvesting of A6 epithelial cells

Immediately following treatment, media was aspirated off flasks containing treated A6 cells and 2 mL of 65% HBSS was added to rinse the cells. The cells were then removed with a cell scraper, suspended in 1 mL of 100% HBSS and transferred to a 1.5 ml microcentrifuge tube. After the cells were pelleted by centrifugation for 1 min at 16, 000 g, the supernatant was removed and cells were stored at -80 °C until protein or RNA isolation.

2.4 Detection of chymotrypsin-like activity

Chymotrypsin-like activity was detected from cultured A6 cells using the Promega Proteasome-Glo cell based luminescent assay kit (Promega Corp., Madison, WI). The Proteasome-Glo cell-based reagents each contain a specific luminogenic proteasome substrate (Suc-LLVY-Glo substrate for chymotrypsin-like activity) in a buffer optimized for cell permeabilization, proteasome activity and luciferase activity. Proteasomal cleavage of the luminogenic molecule generates an aminoluciferin substrate that is consumed by luciferase to produce a luminescent signal at a rate proportional to chymotrypsin-like activity (Promega Corp., 2009). Thawed Proteasome-Glo cell based buffer, lyophilized luciferin detection reagent and Suc-LLVY-Glo substrate were all equilibrated to room temperature in the dark. Once at room temperature the lyophilized luciferin detection buffer was reconstituted in Proteasome-Glo cell based buffer in an amber bottle provided by the manufacturer. Then 50 µL of the Suc-LLVY-Glo substrate was vortexed to remove any precipitate that may have formed. The substrate was then added to the amber bottle containing lyophilized luciferin detection reagent reconstituted in Proteasome-Glo cell based buffer. To remove any free aminoluciferin and reduce background luminescence, the contents of the bottle (prepared proteasome-glo cell based reagent) were then mixed gently and stored at room temperature in the dark for 30 min before use.

Following WA or MG132 treatments, cells were washed with 2 mL of versene and then detached from the flask with 1 mL of 1 X trypsin. Nine mL of fresh L-15 media was then added to the flask and pipetted up and down to rinse the flask surface and allow for even distribution. The cell suspension was then transferred to a 15 mL Falcon tube. Cells were pelleted at 4 °C by gentle centrifugation at 1, 500 g for 5 min. Following centrifugation the

media was removed by aspiration and pellets were washed in 5 mL of fresh L-15 media to remove any trypsin residue and then centrifuged again at 1, 5000 g for 5 min at 4 °C. A6 cells were then resuspended in 5 mL of fresh L-15 media. For each sample, the total number of cells per mL was determined using a Bright-Line haemocytometer (Hausser Scientific, Horsham, PA). Approximately 15,000 were suspended in 100 µL of L-15 media for the proteasome-glo cell based assay.

For the chymotrypsin-like activity assay, 100 µL of each sample was added to the wells of a white-walled 96-well plate. Then 100 µL of Proteasome-Glo cell-based reagent (prepared previously) was added to each sample-containing well. The plate contents were mixed gently in the dark by placing the plate on a rocking platform for 2 min. The samples were then incubated at room temperature for 45 min in the dark, prior to measuring chymotrypsin-like activity. The luminescence of each sample was measured using the Victor3 luminometer at 430 nm (PerkinElmer Inc., Waltham, MA). Values were then compared to a blank sample (Proteasome-Glo cell-based reagent + L-15 media) and a control-treated sample (Proteasome-Glo cell-based reagent + cells treated with DMSO).

2.5 Protein Isolation and Western Blot Analysis

2.5.1 Protein Isolation from A6 Cells

A6 cell pellets were homogenized with 500 µL of lysis buffer (200 mM sucrose, 2 mM EGTA, 1 mM EDTA, 40 mMNaCl, 30 mM HEPES, pH 7.4) containing 1% (w/v) SDS, 1% (w/v) protease inhibitor cocktail (Roche) then sonicated on ice for 15-1 s pulses at 3.5 output using a sonic dismembranator (Model 100, Fisher Scientific, Waltham, MA). It should

be noted that the lysis buffer used for samples in preparation for immunoblotting with a mouse anti-ubiquitin antibody contained 10 mM N-ethylmaleimide (NEM; Sigma) to facilitate the recovery of ubiquitinated protein. Isopeptidases hydrolyze the ubiquitin chain from ubiquitinated proteins and are dependent on active-site cysteinyl residues, which can be inactivated with thiol-blocking compounds such as NEM (Stipanuk et al., 2003). Protein was separated from the cellular debris by centrifugation at 20 800 g for 60 min at 4 °C in an Eppendorf Centrifuge 5810R. After centrifugation, supernatants were transferred to new 1.5 ml microcentrifuge tubes and stored at –20 °C.

2.5.2 Protein Quantification

The concentrations of all isolated proteins were calculated using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Dilutions of bovine serum albumin (BSA; Bioshop, Burlington, ON) ranging from 0 to 2 mg/ml were prepared in distilled water from a 2 mg/ml stock. Aliquots of isolated protein samples were diluted 1:2 in distilled water and 10 µl of each BSA standard and protein sample was loaded onto a 96 well assay plate in triplicate. A 50:1 mixture of BCA reagent A to reagent B (Pierce) was prepared and 80 µl of the mixture was added to each well. The plate was incubated at 37 °C for 30 min then read at 562 nm using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, CA) and the Soft Max Pro software. The BSA standards were used to construct a standard curve in Microsoft Excel that was in turn used to determine the concentration of protein for each sample.

2.5.3 Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) employing 10 -12% polyacrylamide gels was performed using a BioRad Mini Protean III gel system (BioRad, Mississauga, ON). Separating gels [10% (v/v) acrylamide, 0.27% (v/v) n'n'-bismethyleneacrylamide, 0.375 M Tris pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate (APS), 0.1 5% (v/v) n,n,n'n'- Tetramethylethylenediamine (TEMED)] were prepared, poured and allowed to polymerize with a 100% ethanol overlay for 30 min. The ethanol layer was then removed and stacking gels [4% (v/v) acrylamide, 0.11% (v/v) n'n'-bismethyleneacrylamide, 0.125 M Tris pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.2% (v/v) TEMED] were layered on top. Gels were allowed to polymerize for another 30 min with combs inserted to create wells. During gel polymerization, protein samples were aliquoted with 20 – 60 μg of protein 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] to a final concentration of 1 X. All protein samples and protein markers were boiled for 10 min, cooled on ice for 5 min then pulse-spun (6 sec at 9, 000 rpm) in a microcentrifuge before loading into the gel. Gels were electrophoresed with 1 X electrophoresis buffer [25 mM Tris, 0.2 M glycine, 1 mM SDS] at 90 V until the dye reached the separating gel. Electrophoresis was then continued at 150-160 V until the dye front reached the bottom of the gel.

During electrophoresesis, pure nitrocellulose transfer blot membranes (BioRad) and filter paper (BioRad) were cut to 5.5 cm x 8.5 cm, and membranes were equilibrated in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] for 30 min. Following electrophoresis, the stacking gel was cut away and the remaining gel was soaked in transfer

buffer for 10-15 min. Proteins were transferred to the nitrocellulose membrane using a Trans-Blot Semi-dry Transfer Cell (BioRad) at 20 V for 25 min. After transfer, blots were stained with Ponceau-S stain [0.19% (w/v) Ponceau-S, 5% (v/v) acetic acid] for 10 min to determine efficiency of transfer, rinsed twice in water and then scanned with a Hewlett Packard ScanJet 3300C. Blots were then incubated in 5% blocking solution [20 mM Tris (pH 7.5), 0.1% Tween 20 (Sigma), 300 mM NaCl, 5% (w/v) Nestle® Carnation skim milk powder] for 1 h at room temperature. The blocking solution was then replaced with primary antibody in 5% blocking solution for 1 h at room temperature. The antibodies used were either mouse anti-ubiquitin (Zymed, San Francisco, CA), rabbit anti-HSP30 (Fernando and Heikkila, 2000), anti-HSP70 (Gauley and Heikkila) or anti-actin (Sigma) antibodies, prepared for *Xenopus* at dilutions of 1:150; 1:1000, 1:250 and 1:200, respectively. Membranes were washed with 1 X Tris-buffered saline with Tween 20 (TBS-T) [20 mM Tris, 300 mM NaCl, (pH 7.5), 0.1% Tween 20 (Sigma)] once for 15 min and then twice for 10 min to remove excess unbound antibody. Blots were incubated with the secondary antibody conjugate, AP-conjugated goat-anti-rabbit (BioRad) at a 1:3000 dilution or AP-conjugated goat-anti-mouse (BioRad) at a 1:1000 dilution in 5% blocking solution for 1 h. The membranes were washed with fresh TBS-T once for 15 min and then twice for 5 min. For detection, the blots were immersed in alkaline phosphatase detection buffer (50 mM Tris, 50 mM NaCl, 25 mM MgCl2, pH 9.5) with 0.33% nitrobluetetrazolium chloride (NBT; Roche) and 0.17% 5-bromo- 4-chloro-3-indolyl phosphate, toluidine salt (BCIP; Roche) until the bands were visible, then removed before saturation of the bands occurred. Images were scanned using a Hewlett Packard ScanJet 3300C.

2.5.4 Densitometric statistical analysis

Densitometric analyses were performed in triplicate of appropriately stained blots (within the range of signal linearity) using ImageJ (Version 1.38) software. In using this software, equal sized boxes were drawn around all protein bands detected with ant-HSP30, anti-HSP70 or anti-ubiquitin. Average densitometric values were expressed as a percentage of the maximum hybridization band or as percent inhibition for KNK437 experiments. The data were graphed with standard error represented as vertical error bars. The level of significance of the differences between the samples was calculated using one-way analysis of variance (ANOVA) with a Tukey's post-test. Confidence levels used were 95% ($p<0.05$) or 90% ($p<0.1$) as indicated.

2.6 Immunocytochemistry and laser scanning confocal microscopy

For immunofluorescence analysis, A6 cells were grown on glass coverslips in Petri dishes. Coverslips were first base washed in small staining jars (Thomas Scientific Apparatus, Philadelphia, PA) to ensure full contact with the base solution [49.5% (v/v) ethanol, 0.22 M NaOH] for 30 min with periodic shaking at room temperature. The coverslips were then rinsed with running distilled water for 3 h and dried on 3MM Whatman paper. Finally, the coverslips were flame sterilized in the laminar flow hood then placed in new sterile Petri dishes. A6 cell suspension was added to the Petri plate and allowed to attach for 24 h at 22 °C. For WA treatments, cells were treated directly in the Petri dishes at 22 °C. In heat shock experiments, the Petri dishes were wrapped with parafilm® (Pechiney Plastic Packaging, Menasha, WI), sealed in a plastic bag and then placed in a heated water bath. Following treatment, the L-15 media was removed and the cells were rinsed twice in

phosphate-buffered saline (PBS; 1.37 M NaCl, 67 mM Na2HPO4, 26 mM KCl, 14.7 mM H2PO4, 1 mM CaCl2, 0.5 mM MgCl2, pH 7.4) and the coverslips were transferred to new small Petri dishes. Cells were then fixed in 3.7% paraformaldehyde (BDH, Toronto, ON) for 15 min followed by three washes with PBS for 5 min each. After permeabilized with 0.3% Triton X-100 (Sigma- Aldrich) in PBS for 10 min, cells were rinsed three times with PBS for 5 min. Cells were then incubated with 3.7% bovine serum albumin fraction V (BSA fraction V; FisherScientific, Ottawa, ON) for 1 h at room temperature or overnight at 4 °C followed by with affinity-purified rabbit anti-*Xenopus* HSP30 antibody (1:500) in 3.7% BSA for 1 h. BSA fraction V was filter-sterilized with a 0.45 μm filter to remove particulate that might affect image quality before use. Three washes for 3 min each in PBS, was followed by indirect labeling of cells with a fluorescent-conjugated secondary antibody, goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR), at 1:2000 in 3.7% BSA for 30 min in the dark. The cells were subsequently probed for F-actin with rhodamine-tetramethylrhodamine-5-isothiocyanate phalloidin conjugated TRITC (Molecular Probes) at 1:60 in PBS for 15 min in the dark followed by three PBS washes for 5 min each. Coverslips were mounted on glass slides with VectaShield mounting media containing 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Inc., Burlingame, CA), which stains nucleic acids allowing visualization of nuclei. Coverslips were permanently attached and sealed to slides using clear nail polish and then stored at 4 °C for no longer than three weeks. Slides were evaluated by laser scanning confocal microscopy (LSCM) using a Zeiss Axiovert 200 microscope and LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, ON) and the 63 X oil objective. The multi-channel configuration was used to collect emission spectra for DAPI (405), Alexa 488

(488 nm), and TRITC (533 nm) on multiple tracks to minimize cross-channel contamination of fluorescence emissions.

2.7 Antisense riboprobe production and northern blot hybridization analysis

2.7.1 *hsp30C* template construction

Previously in our laboratory, the coding region of *hsp30C* genomic DNA was inserted into the pRSET expression vector (Invitrogen, Carlsbad, CA) by Pasan Fernando (Fernando and Heikkila, 2000). Plasmids containing the *hsp30C* insert were then transformed into *Escherichia coli* DH5α cells where individual colonies were inoculated in 5 mL of LB broth [1% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5] containing 100 µg/ml ampicillin (Bioshop, Burlington, ON) and grown overnight at 37 °C for 14-16 h with vigorous shaking.

2.7.2 *hsp70* template construction

The open reading frame of the *hsp70* gene was previously isolated (Lang et al., 2000) and inserted into the plasmid pSP72 (Promega, Napean, ON). Plasmids containing the *hsp70* insert were also inoculated in 5 mL of LB broth containing 100 ìg/ml ampicillin and grown overnight at 37 °C for 14-16 h.

2.7.3 Isolation of plasmid DNA

After overnight incubation, bacterial cells were collected by centrifugation (Eppendorf Centrifuge 5810R; Brinkmann Instruments Ltd., Mississauga, ON) at 2, 600 g for 5 min at 4 °C. After removing the media, pelleted cells were resuspended in 200 µl of ice-cold alkaline lyisis solution #1 [50 mM glucose, 25 mM Tris (pH 8.0) and 1 mM EDTA (pH

8.0)] and then transferred into a microcentrifuge tube. The cells were lysed by gentle mixing with 200 µl of alkaline lysis solution #2 [0.2 M NaOH and 1% (w/v) SDS (sodium dodecyl sulfate)]. The microcentrifuge tubes were kept on ice for the remainder of the protocol. After adding 200 µl of ice-cold alkaline solution #3 [3 M potassium acetate and 5 M glacial acetic acid] the tubes were mixed gently and placed on ice for 5 min. To separate cellular debris, the samples were centrifuged at 20, 800 g for 5 min at 4 °C and the supernatant was transferred to fresh microcentrifuge tubes. This was followed by RNase A treatment (10 µg/mL; BioShop, Burlington, ON) for 1 h at 37 °C to remove unwanted RNA from the samples.

After removing samples from the 37 °C water bath, 600 µl of phenol:chloroform (1:1) was added and the samples were mixed by vortexing then centrifuged at 20, 800 g for 3 min at 4 °C. The upper aqueous layer was transferred to new microcentrifuge tubes to which 600 µL of isoamyl alcohol:chloroform (1:24) was added These samples were then vortexed and centrifuged at 20, 800 g for 3 min at 4 °C. Again the upper aqueous layer was transferred to fresh microcentrifuge tubes. To precipitate the nucleic acids, 600 µl of isopropanol was added to each microcentrifuge tube. The samples were then mixed by vortexing and allowed to stand at room temperature for 2 min, followed by centrifugation at 20, 800 g for 5 min at room temperature. The supernatant was gently removed and 1 ml of 70% ethanol was added to wash the pellets. The samples were then centrifuged at 20, 800 g for 2 min at 4 °C. Then the ethanol was removed and the previous step was repeated. The pellets were allowed to air dry and then resuspended in 50 µl distilled water and stored at -20 °C.

2.7.4 In vitro transcription

The isolated plasmid containing the *hsp30C* or *hsp70* insert as mentioned above was linearized using the PvuII or MluNI restriction enzymes, respectively (Roche, Laval, QU). The linearized plasmids were then electrophoresed on a 1% (w/v) agarose gel in 1 X modified tris-acetate EDTA buffer (TAE; Millipore corp., Bedford, MA). The DNA band corresponding to the *hsp30C* or *hsp70* insert, when compared to size standards, was visualized using a UV lamp. The DNA band was excised from the gel using a razor blade. Plasmid DNA was collected from the gel using the Montage DNA gel Extraction Kit (Millipore). The gel slice containing the plasmid DNA was placed into a gel nebulizer, containing a microporous membrane filter (Millipore), which was then centrifuged at 5, 000 g for 10 min at 4 °C. The filter was removed and 100% ice-cold filtered ethanol (2x volume) and 3 M sodium acetate (pH 5.2, 1/10 volume) were added to precipitate the DNA. The tube was then placed at -20 °C for 30 min followed by centrifugation at 20, 800 g for 10 min at 4 °C. After the supernatant was discarded, the pellet was washed twice with 1 ml of 70% ice-cold filtered ethanol and centrifuged at 20, 800 g for 10 min at 4 °C. The ethanol was then discarded and the pellet was air dried and resuspended in 20 µl of sterile water and stored at -20 °C.

In vitro transcription was used to generate digoxygenin (DIG)-labelled riboprobes. Each in vitro transcription reaction containing 4 µl of linearized DNA template, 4 µl of rNTP mix [2.5 mM rGTP, 2.5 mM rATP, 2.5 mM rCTP, 1.625 mM rUTP (Promega, Nepean, ON), 0.875 mM DIG-11-UTP (Roche), 1.5 µl diethylpyrocarbonate (DEPC, Sigma)-treated water, 4 µl of 100 mM diatholthreitol (DTT; Promega), 4 µl of 5 X transcription buffer (Fermentas, Burlington, ON), 0.5 µl RNase inhibitor (Fermentas) and 40 IU of SP6 RNA polymerase

(Roche)], was carried out in a 1.5 ml microcentrifuge tube for 1 h at 37 °C. To remove any remaining DNA template, reactions were incubated with 2 µl of RNase-free DNase 1 (Roche) for 10 min at 37 °C. In vitro transcripts were then precipitated by the addition of 10 µl of 3 M sodium acetate (pH 5.2), 80 µl of TES [10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 1% (w/v) SDS] and 220 µl of ice-cold 100% filtered ethanol. The reaction was incubated at -80 ˚C for 30 min and then centrifuged at 20, 800 g for 15 min at 4 °C. After the supernatant was removed and the pellet was air-dried the RNA was resuspended in 21 µl of DEPC-treated water and stored at -80 °C until use in northern blot hybridization analysis. Two µl of the sample was electrophoresed to verify the presence of the in vitro transcript.

2.7.5 RNA isolation

RNA was isolated from pelleted A6 cells using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, ON) according to the manufacturer's protocol outlined in the RNeasy Mini Handbook (2009). Isolated RNA was suspended in 30 µl of DEPC-treated water and quantified using the NanoDrop ND-1000 (NanoDrop, Waltham, MA) spectrophotometer. RNA integrity was evaluated by electrophoresis using 2 µg of each RNA sample on a 1.2% formaldehyde agarose gel [1.2% (w/v) agarose, 10% (v/v) 10 X MOPS, 16% (v/v) formaldehyde]. Samples were prepare for electrophoresis by the addition of 10 µl of loading buffer [1 µl 10 X MOPS, 1.6 µl formaldehyde, 2 µl RNA loading dye (0.2% bromophenol blue, 1 mM EDTA (pH 8.0), 50% (v/v) glycerol), 5 µl formamide, 0.5 µg/ml ethidium bromide] followed by heat denaturation in a 68 °C water bath for 10 min then immediately cooled on ice for 5 min. The samples were then electrophoresed at 90-100 V for approximately 1 h.

2.7.6 Northern blot hybridization analysis

Ten μg of each RNA sample was separated by 1.2% formaldehyde agarose gel electrophoresis as previously described with the following exceptions. Ethidium bromide was omitted from the loading buffer and the gel was electrophoresed for 3-4 h at 90 V. Following electrophoresis the gel was soaked in 0.05 NaOH for 20 min at room temperature to denature the RNA. Next, the gel was rinsed in DEPC-treated water and soaked twice for 20 min each in fresh 20 X SSC buffer [3 M sodium chloride, 300 mM sodium citrate]. The RNA was then transferred overnight to a positively charged nylon membrane (Roche) by capillary action as described below. A piece of SSC pre-soaked 3MM Whatman filter paper was placed on a plexiglass support over a Pyrex® dish containing approximately 500 mL of 20 X SSC to act as a wick. The gel was inverted onto the wick, then topped with an appropriate sized piece of nylon membrane and two pieces of 3MM Whatman filter paper. Paper towels were next stacked about 7-8 cm high on top of the filter paper followed by a plexiglass support and a weight of approximately 250 g to aid transfer. The wick was covered in parafilm® to avoid excessive evaporation. Following transfer, the RNA was UV-crosslinked twice to the membrane using a UVC-515 Ultraviolet Multilinker (UltraLum Inc., Claremont, CA) at 12,000 MicroJ/CM2. The membrane was then soaked in 10% (v/v) glacial acetic acid for 5 min before staining with 1 X Blue Reversible Northern Blot Staining Solution (Sigma) for 10 min to assess the quality of the transfer. After rinsing with DEPC-treated water, the stained membrane was scanned using a Hewlett Packard ScanJet 3300C.

The membrane was then incubated in 50 ml of pre-heated prehybridization buffer [50% (v/v) formamide, 5 X SSC, 0.02% (w/v) SDS, 0.01% (w/v) N-laurylsarcosine, 2% (w/v) blocking reagent (Roche)] in a hybridization bag at 68 °C for 4 h in a pre-heated

hybridization oven (Boekel Scientific, Feasterville, PA). Prehybridization buffer was replaced with hybridization buffer (same components as prehybridization buffer) containing 20 µl of either *hsp30* or *hsp70* DIG-labelled antisense riboprobe. The membrane was returned to the hybridization oven and incubated overnight at 68 °C.

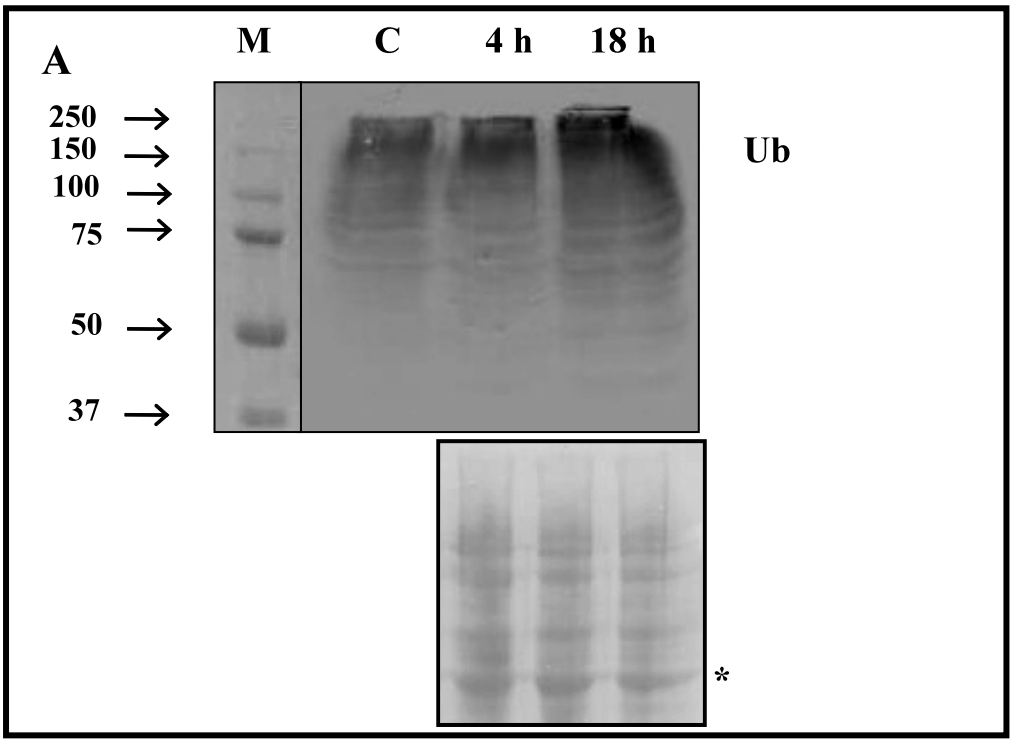
Following hybridization, any unbound probe was removed by washing the membrane. Initially, the membrane was washed twice in 2 X SSC with 0.1% (w/v) SDS at room temperature for 5 min. This was followed by a 15 min wash in 0.5 X SSC and 0.1% (w/v) SDS at 68 °C then a 15 min wash in 0.1 X SSC and 0.1% (w/v) SDS at 68 °C. The blot was then equilibrated for 1 min at room temperature in washing buffer [100 mM maleic acid buffer, 0.3% (v/v) Tween-20] then blocked using blocking solution [2% (w/v) blocking reagent (Roche), 10% (v/v) maleic acid buffer] for 1 h at room temperature. After blocking, the membrane was incubated in blocking solution containing 1:8000 alkaline phosphatase conjugated anti-DIG-Fab fragments (Roche) for 30 min. After the membrane was washed twice in washing buffer for 10 min each, it was equilibrated in detection buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl] for 2 min. CDP-star (Roche), a chemiluminescent reagent, which was applied to the membrane and allowed to develop in a hybridization bag for 10 min in the dark. Bound probe was detected using a DNR Chemiluminescent Imager (DNR Bioimaging Systems, Kirkland, QU) with the enzymatic chemiluminescence (ECL) plate for up to 12 min depending on the strength of the signal.

3 Results

3.1 Inhibition of Proteasome Activity by WA in A6 cells

Previous studies suggested that WA inhibited proteasome activity in mammalian cells (Yang et al., 2007). The impact of WA on proteasome activity in *Xenopus laevis* A6 cells was determined by examining the relative levels of ubiquitinated protein and chymotrypsin-like activity. The relative level of ubiquitinated protein in A6 cells treated with 5 μM WA for 4 h, as determined by immunoblot analysis using an anti-ubiquitin antibody, was not significantly enhanced compared to control cells (Fig. 4a). However, densitometric analysis (Fig. 4b) revealed that cells exposed to 5 μM WA for 18h exhibited a 2-fold increase in the relative levels of ubiquitinated protein, compared to control treated cells. These results are comparable to those observed with the proteasome inhibitor MG132, previously characterized in *Xenopus laevis* A6 cells (Young and Heikkila, 2010). Chymotrypsin-like activity of WA-treated A6 cells was determined by means of a cell-based assay. Chymotrypsin-like activity decreased by approximately 45% and 70% in A6 cells treated with 5 μM WA for 4 and 18 h, respectively, compared to control cells (Fig. 5). Similar results were obtained with cells subjected to different concentrations of WA for 18 h. A6 cells exposed to 2 and 5 μM WA exhibited a 38% and 80% decrease in chymotrypsin-like activity, respectively, compared to controls (Fig. 6). In comparison, treatment of cells with 30 μM MG132, a proteasome inhibitor, displayed an 83% to 98% reduction in chymotrypsin-like activity (Fig. 5 and Fig. 6).

Figure 4. Effect of WA on the relative levels of ubiquitinated protein (Ub) in A6 kidney epithelial cells. A) Cells were maintained at 22 °C and treated with DMSO (C) or with 5 μM WA for 4 or 18 h. After treatment, cells were harvested and total protein was transferred to nitrocellulose membranes from SDS-polyacrylamide gels and probed with a mouse anti-ubiquitin monocolonal antibody as described in Materials and methods. The positions of molecular mass standards in kDa are shown in the first lane (M). A sample of a representative Ponceau S stained membrane (lower panel) that brackets a 42-kDa band (*) is included to demonstrate efficient protein transfer. B) Image J software was used to perform densitometric analysis of the signal intensity for protein of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band while the standard error is represented by vertical error 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 (C) and cells exposed to 5 μM WA for 18 h are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
M
C
4 h
18 h
250
150
100
75
50
37
Ub
*

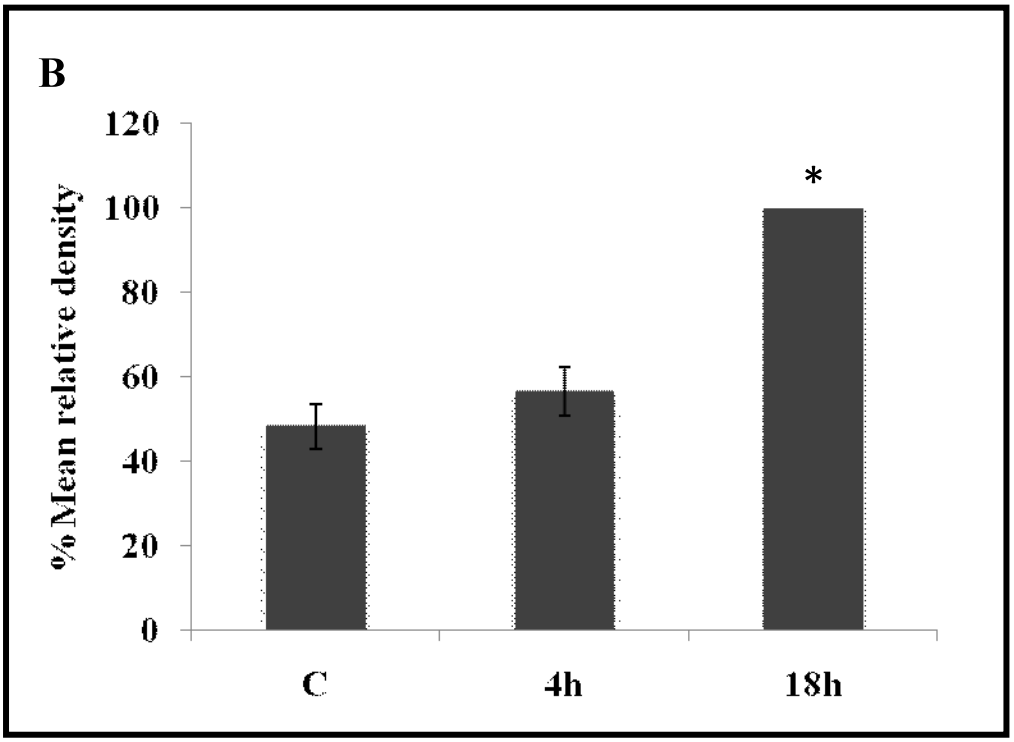

B
% Mean relative density
120
100
80
60
40
20
0
*
C
4h
18h

Figure 5. Inhibition of chymotrypsin-like activity of A6 cells treated with WA for 4 and 18 h. A6 cells were maintained at 22 °C and incubated with DMSO (C), or with 5 μM WA for 4 or 18 h or 30 μM MG132 for 18 h. The relative levels of chymotrypsin (CT)-like activity were determined and expressed as a percentage of control while the standard error is represented by vertical error 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 cells and A6 cells treated with MG132 or WA are indicated as * ($p<0.05$). These data were derived from three different experiments.

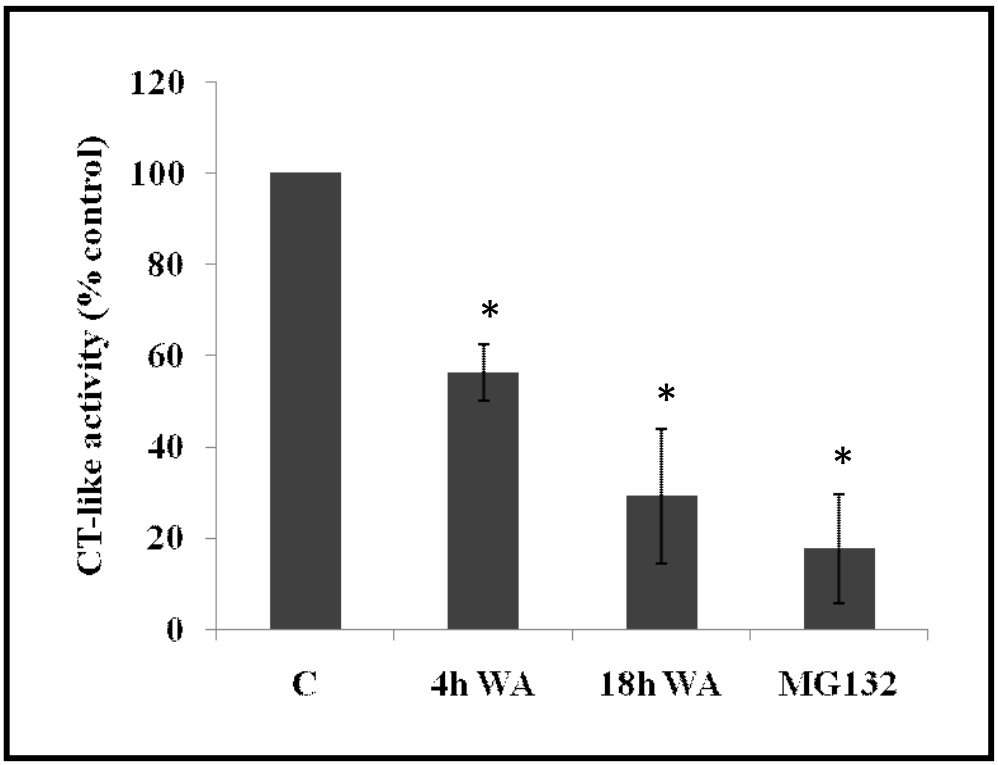
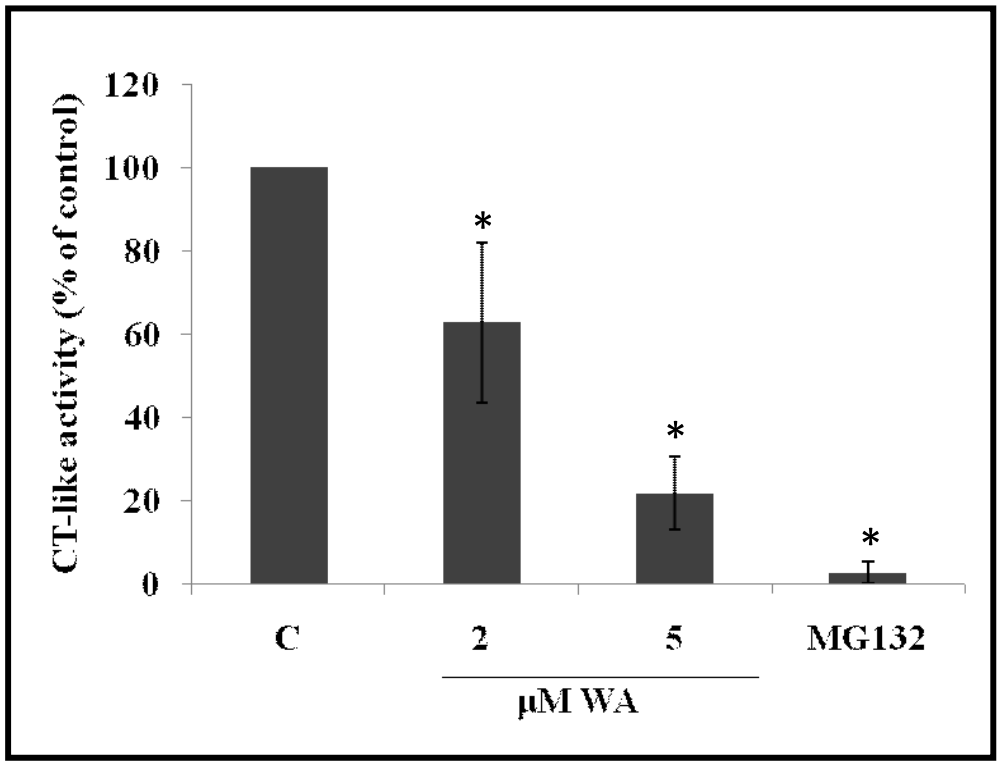

CT-like activity (% control)
120
100
80
60
40
20
0
*
*
*
C
4h WA
18h WA
MG132

Figure 6. Inhibition of chymotrypsin-like activity in A6 cells treated with 2 and 5 μM WA. Cells were maintained at 22 °C and incubated with DMSO (C) or with 2, or 5 μM WA or 30 μM MG132 for 18 h. The relative levels of chymotrypsin (CT)-like activity were determined and expressed as a percentage of control while the standard error is represented by vertical error 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 cells and A6 cells treated with MG132 or WA are indicated as * ($p<0.05$). These data were derived from three different experiments.


CT-like activity (% of control)
120
100
80
60
40
20
0
*
*
*
C
2
5
MG132
μM WA

3.2 Effect of WA on HSP30 and HSP70 accumulation

Accumulation of HSP30 and HSP70 in response to WA treatment was determined by immunoblot analysis using anti-*Xenopus* HSP30 and anti-*Xenopus* HSP70 polyclonal antibodies. These antibodies were previously used to detect HSP30 and HSP70 accumulation in A6 cells in response to a variety of stressors including heat shock (Young et al., 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). Figure 7 displays a typical result in which the relative levels of HSP30 and HSP70 were enhanced in A6 cells treated with a 33 °C heat shock compared to cells maintained at 22 °C. The effect of different concentrations of WA on HSP accumulation in A6 cells is shown in Figure 8. Maximal accumulation of HSP30 was detected in cells incubated for 18 h with 5 μM WA and levels remained elevated at 10 μM WA. Compared to cells treated with 2 μM WA, exposure to 5 and 10 μM WA, resulted in an 11- and 10-fold increase, respectively, in HSP30 accumulation. In contrast, cells exposed to 2 μM and 5 μM WA for 18 h exhibited an 8-fold increase in HSP70 accumulation, compared to control. The multiple protein bands observed in Figure 8a (top panel) may represent additional HSP30 family members that are detected by the anti-HSP30 antibody. In addition, variation in the relative intensity exists with respect to the different HSP30 bands. As WA concentration increased, the intensity of the top band increased while the intensity of the lower band decreases. The cause of this is not known, however, variations in the accumulation of HSP30 family members or post-translational modifications may exist in cells treated with different WA concentrations. It is unlikely that this variation is the result

Figure 7. Effect of heat shock on HSP30 and HSP70 accumulation in A6 cells. Cells were maintained at 22 °C or heat shocked at 33 °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 anti-HSP30, anti-HSP70 or anti-actin polyclonal antibodies. These data are representative of three separate experiments.

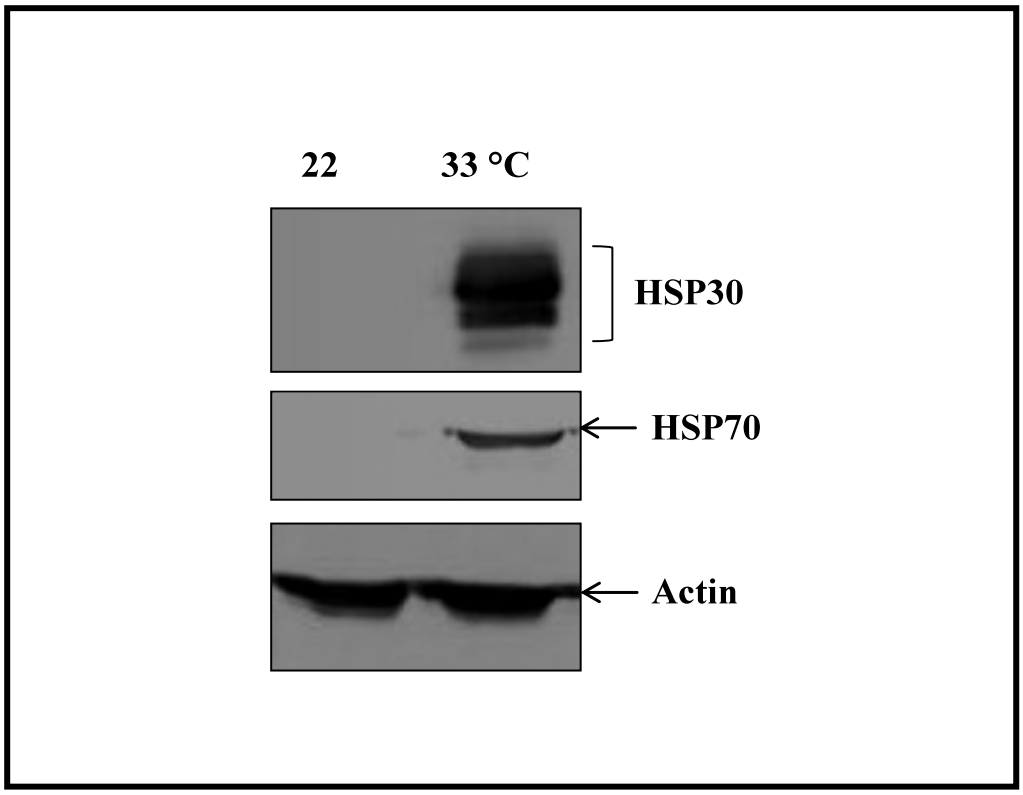
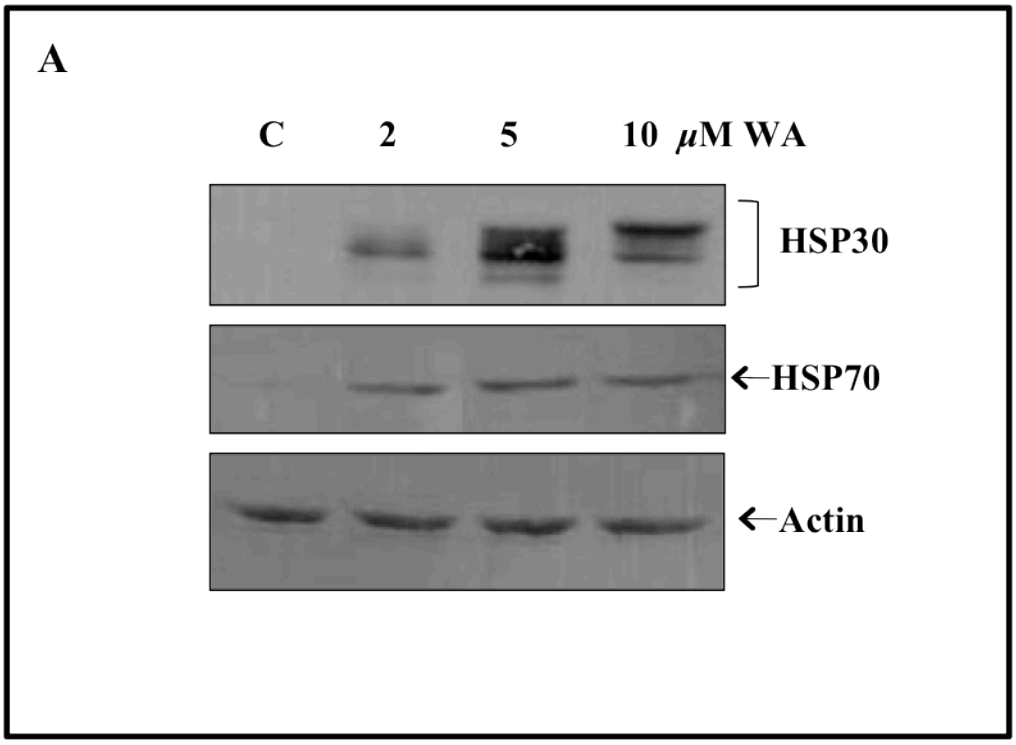
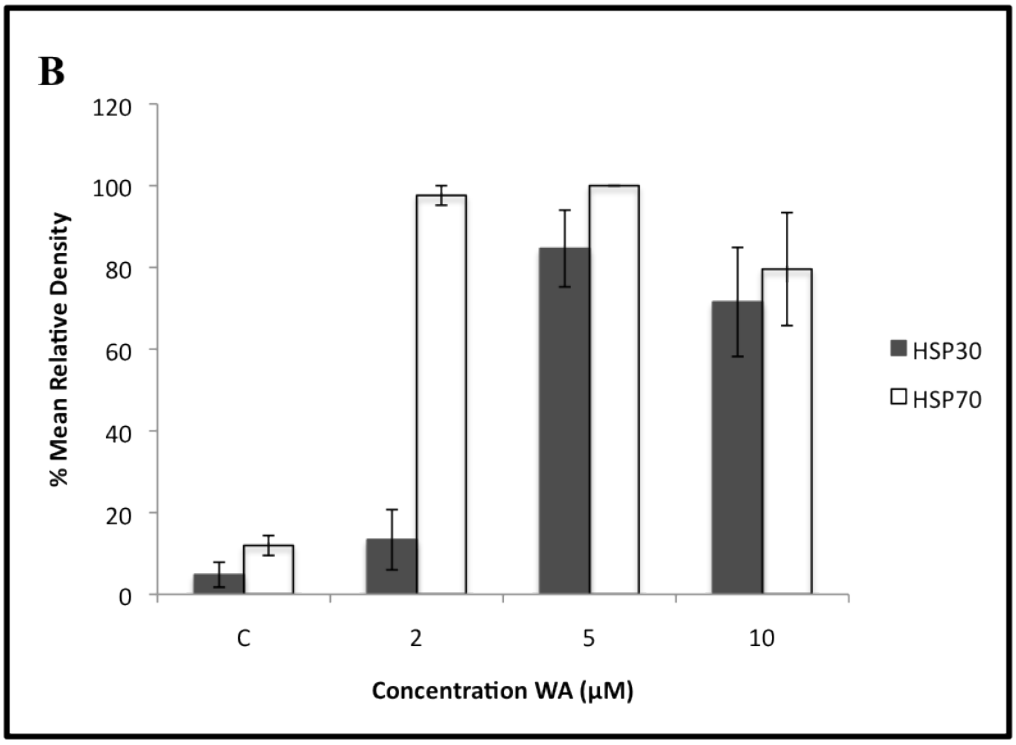

22
33 °C
HSP30
HSP70
Actin

Figure 8. Effect of different concentrations of WA on HSP30 and HSP70 accumulation in A6 cells. A) Cells were maintained at 22 °C and treated with DMSO (C) or 2, 5 or 10 μM WA for 18 h. Total protein was isolated and subjected to immunoblot analysis employing anti-HSP30, anti-HSP70 or anti-actin polyclonal antibodies. B) Image J software was used to perform densitometric analysis of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band (5 μM WA or 10 μM WA for HSP30 and 5 μM for HSP70) while the standard error is represented by vertical error 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 (C) and 5 and 10 μM WA are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
C
2
5
10 μM WA
HSP30
HSP70
Actin


B
120
100
80
60
40
20
0
% Mean Relative Density
C
2
5
10
Concentration WA (μM)
HSP30
HSP70

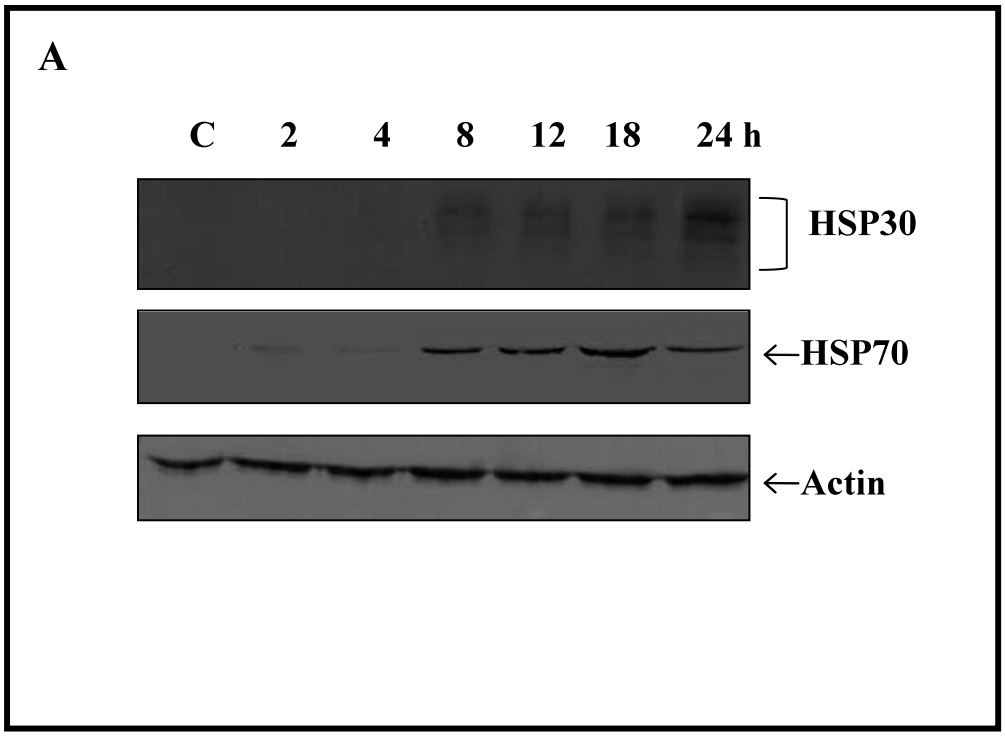
of phosphorylation as HSP30 was only found to be mono-phosphorylated by MAPKAPK-2, adding approximately 79 Da to its size, which cannot be separated by the SDS-PAGE protocol used in this experiment. To further explore this possibility, additional HSP30 post-translational modifications should be examined. Densitometric analysis also revealed that in cells treated with 10 μM WA for 18 h HSP70 accumulation decreased 20% compared to cells treated with 5 μM WA.

In time course studies, immunoblot analysis revealed that HSP30 and HSP70 levels gradually increased with extended incubation time up to 24 h in A6 cells exposed to 5 μM WA (Fig. 9). A significant increase ($p<0.05$) of 31% and 52% in the relative levels of HSP30 and HSP70, respectively, occurred in cells treated with 5 μM WA for 8 h compared to control.

3.3 Analysis of the regulation of WA-induced *hsp30* and *hsp70* gene expression

Previous studies with *Xenopus laevis* A6 cells indicated that induction of *hsp* genes by various stressors including heat shock, sodium arsenite, cadmium chloride, celastrol and MG132 were regulated, at least in part, at the level of transcription (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008, Young et al., 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). HSF1-mediated *hsp* gene expression results in the synthesis of *hsp* mRNA. Northern blot hybridization analysis revealed that WA treatment induced the accumulation of both *hsp30* and *hsp70* mRNA. Relatively low levels of *hsp30* and *hsp70* mRNA were observed in cells exposed to 5 μM WA for 4 h, which increased with prolonged incubation time up to 12 h (Fig. 10).

Figure 9. Temporal pattern of HSP30 and HSP70 levels in A6 cells treated with WA. A) Cells were maintained at 22 °C or treated with 5 μM WA for 2 to 24 h. Total protein was isolated and subjected to immunoblot analysis employing anti-HSP30, anti-HSP70 and anti-actin polyclonal antibodies. B) Image J software was used to perform densitometric analysis of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band (24 h and 18 h for HSP30 and HSP70, respectively). The standard error is represented by vertical error 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 (C) cells and WA treated cells are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
C
2
4
8
12
18
24 h
HSP30
←HSP70
←Actin

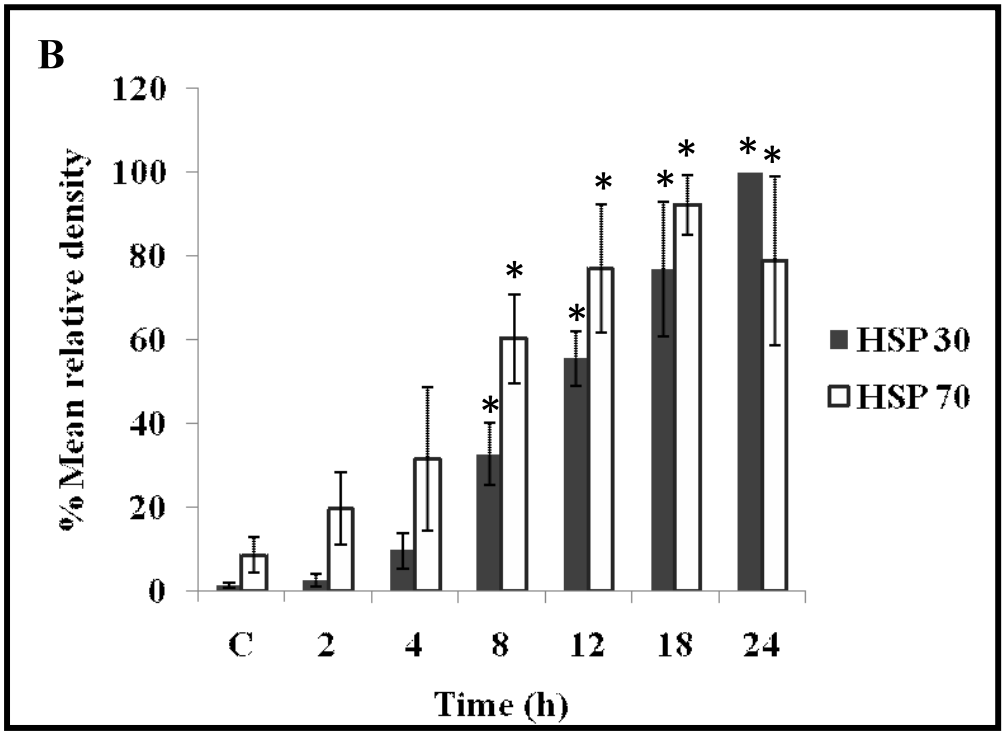
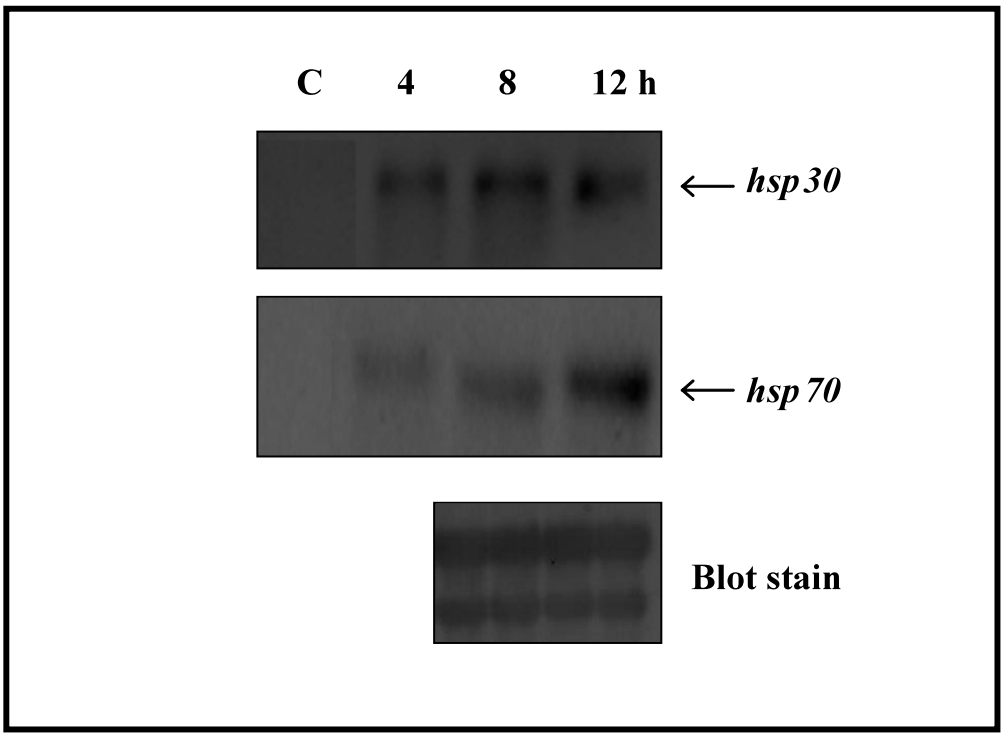

B
120
100
80
60
40
20
0
% Mean relative density
C
2
4
8
12
18
24
Time (h)
HSP 30
HSP 70

Figure 10. *hsp30* and *hsp70* mRNA accumulation in A6 cells treated with WA. Cells were maintained at 22 ºC and incubated with DMSO (C) or exposed to 5μM WA for 4, 8 or 12 h. Cells were harvested and total RNA was isolated and quantified. Total RNA (10 μg) was analysed by northern hybridization analysis using *hsp30* and *hsp70* antisense riboprobes. The bottom panel exhibits a reversible blot stain which confirms quality of transfer. These results are representative of at least 3 different experiments.

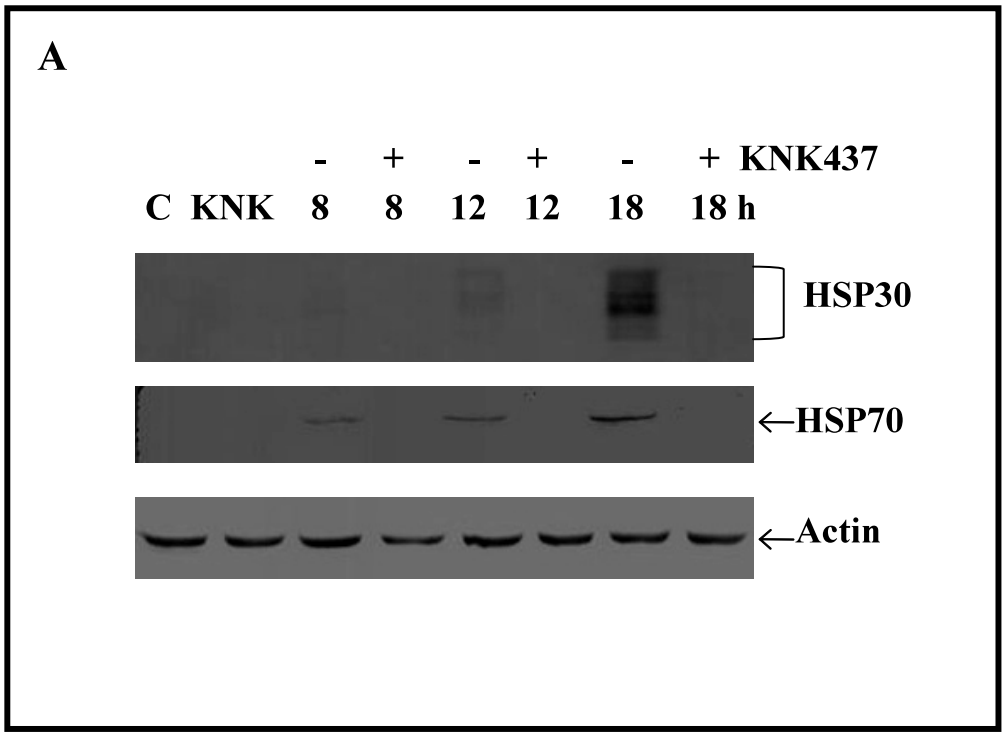

C
4
8
12 h
hsp 30
hsp 70
Blot stain

Additionally, KNK437, a HSF1 inhibitor, was utilized to determine if the WA-induced *hsp30* and *hsp70* gene expression in A6 cells was due to HSF1 activation (Fig. 11). Cells were pretreated with 100 μM KNK437 for 6 h prior to exposure to 5 μM WA for 8, 12 or 18 h. Immunoblot and subsequent densitometric analysis demonstrated that WA-induced HSP30 accumulation was inhibited in cells treated with WA for 8, 12 and 18 h by 95%, 90% and 98% respectively, compared to WA-treated cells without KNK437. Similarly, following pretreatment with KNK437, HSP70 levels decreased in cells incubated with WA for 8, 12 and 18 h by 98%, 96% and 94%, respectively.

3.4 The localization of HSP30 in A6 cells treated with WA

The accumulation and intracellular localization of HSP30 induced by heat shock or WA treatment was analyzed 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 Figure 12, accumulation of HSP30 was not detected in control cells maintained at 22 °C or in cells treated with a 30 °C heat shock. However, HSP30 was detected in approximately 99% of A6 cells exposed to a 33 °C heat shock. Treatment of cells with 5 μM WA for 8, 12 or 18 h resulted in the accumulation of HSP30 in approximately 20% of cells in a granular or punctate pattern primarily in the cytoplasm with a small amount in the nucleus (Fig. 13). Examination of the general morphology and F-actin organization revealed that cells exposed to WA for 8 h

Figure 11. Effect of KNK437 on WA-induced HSP30 and HSP70 accumulation in A6 cells. A) Cells were maintained at 22 °C, treated with DMSO (C), pretreated with 100 μM KNK437 for 6 h or treated with 5 μM WA for 8 – 18 h with or without 100 μM KNK pretreatment for 6 h. Total protein was isolated and analyzed by immunoblotting using anti-HSP30, anti-HSP70 or anti-actin polyclonal antibodies. B) Densitometric analysis was performed, using Image J software, of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots. The data are expressed as percent inhibition of WA-induced HSP at each time point and the standard error is represented by vertical error 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 cells exposed to WA with or without a 6 h KNK437 pretreatment are indicated as * ($p<0.05$) or Δ ($p<0.1$). These data are representative of three separate experiments.


A
- + - + - + KNK437
C KNK 8 8 12 12 18 18 h
HSP30
←HSP70
←Actin

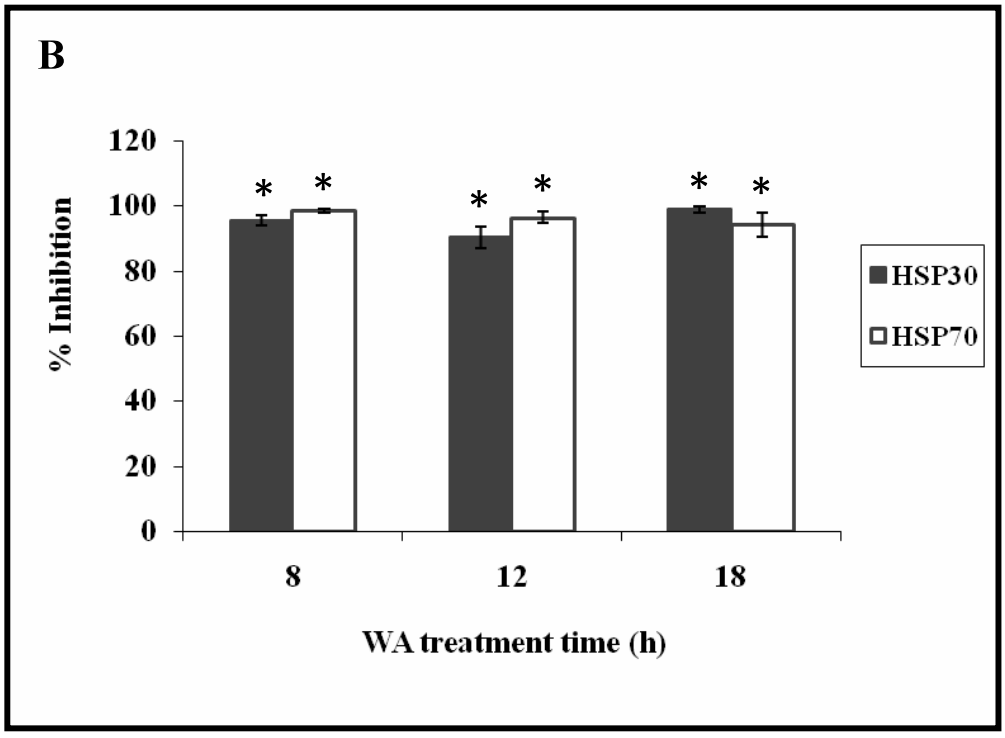

B
120
100
80
60
40
20
0
% Inhibition
8
12
18
WA treatment time (h)
HSP30
HSP70

Figure 12. Localization of HSP30 accumulation in A6 cells in response to heat shock. Cells were cultured on base-washed glass coverslips and maintained at 22 °C or heat shocked at 30 °C or 33 °C for 2 h followed by a 2 h recovery at 22 °C. 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 10 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of three separate experiments.

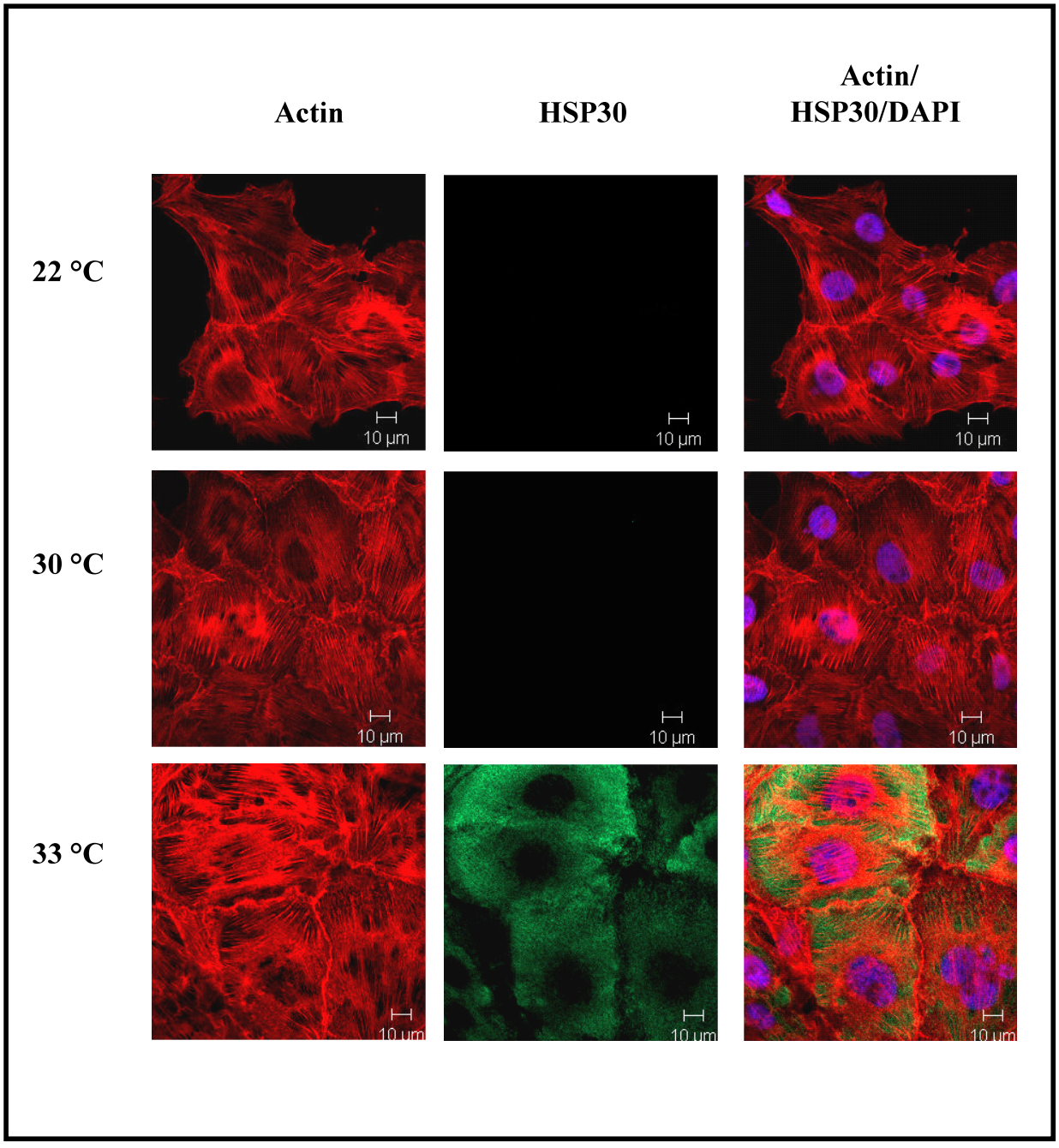
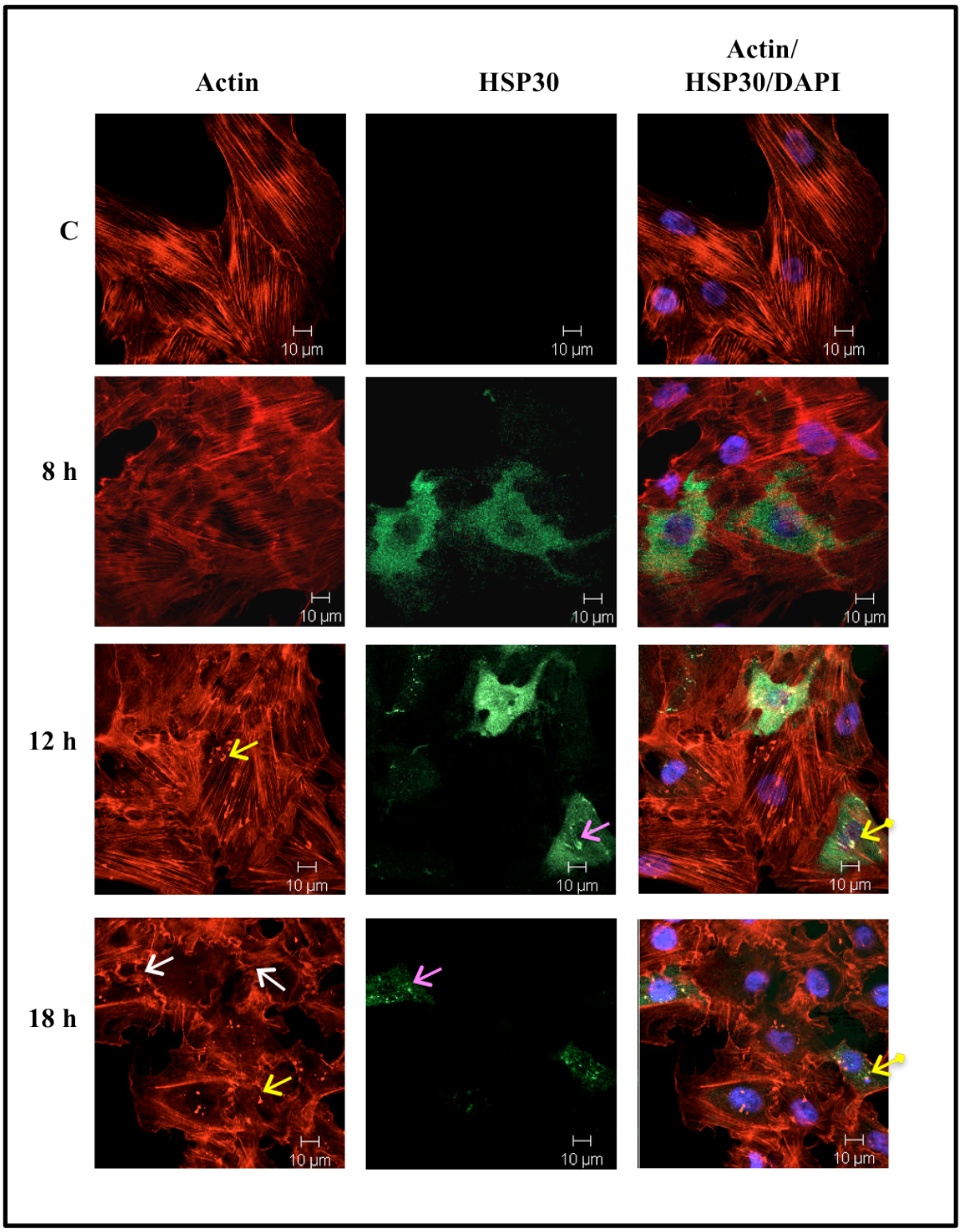

Actin
HSP30
Actin/
HSP30/DAPI
22 °C
30 °C
33 °C
10 µm

Figure 13. Localization of WA-induced HSP30 accumulation in A6 cells. Cells were cultured on base-washed glass coverslips and maintained at 22 °C or incubated with 5 μM WA for 8, 12 or 18 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 white arrows indicate ruffled edges of the actin cytoskeleton. Actin aggregates are indicated by a yellow arrow, the pink arrows point to HSP30 associated foci and the yellow arrows with diamond ends indicate co-localization of actin aggregates and HSP30 associated foci. The 10 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
C
8 h
12 h
18 h
10 μm

displayed an intact cytoskeleton and visible stress fibres, similar to control cells. In contrast, cells treated with prolonged WA exposure displayed some actin disorganization. After 12 and 18 h, WA-treated cells displayed distinct areas of actin aggregation, indicated by the yellow arrows. In addition, ruffled edges at the cellular periphery, resulted from exposure to WA for 18 h, as indicated by white arrows. Figure 14 displays an enlarged image of an A6 cell treated with 5 μM WA for 18 h. Orange staining in the far right panel indicates areas of actin aggregation co-localized with large HSP30 associated granules.

3.5 The effect of mild heat shock on WA-induced HSP30 and HSP70 accumulation

In previous studies, mild heat shock treatments enhanced sodium arsenite-, celastrol- and MG132-induced *hsp* gene expression in A6 cells (Young et al., 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). In the present study, the effect of a mild heat shock temperature on WA-induced HSP30 and HSP70 accumulation was examined by immunoblot analysis. HSP30 accumulation was not detected in cells exposed singly to mild heat shock (30 °C) nor in cells treated with 2 μM WA for 8 h (Fig. 15). Similarly, low levels of HSP70 were detected in heat shock-treated and WA-treated cells. In contrast, concurrent treatment of mild heat shock with 2 μM WA, resulted in enhanced levels of HSP30 and HSP70 accumulation that was greater than the sum found with each stress individually. Compared to cells exposed singly to 2 μM WA and 30 °C, HSP30 accumulation increased, in cells treated with concurrent stress, by 99% and 98%, respectively and the level of HSP70 increased 67% and 90%, respectively. Similarly, co-treatment with mild heat shock and 5 μM WA significantly ($p<0.05$) increased the levels of HSP30 (99% and 98%, respectively)

Figure 14. Co-localization of actin aggregates with HSP30 associated foci. Cells were cultured on base-washed glass coverslips and incubated with 5 μM WA for 18 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). Actin aggregates are indicated by a yellow arrow, the pink arrows point to HSP30 associated foci and the yellow arrows with diamond ends indicate co-localization of actin aggregates and HSP30 associated foci. The 5 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of three separate experiments.

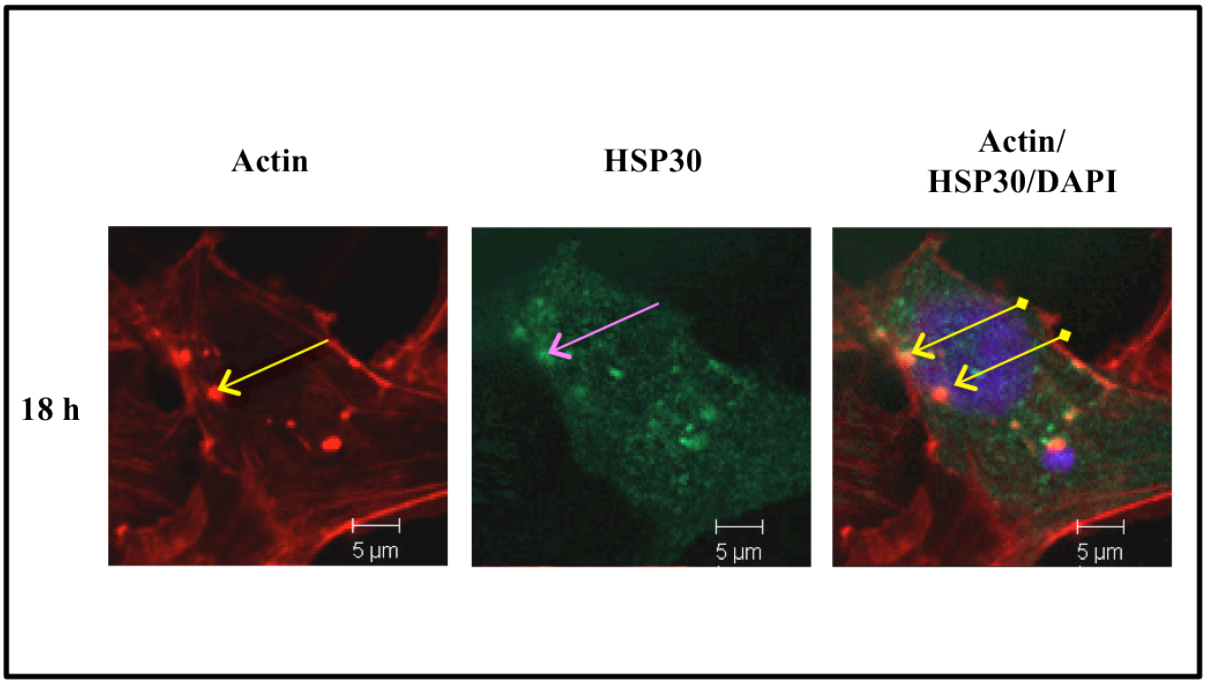
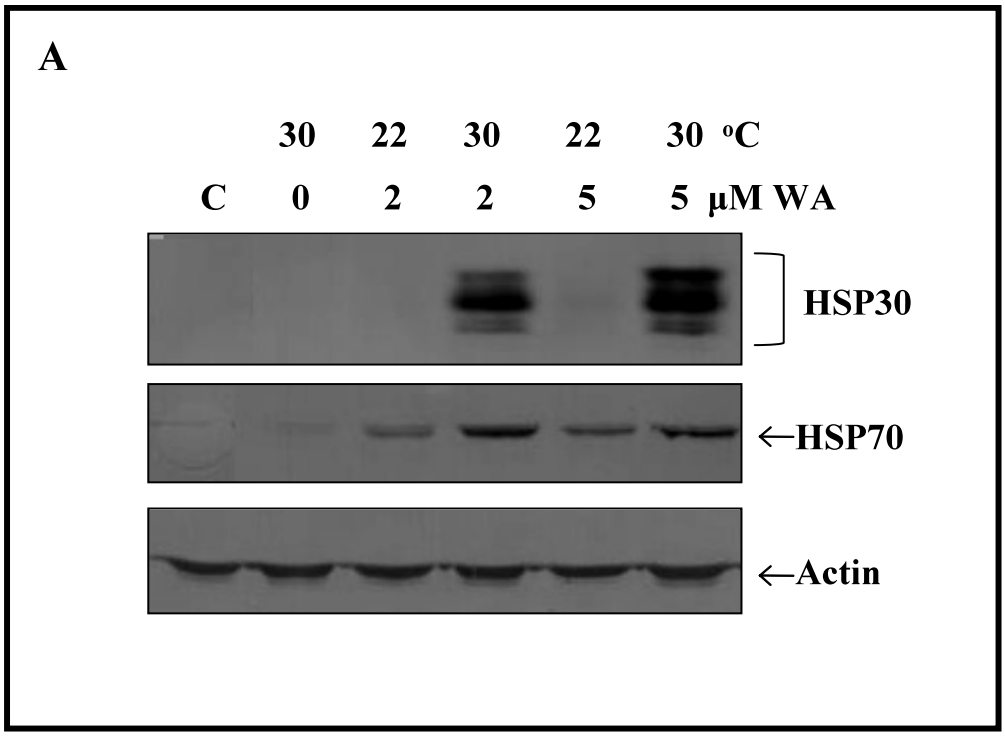
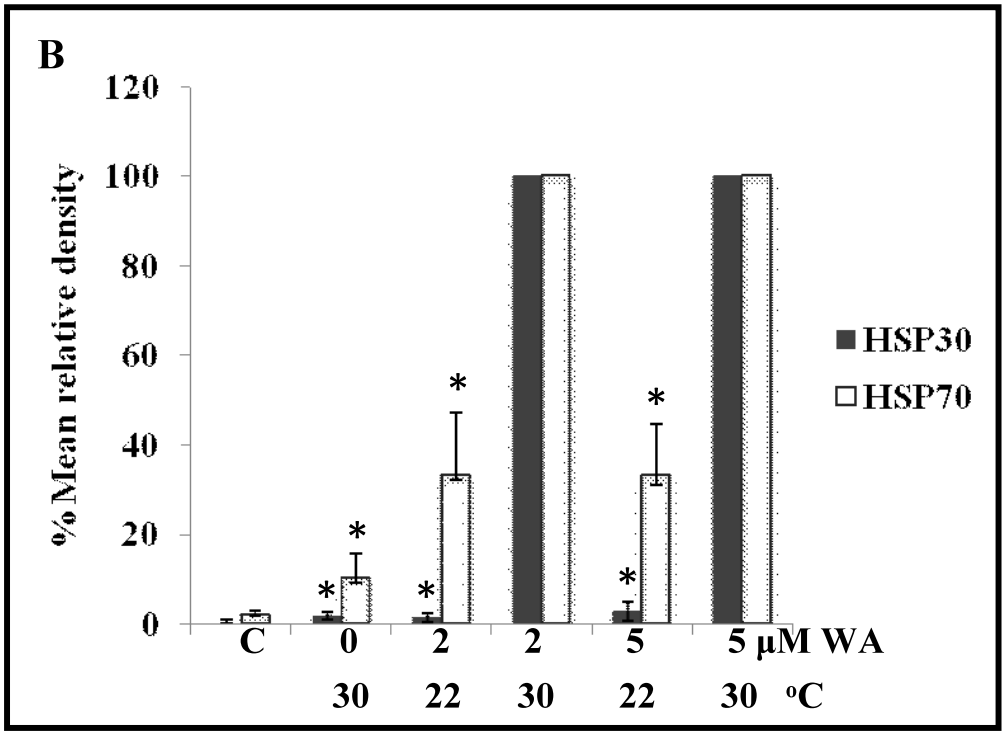

Actin
HSP30
Actin/
HSP30/DAPI
18 h
5 µm
5 µm
5 µm

Figure 15. Effect of mild heat shock on WA-induced HSP30 and HSP70 accumulation in A6 cells. A) Cells were maintained at 22 °C and treated with DMSO (C), or exposed to 2 or 5 μM WA singly or with a mild heat shock at 30 °C for 8h. Following treatment, cells were harvested and total protein isolated. Immunoblot analysis was performed using anti-HSP30, anti-HSP70 and anti-actin polyclonal antibodies. B) Image J software was used to perform densitometric analysis of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band (combined WA and mild heat shock treatment) at each WA concentration. The standard error is represented by vertical error 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 the maximum signal for each concentration are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
30 22 30 22 30 °C
C 0 2 2 5 5 µM WA
HSP30
←HSP70
←Actin


B
% Mean relative density
120
100
80
60
40
20
0
HSP30
HSP70
C 0 2 2 5 5 µM WA
30 22 30 22 30 °C

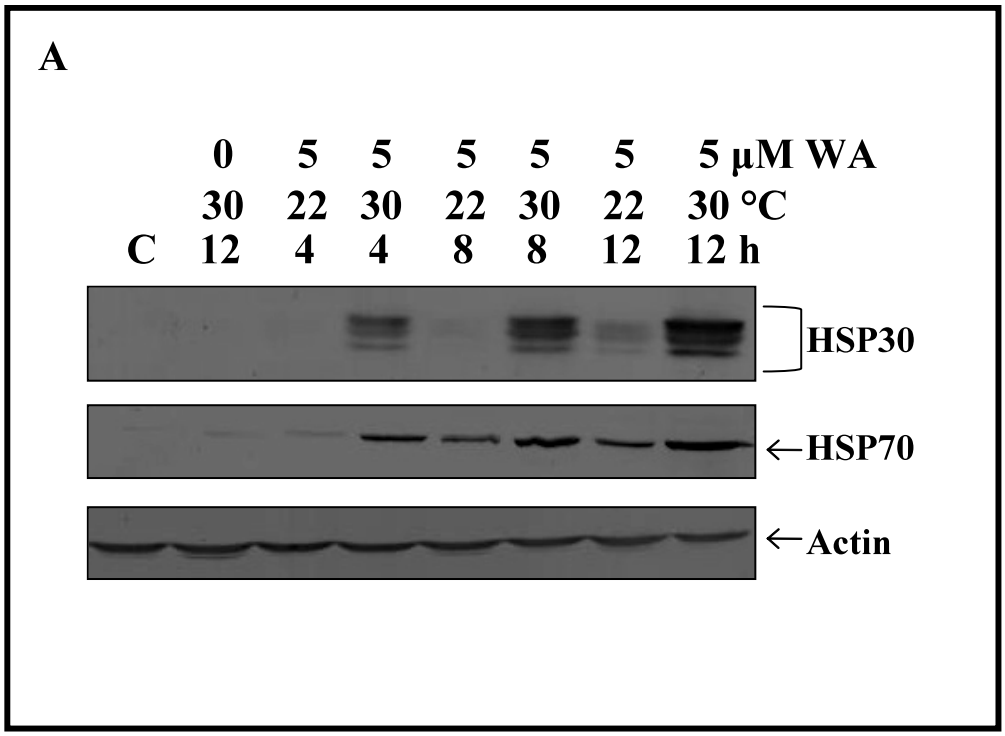
and HSP70 (67% and 90%, respectively) compared to cells exposed to mild heat shock or WA alone.

Additionally, time course studies showed elevated levels of HSP30 and HSP70 in A6 cells treated with 5 μM WA and mild heat shock for 4, 8 and 12 h compared to each stressor individually (Fig. 16). Densitometric analysis revealed that HSP30 and HSP70 accumulation in cells treated with 5 μM WA at 30 °C was greater than the sum of either WA or mild heat shock alone at each time point. For example, compared to cells treated with either 5 μM WA or 30 °C for 4 h, cells exposed to both stressors simultaneously increased the level of HSP30 by 14.5- and 26.3-fold, respectively. Similarly HSP70 accumulation in A6 cells treated concurrently with both stressors increased by 22.6- and 5.5-fold, respectively, compared to cells exposed to WA or mild heat shock alone.

3.6 HSP30 localization in A6 cells exposed concurrently to WA and heat shock treatment

The effect of heat shock on WA-induced HSP30 accumulation and localization in A6 cells was examined by immunocytochemistry and LSCM. Cells were grown on base washed coverslips and maintained at 22 °C or incubated with 5 μM WA with or without heat shock (30 °C) for 8 h. HSP30 accumulation was not detected in cells exposed to a mild heat shock, while HSP30 was detected in approximately 20% of cells exposed to 5 μM WA for 8 h. An increase in HSP30 accumulation (approximately 99% of cells) was observed in A6 cells treated concurrently with 5 μM WA and mild heat shock, compared to either stressor alone (Fig. 17). The actin cytoskeleton of cells treated with both stressors resembled control cells,

Figure 16. Temporal pattern of HSP30 and HSP70 accumulation A6 cells treated concurrently with WA plus mild heat shock. A.) Cells were maintained at 22 °C and treated with DMSO (C), or exposed to 5 μM WA singly or with a mild heat shock at 30 °C for 4, 8 or 12 h. Following treatment, cells were harvested and total protein isolated. Immunoblot analysis was performed using anti-HSP30, anti-HSP70 and anti-actin polyclonal antibodies. B) Image J software was used to perform densitometric analysis of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band (combined WA and mild heat shock treatment) at each time point. The standard error is represented by vertical error 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 the maximum signal at each time point are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
0 5 5 5 5 5 5 µM WA
30 22 30 22 30 22 30 °C
C 12 4 4 8 8 12 12 h
HSP30
←HSP70
←Actin

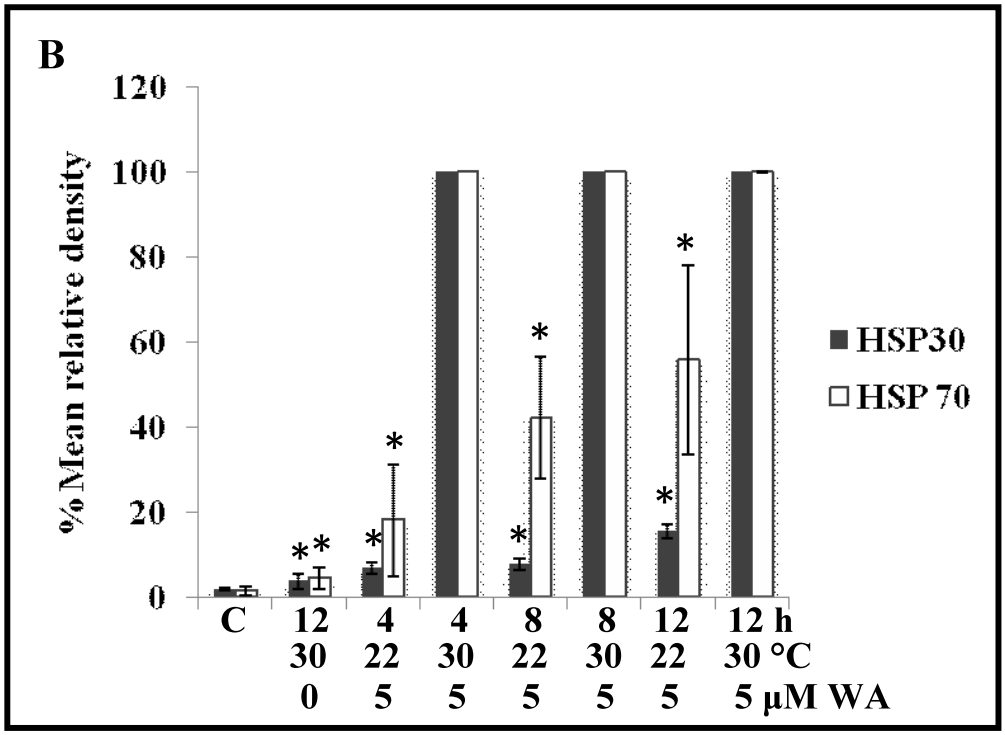
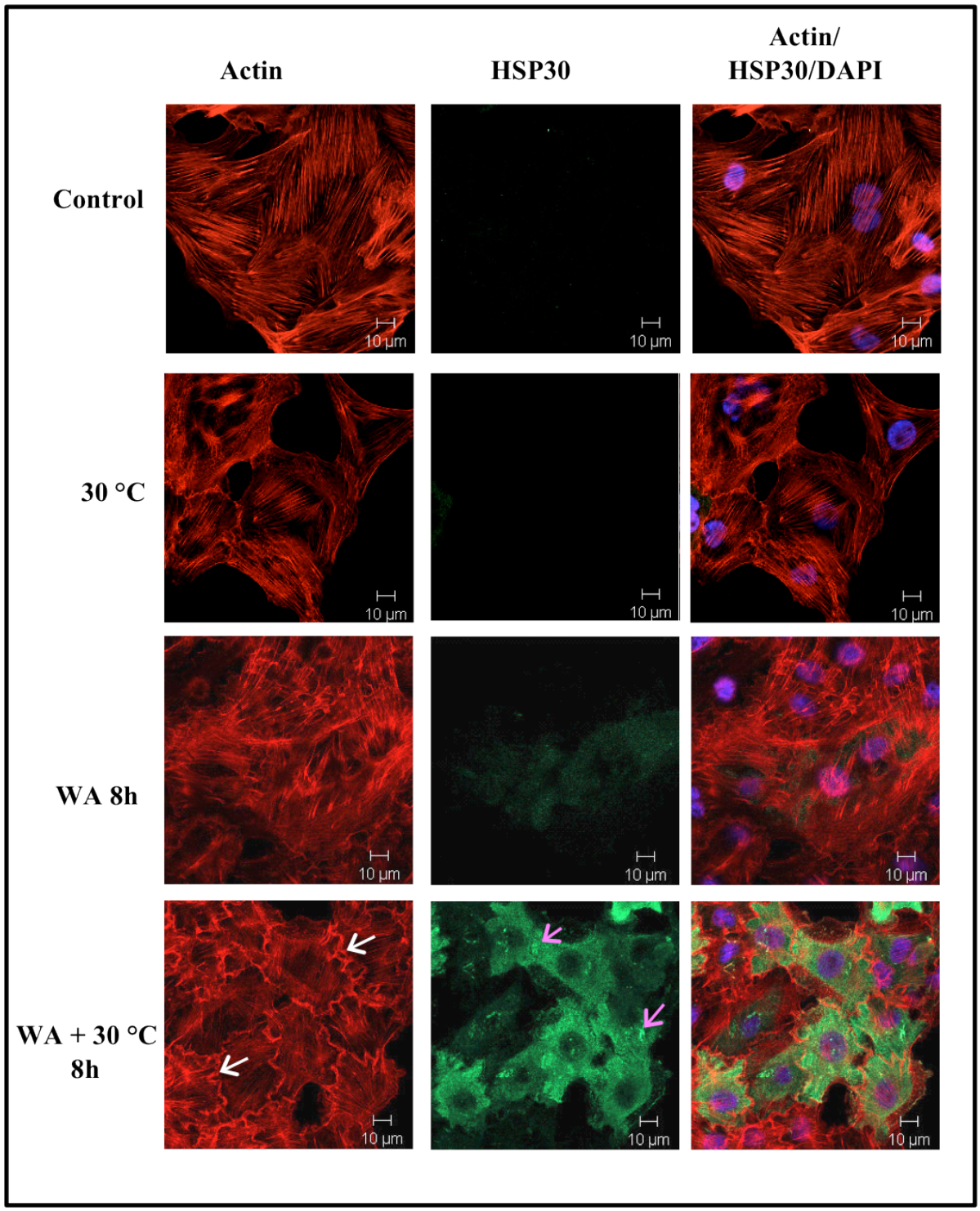

B
% Mean relative density
120
100
80
60
40
20
0
HSP30
HSP 70
C 12 4 4 8 8 12 12 h
30 22 30 22 30 22 30 °C
0 5 5 5 5 5 5 µM WA

Figure 17. Localization of HSP30 in A6 cells treated with concurrent stress. Cells were cultured on base-washed glass coverslips and maintained at 22 °C or 30 °C for 8 h or incubated with 5 μM WA for 8 h at 22 °C or 30 °C. 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 white arrows indicate ruffled edges of the actin cytoskeleton and the pink arrows point to HSP30 associated foci. The 10 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
30 °C
WA 8h
WA + 30 °C
8h
10 µm

however a slight ruffling of the edges was present as indicated by white arrows. As well, large HSP30 associated aggregates or foci were observed in some cells exposed to concurrent WA and mild heat shock (pink arrows). Similar structures were observed in A6 cells treated with celastrol and MG132 (Walcott and Heikkila, 2010; Young and Heikkila, 2010).

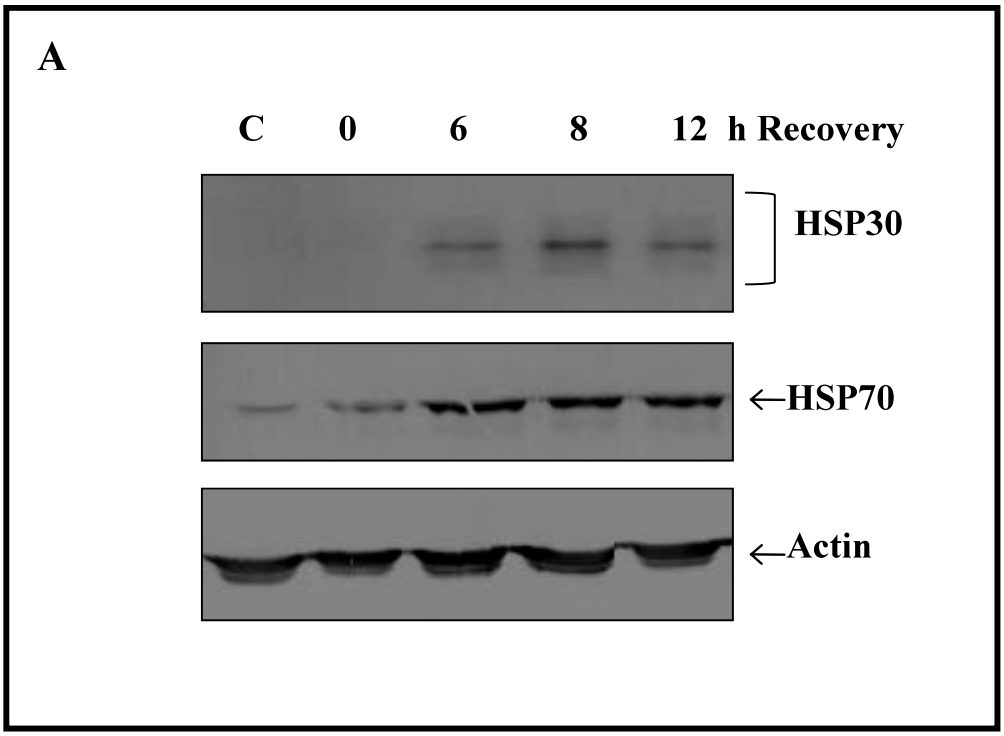
3.7 Analysis of HSP30 and HSP70 accumulation in A6 cells recovering from WA treatment

In recovery experiments, cells were treated with 5 μM WA for 4 h and were then allowed to recover in fresh L-15 media for 6, 8 or 12 h. The relative levels of both HSP30 and HSP70 protein were assessed by immunoblot analysis (Fig. 18a) and densitometry (Fig. 18b). HSP30 and HSP70 accumulation increased in WA-treated cells allowed to recover for 6 h and remained elevated until 12 h, compared to cells exposed to WA without a recovery period. Densitometric analysis revealed that exposure of cells to 5μM WA for 4 h followed by a 6 h recovery period, resulted in a 6.6-fold increase in HSP30 accumulation and a 4.4-fold increase in HSP70 accumulation, compared to non-recovered, WA-treated cells.

3.8 The effect of WA on the acquisition of thermotolerance in A6 cells

In previous studies, it was established that pretreatment of A6 cells with a 33 °C heat shock or MG132 resulted in the accumulation of HSPs and an acquired state of thermotolerance (Manwell and Heikkila, 2007; Young and Heikkila, 2010). To assess whether WA can produce the same effect, A6 cells were pretreated with WA prior to a thermal challenge at 37 °C (Fig. 19). Cells treated with 2 μM WA for 6 h with a 12 h recovery period had relatively high accumulation of HSP30, approximately 50% of cells,

Figure 18. Effect of extended recovery on HSP30 and HSP70 accumulation in WA-treated A6 cells. A) Cells were maintained at 22 °C and treated with DMSO (C), or with 5 μM WA for 4 h followed by 0, 6, 8 or12 h recovery in WA-free L-15 media. Following recovery, cells were harvested and total protein was isolated. Immunoblot analysis was performed using anti-HSP30, anti-HSP70 and anti-actin polyclonal antibodies. B) Image J software was used to perform densitometric analysis of the signal intensity for HSP30 (grey) and HSP70 (outline) protein bands of the immunoblots as described in Materials and methods. The data are expressed as a percentage of the maximum band while the standard error is represented by vertical error 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 cells and those exposed to 5 μM WA are indicated as * ($p<0.05$). These data are representative of three separate experiments.


A
C
0
6
8
12 h Recovery
HSP30
←HSP70
←Actin

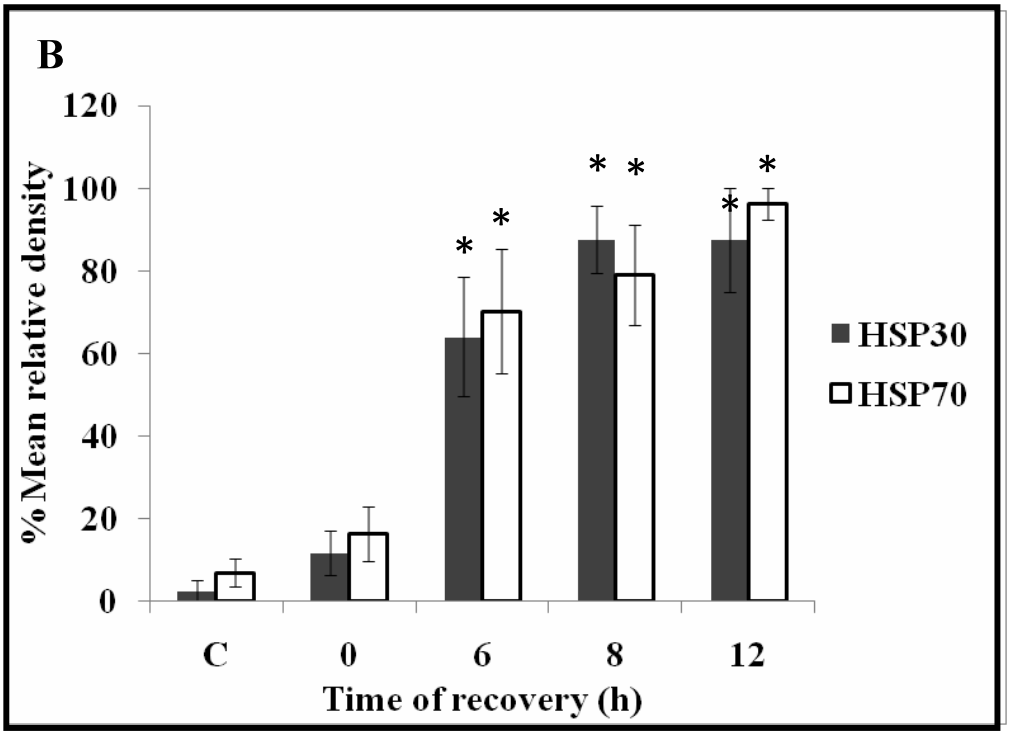
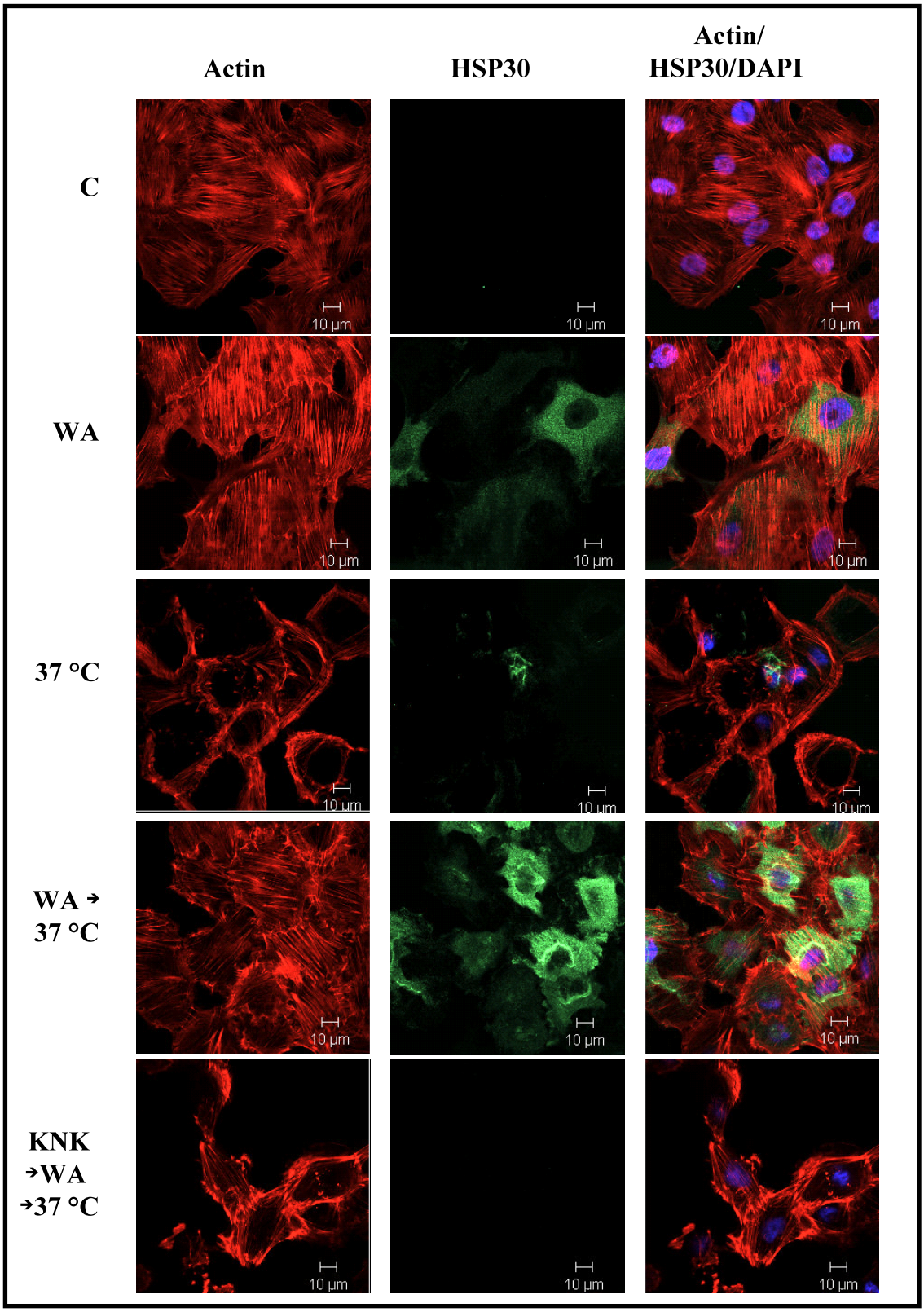

B
120
100
80
60
40
20
0
% Mean relative density
C
0
6
8
12
Time of recovery (h)
HSP30
HSP70

Figure 19. Cytoprotective effects of pretreating A6 cells with WA prior to a 37 °C thermal challenge. Cells were cultured on base-washed glass coverslips and maintained at 22 °C (C), exposed to 2 μM WA for 6 h with an12h recovery at 22 °C in WA-free media followed by a 37 °C heat shock for 1 h with a 6 h recovery at 22 °C. In the last row, cells were pretreated with 100 μM KNK437 for 6 h before the WA treatment and subsequent thermal challenge. 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 pink arrows indicate HSP30 associated foci. The 10 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of 3 separate experiments.


Actin
HSP30
Actin/
HSP30/DAPI
C
WA
37 °C
WA →
37 °C
KNK
→WA
→37 °C
10 µm

therefore these conditions were employed in the present study. Shifting the incubation temperature from 22 °C directly to a 37 °C thermal challenge for 1 h resulted in the collapse of the actin cytoskeleton and distinct cell rounding. However, cells exposed to a WA pretreatment before the thermal challenge maintained their cytoskeletal organization and stress fibres. Similar results were obtained in cells pretreated with concurrent 2 μM WA and 30 °C heat shock prior to a 37 °C thermal challenge in that all of the cells exhibited control like stress fibres and an intact cytoskeleton (Fig. 20). In contrast, cells heat shocked at 30 °C prior to a 37 °C thermal challenge had a disorganized actin cytoskeleton and slightly rounded morphology (Fig. 21).

Figure 20. Cytoprotective effects of pretreating A6 cells concurrently with WA and mild heat shock prior to a 37 °C thermal challenge. Cells were cultured on base-washed glass coverslips and maintained at 22 °C (C), exposed to 2 μM WA for 6 h at 30 °C followed by a 12 h recovery period in WA-free media at 22 °C with or without a 37 °C heat shock for 1 h with a 6 h recovery at 22 °C. In the last row, cells were pretreated with 100 μM KNK437 for 6 h before the combined treatment and subsequent thermal challenge. 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 10 μM white scale bars are indicated at the bottom right section of each panel. These data are representative of three separate experiments.

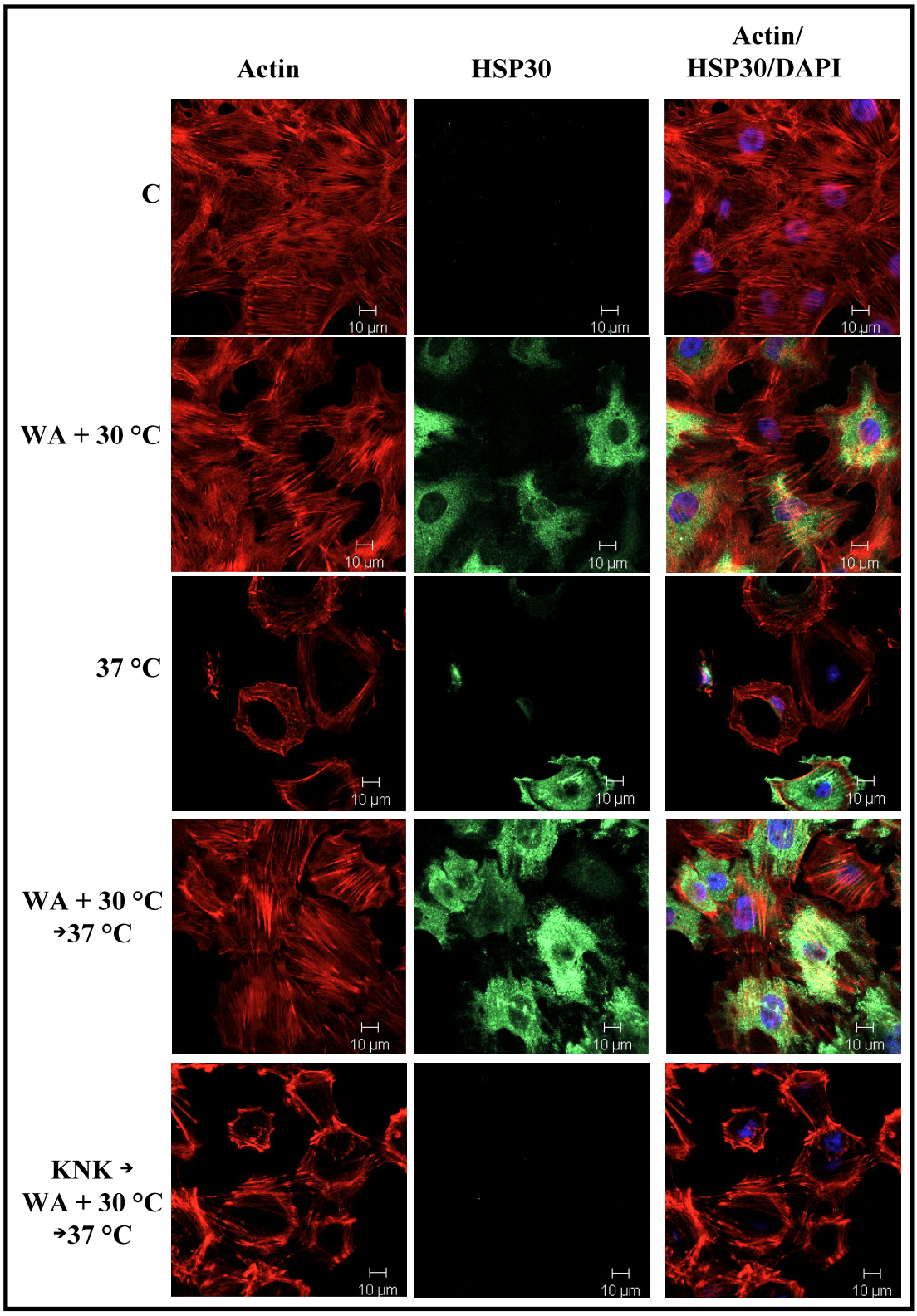
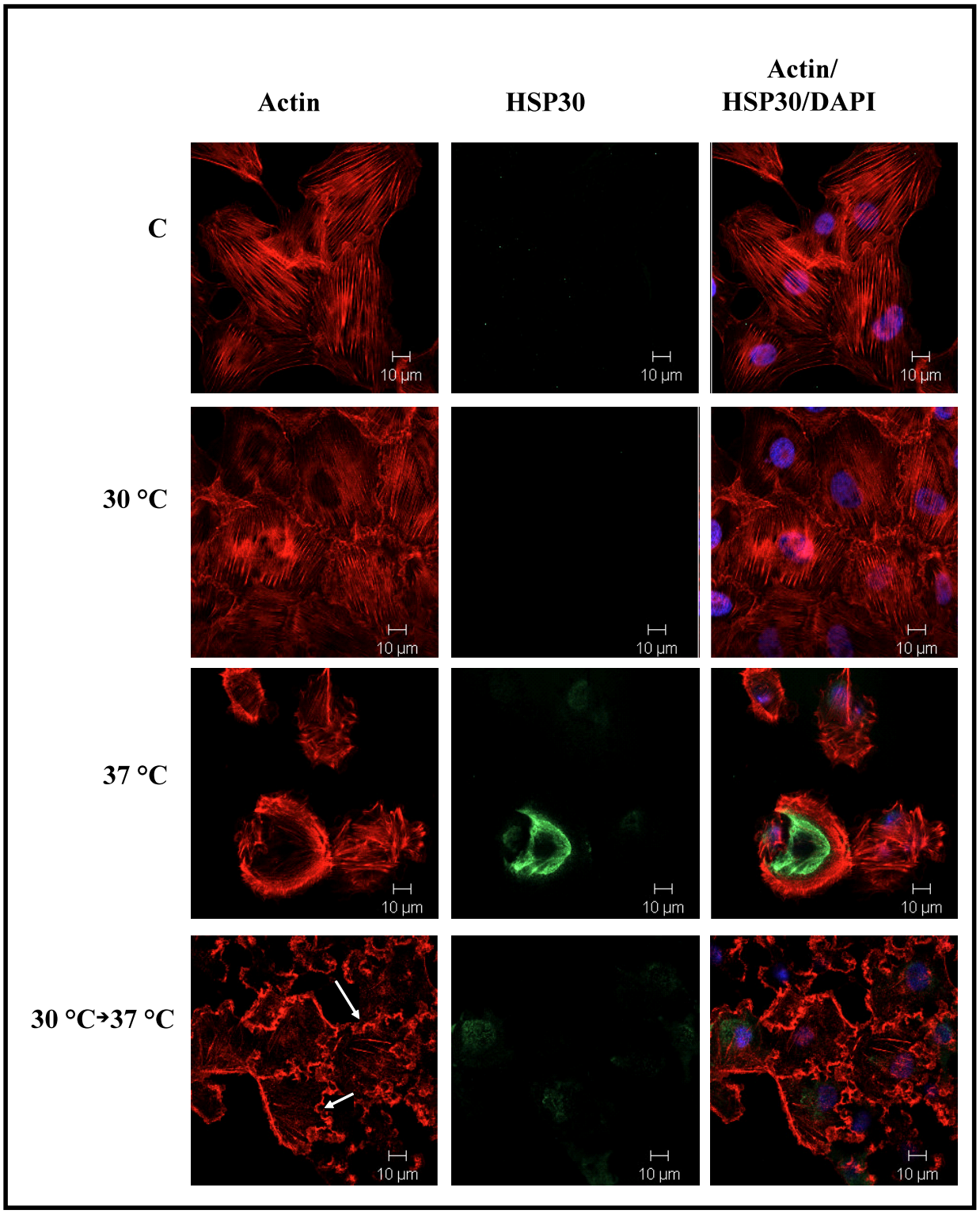

Actin
HSP30
Actin/
HSP30/DAPI
C
WA + 30 °C
37 °C
WA + 30 °C
→37 °C
KNK →
WA + 30 °C
→37 °C
10 µm

Figure 21. Effects of pretreating A6 cells with mild heat shock prior to a 37 °C thermal challenge. Cells were cultured on base-washed glass coverslips and maintained at 22 °C (C), or 30 °C for 6 h followed by a 12 h recovery period at 22 °C with or without a 37 °C heat shock for 1 h with a 6 h recovery at 22 °C. 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 white arrows indicate ruffled edges of the actin cytoskeleton. The 10 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
C
30 °C
37 °C
30 °C→37 °C
10 µm

4 Discussion

The present study determined that WA inhibited proteasomal activity and induced *hsp30* and *hsp70* gene expression in *Xenopus laevis* A6 kidney epithelial cells. Initial studies demonstrated that exposure of A6 cells to 5 μM WA enhanced the relative levels of ubiquitinated protein. These results were comparable to the effects of the proteasomal inhibitor, MG132, documented previously in our laboratory (Young and Heikkila, 2010). Additionally, cells treated with 5 μM WA exhibited a 45% to 70% decrease in chymotrypsin-like activity. Taken together the WA-induced increase in the accumulation of ubiquitinated protein and decreased chymotrypsin-like activity in A6 cells, is an indicator of proteasomal inhibition. Similarly, treatment of human prostate cancer (PC-3) cells with 5 to 20 μM WA for 4 h resulted in an increase in ubiquitinated protein in a dose dependent manner (Yang et al., 2007). Also, chymotrypsin-like activity of PC-3 cells was inhibited by 50% after treatment with 20 μM WA for 16 h. Yang et al. (2007) suggested that WA-induced proteasomal inhibition resulted from the direct interaction between WA and the β5 subunit, with chymotrypsin-like activity, of the 20S proteasome. In silico docking and nucleophilic attack studies were used to produce a computer model of the interaction and reaction between WA and the 26S proteasome (Yang et al., 2007). C1 and C24 of WA are in range for direct interaction with the N-terminal threonine, the catalytically active amino acid, of the β5 subunit. This indicates that WA may interact directly interact with the 20S proteasome to inhibit its chymotrypsin-like activity.

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 the cell 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). Since misfolded proteins possess exposed hydrophobic amino acid residues, increased concentrations of these 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). Under stress conditions, unfolded protein is thought to be a signal for the activation of *hsp* gene expression (Voellmy et al., 2004).

As mentioned above, treatment of A6 cells with WA resulted in the accumulation of *hsp30* and *hsp70* mRNA as well as HSP30 and HSP70 protein. In these experiments, WA had no detectable effect on actin protein levels. Moreover, the accumulation of HSP30 and HSP70 increased with increasing time up to 24 h. These findings are in agreement with previous studies in our laboratory, which demonstrated that proteasomal inhibition by MG132 and celastrol induced *hsp* gene expression in A6 cells (Walcott and Heikkila, 2010; Young and Heikkila, 2010). In addition to proteasomal inhibition, WA-induced ROS generation may result in increased cellular unfolded protein (Malik et al., 2007). This suggests that various avenues exist for WA-induced *hsp* gene expression. The effects of WA on *hsp* gene expression were only examined in one other system, human pancreatic cells, in which their treatment with 10 μM WA increased the protein level of HSP70 by 13.47-fold after 6 h (Yu et al., 2010).

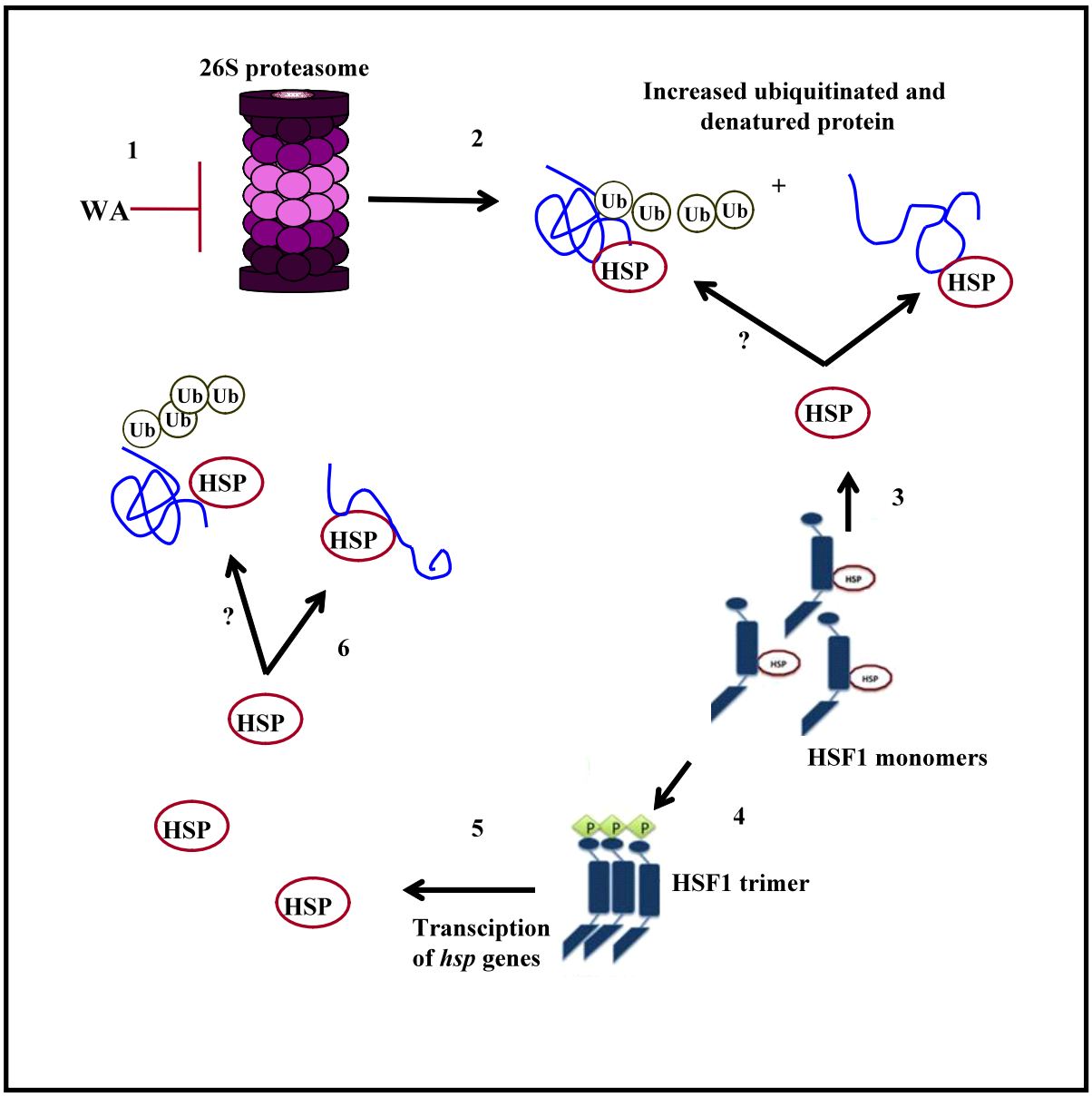
In experiments employing various concentrations of WA, HSP70 was detected in cells treated with 2 μM whereas HSP30 was first detected in cells treated with 5 μM. Similarly, HSP70 was detected earlier than HSP30 in cells treated with 5 μM WA. This discrepancy may be due to variation in the synthesis or processing of HSP30 compared to HSP70 or the sensitivity of the antibodies used to detect the protein accumulation. Previous studies in our laboratory found a similar anomaly in A6 cells treated with heat shock, celastrol and MG132 (Young et al., 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). For example, experiments employing MG132 found that HSP70 was detected in A6 cells treated with lower concentrations than was HSP30. Also, HSP70 was detected earlier than HSP30 in cells treated with 50 μM MG132 (Young and Heikkila, 2010).

In time course experiments HSP30 and HSP70 accumulation increased significantly in cells exposed to 5 μM WA, from 8 to 24 h. Comparatively, in A6 cells exposed to continuous heat shock HSP30 and HSP70 accumulation was detected after 1 h and maximal levels were observed after 2 h (Darasch et al., 1988). Reasons for the variation amongst temporal patterns of HSP accumulation in A6 cells subjected to heat shock and WA are unknown. The delay in elevated HSP accumulation in A6 cells treated with WA may be due to the time required for WA to enter the cell and subsequently increase the unfolded protein levels required to initiate HSF1-HSE-binding activity. In support of this concept, a delayed accumulation of HSPs compared to heat shock was observed in A6 cells exposed to sodium arsenite, cadmium chloride, celastrol, MG132, or herbymicin A (Darasch et al., 1988; Ohan et al., 1998; Gauley and Heikkila, 2006; Woolfson and Heikkila, 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). For example, celastrol-treated A6 cells displayed detectable HSP70 and HSP30 accumulation after 2 and 6 h, respectively, with maximal HSP

accumulation occurring after 18 h (Walcott and Heikkila, 2010). Also, in A6 cells exposed to MG132, HSP70 and HSP30 were detected after 4 and 8 h, respectively with maximal HSP levels occurring after 24 h (Young and Heikkila, 2010).

The molecular and cellular mechanisms associated with the stress-inducible *hsp* gene expression during proteasomal inhibition are unclear. A simplified model of how WA interacts with the proteasome to induce hsp gene expression is shown in Figure 22. The proteasome degrades 80-90% of all proteins and inhibition of its activity results in a substantial increase in the concentration of total cellular protein (Lee and Goldberg, 1998). Excess proteins, particularly damaged or aged, are prone to misfolding, which may expose their hydrophobic amino acid residues and lead to aggregation. In *Xenopus*, the stress-induced expression of *hsp* genes is mediated by HSF1-HSE-binding activity and the accumulation of unfolded protein by proteasomal inhibition may trigger HSF1 activiation (Morimoto and Santoro, 1998; Heikkila, 2003; Voellmy, 2004; Morimoto, 2008; Heikkila, 2010). The involvement of HSE-HSF1-binding activity in WA-induced expression of *hsp* genes was determined using the HSF1 inhibitor, KNK437. Treatment of A6 cells with KNK437, prior to the addition of WA, inhibited the accumulation of both HSP30 and HSP70. This suggested that WA-induced *hsp* gene expression in *Xenopus* A6 cells was transcriptionally mediated, at least in part, via the activation of HSF1 DNA-binding. Similar results were found in previous studies in *Xenopus* A6 cells, demonstrating that pretreatment

Figure 22. Model of the effect of WA on proteasomal activity and hsp gene expression. WA inhibits proteasomal activity (1) which leads to a build up of ubiquitinated and denatured protein (2). HSPs are recruited away from inactive HSF1 monomers to bind unfolded and possibly ubiquitinated protein (3). HSF1 monomers are then free to trimerize (4). Active trimers translocate to the nucleus where they activating the synthesis of *hsp* genes (5). Newly synthesized HSPs bind to the excess unfolded and possibly ubiquitinated protein in the cell and aids in protein refolding once normal conditions have returned (6).


26S proteasome
1
WA
2
Increased ubiquitinated and denatured protein
Ub
Ub
Ub
Ub
+
HSP
HSP
?
HSP
3
HSF1 monomers
4
HSF1 trimer
P
P
P
5
Transciption of *hsp* genes
HSP
HSP
6
?
Ub
Ub
Ub
Ub
HSP
HSP

with KNK437, repressed heat shock-, chemical stress- and celastrol-induced *hsp* gene expression (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008; Walcott and Heikkila, 2010; Young and Heikkila, 2010). This study shows for the first time that WA-induced *hsp30* and *hsp70* gene expression is controlled, at least in part, by HSF1-HSE binding activity.

In A6 cells, concurrent treatment with both mild heat shock and WA induced elevated levels of HSP30 and HSP70 accumulation. In fact, the relative levels of HSP30 and HSP70 accumulation induced by the concurrent stresses were greater than the sum of the levels found with each stress individually. Enhanced induction of *hsp* gene expression was previously reported in *Xenopus* A6 kidney epithelial cells treated simultaneous with relatively mild heat shock temperatures and herbimycin A, hydrogen peroxide, cadmium chloride, sodium arsenite or celastrol (Briant et al., 1997; Muller et al., 2004; Woolfson and Heikkila, 2009, Young et al., 2009; Walcott and Heikkila, 2010).The mechanism responsible for elevated levels of HSP30 and HSP70 in A6 cells induced by combined WA and mild heat shock is not clear. As mentioned previously, an increase in the relative levels of ubiquitinated protein destined for degradation resulted from WA-induced proteasomal inhibition. In addition, elevated temperatures can induce a generalized unfolding of intracellular proteins. Thus, it is possible that exposure of A6 cells to a combined mild heat shock and WA treatment elevated the total level of unfolding protein to a threshold point necessary for HSF1 activation. Support for the existence of a threshold level for HSF1 activation has been demonstrated in several organisms including mouse T-lymphocytes and testis, *Xenopus*,, HeLa cells and intertidal mussels (Lee et al., 1995; Sarge, 1995; Ali et al., 1997; Buckley et al., 2001; Gothard et al., 2003).

Immunocytochemistry and LSCM were employed to examine the localization of HSP30 in A6 cells treated with WA. Cells exposed to 5 μM WA displayed HSP30 accumulation primarily in the cytoplasm in a punctate pattern with a lesser amount in the nucleus. The granular or punctate pattern of HSP30 accumulation may be due to the stress-induced formation of HSP30 multimeric structures that are required for sHSP function (Ohan et al., 1998; MacRae, 2000; Van Montfort et al., 2001). Additionally, relatively large HSP30 staining foci were detected in cells exposed to 5 μM WA for 18 h. Similar structures were reported in A6 cells exposed to cadmium chloride, sodium arsenite, celastrol or MG132 (Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009; Walcott and Heikkila, 2010; Young and Heikkila, 2010). Although the identity of these large structures is unknown, it has been suggested that they are inclusion bodies containing HSP30 bound to unfolded proteins and may be associated with the molecular chaperone function of HSP30 (Fernando and Heikkila, 2000; Heikkila, 2003; Heikkila, 2004; Young and Heikkila, 2010). In support of this possibility, previous studies have determined that treating cells with compounds that inhibit proteasome activity significantly increased the formation of cytosolic aggresomes, proteinaceous inclusion bodies that form as a general cellular response to aggregated proteins (Garcia-Mata et al., 1999). Previously, our laboratory determined that these HSP30 staining foci occurred in response to high concentrations of MG132 (Young and Heikkila, 2010). Lastly, the current study determined that treatment of A6 cells with WA for 8 h did not visibly disrupt the actin cytoskeleton, which has been used as an indicator of cellular viability and health (Wiegant et al., 1987; Ohtsuka et al., 1993). However, after extended WA treatment A6 cells showed some actin disorganization with the formation of numerous punctate areas of actin aggregation and peripheral ruffled edges. Enhanced accumulation of

HSP30 in A6 cells treated concurrently with WA and a mild heat shock, compared to the stresses individually, was verified by immunocytochemistry and LSCM. HSP30 accumulated primarily in the cytoplasm in a punctate pattern with some in the nucleus. It was also noted that the combined stress conditions caused membrane ruffling and HSP30 associated foci.

WA-induced actin aggregation was previously reported in mammalian systems and may be due to the interaction of WA with the intermediate filament protein vimentin, and annexin II, which has basal F-actin cross-linking activity. In support of this possibility, Bargagna-Mohan et al. (2007) demonstrated that bovine aortic endothelial cells exposed to 3 μM WA for 18 h showed the presence of numerous vimentin-positive staining particles in the cytoplasm, which strongly co-stained for actin. In addition, annexin II-transduced WI-38 human fibroblasts displayed increased levels of F-actin aggregation after treatment with 4 μM WA for 2 h (Borm et al., 2005). Several of these compact areas of actin co-localized with the HSP30 associated foci (Fig. 15). In previous studies, the actin cytoskeleton of sodium arsenite-treated A6 cells displayed some disorganization that co-localized with areas of HSP30 enrichment (Gallalchew and Heikkila, 2005).

The mechanism responsible for WA-induced cell membrane ruffling is not well understood. In general ruffling is the formation of a motile cell surface that contains a meshwork of newly polymerized actin filaments under the plasma membrane and may indicate global instability of cellular adhesion (Borm et al., 2005). Previous studies have demonstrated that membrane ruffling was induced by extracellular influences such as growth factors and heat shock (Mellstrom et al., 1988; Chan et al., 1998; Svitkina and Borisy, 1999; Borisy and Svitkina, 2000; Cooper and Schafer, 2000; Hamilton and Heikkila, 2005). For

example, one of the earliest effects of platelet-derived growth factor on human fibroblasts in culture is an induction of membrane ruffling (Mellstrom et al., 1988).

The present study also investigated the pattern of HSP accumulation in *Xenopus* A6 cells recovering from WA treatment. Relative levels of HSP30 and HSP70 accumulation remained elevated for 6 to 12 h after the removal of WA. These findings were similar to results described in A6 cells recovering from celastrol, in which the relative levels of HSP30 and HSP70 remained elevated for 18 to 24 h (Walcott and Heikkila, 2010). Additionally, a prolonged accumulation of HSPs was reported in Chinese hamster ovary cells recovering from proteasomal inhibition (Kovacs et al., 2006). The lingering accumulation of HSPs in A6 cells recovering from WA may be due to continued *hsp* gene transcription or an increase in *hsp* mRNA or HSP stability, as suggested previously for sodium arsenite (Darasch et al., 1988). Even so, it is likely that elevated levels of HSPs are beneficial to WA-treated cells since relatively high concentrations of ubiquitinated cellular proteins that accumulate within the cytosol may require an extended amount of time to re-establish protein homeostasis by degradation (Bush et al., 1997; Lee and Goldberg, 1998; Liao et al., 2006).

The present study also determined that A6 cells acquired a state of thermotolerance when treated with WA, such that they were able to maintain their cytoskeletal organization after a subsequent thermal challenge at 37 °C, which is normally cytotoxic (Fig. 20). Pretreatment with KNK437 inhibited HSP30 accumulation and failed to result in WA-induced thermotolerance as indicated by the disruption of the actin cytoskeleton. This signifies that HSPs, including HSP30, are required for the acquisition of thermotolerance in A6 cells treated with WA. A cytoprotective effect was also demonstrated in cells treated with

concurrent WA and mild heat shock (Fig. 21) but not in mild heat shock alone (Fig. 22), prior to a 37 °C thermal challenge.

In summary, this study has shown for the first time that the natural product, WA, induced *hsp30* gene expression. In addition, this study has demonstrated for the first time, in an amphibian system, that WA induced *hsp70* gene expression, increased the accumulation of ubiquitinated proteins, inhibited chymotrypsin-like activity and partially disrupted the actin cytoskeleton. Understanding the effect that WA has on *Xenopus hsp* gene expression is of importance given the potential therapeutic role for HSPs in various human diseases. Proteasome dysfunction and *hsp* gene expression have been implicated in numerous neuropathologies, including Alzheimer's disease (Morimoto, 2008). A consistent feature of Alzheimer's disease is the presence of ubiquitinated tau-positive inclusion bodies containing HSPs in oligodendrocytes (Goldbaum and Landsberg, 2004). This suggests that the proteasome and HSPs have an essential role in maintaining tau protein homeostasis and malfunction of these processes could lead to disease states.

This study leaves some unanswered questions about the effects of WA on *hsp* gene expression. Therefore, further analysis on the relationship between WA-induced proteasome inhibition and the accumulation of HSPs is required. Future studies should examine the effect of WA on other *Xenopus hsp* genes such as *hsp27*, *BiP*, *hsp47*, *hsp90* and *hsp110*. As well, further experiments are needed to determine if the multiple bands detected with anti-HSP30 are the products of different *hsp30* family members or post-translationally modified proteins. Mammalian studies have suggested that proteasome inhibition may have an impact on molecular chaperones in the endoplasmic reticulum and HSP90 (Bush et al., 1997; Banerji,

2009). Given the advantages of the *Xenopus* embryonic system including microinjections and transgenic methodology, future experiments should examine the effects of WA on *hsp* gene expression during early development. Also, the effect of proteasome inhibition on *hsp* gene expression during animal development has not been investigated. Additionally, experimental evidence is needed to explain the large cytoplasmic foci containing HSP30 observed in A6 cells exposed to WA. Formation of aggresomes in mammalian cells was paralleled by the redistribution of the intermediate filament protein vimentin as well as by the recruitment of the proteasome, and the *hsp70* systems of chaperones (Garcia-Mata et al., 1999). Therefore, a test for co-localization of vimentin or HSP30, utilizing immunocytochemistry and LSCM, would help to determine if these foci are stress granules or inclusion bodies. Previous studies have also shown that WA can induce apoptosis through inhibition of the NF-kβ signaling pathway (Mohan et al., 2004j; Malik et al., 2007; Idris et al., 2009). Therefore, the potential induction of apoptosis in WA-treated *Xenopus* A6 cells and embryos should also be investigated.

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