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Curcumin-induced inhibition of proteasomal activity, enhanced HSP accumulation and the acquisition of thermotolerance in *Xenopus laevis* A6 cells

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ABSTRACT

In the present study, curcumin, a phenolic compound with anti-inflammatory, anti-tumor and anti-amyloid properties, inhibited proteasomal activity and induced the accumulation of HSPs in the frog model system, *Xenopus laevis*. Treatment of A6 kidney epithelial cells with curcumin enhanced ubiquitinated protein levels and inhibited chymotrypsin-like activity. Furthermore, exposure of cells to 10–50 μM curcumin for 24 h induced HSP30 and HSP70 accumulation. This phenomenon was controlled at the transcriptional level since pre-treatment of cells with KNK437, a heat shock factor 1 (HSF1) inhibitor, repressed HSP accumulation. Additionally, elevation of the incubation temperature from 22 to 30 °C greatly enhanced the curcumin-induced accumulation of HSP30 and HSP70. Immunocytochemical analysis revealed that curcumin-induced HSP30 was detectable primarily in the cytoplasm in a punctate pattern with minimal detrimental effects on the actin cytoskeleton. Finally, prior exposure of cells to curcumin conferred a state of thermotolerance since it protected them against a subsequent cytotoxic thermal challenge. These findings are of importance given the interest in identifying agents that can upregulate HSP levels with minimal effects on cell structure or function as a therapeutic treatment of protein folding diseases.

1. Introduction

Organisms are constantly challenged by conditions that cause chronic or acute stress and have evolved networks of responses that identify, monitor and respond to these stressful stimuli (Morimoto, 2008). At the molecular level, biochemical mechanisms responsible for protein function and stability are disrupted as a result of exposure to stressful stimuli, both environmental and physiological, causing the proteins to unfold into unstable conformations, which are then targeted for degradation (Morimoto, 1998; Balch et al., 2008). The ATP-dependent ubiquitin–proteasome system (UPS) is responsible for the majority (80–90%) of protein hydrolysis in the cell (Lee and Goldberg, 1998a). It is essential for many fundamental cellular processes including cellular differentiation, cell cycle progression, proliferation and apoptosis (Mani and Gelmann, 2005; Landis-Piwowar et al., 2006). The pathway is broken down into two successive steps: addition of ubiquitin molecules onto the protein targeted for degradation and the subsequent degradation of the ubiquitinated proteins by the 26S proteasome (Yang et al., 2008). The proteasome is capable of hydrolyzing most unfolded proteins and contains multiple peptidases including chymotrypsin-like (CT-like) activity (Groll et al., 1997; Ciechanover, 1998). Inhibition or malfunction of the UPS has been associated with a variety of neurological and protein-misfolding diseases including Alzheimer's, Parkinson's and Huntington's (Masliah et al., 2000; Ross and Pickart, 2004). In eukaryotic organisms, inhibition of the proteasome has also been associated with an increase in heat shock protein (*hsp*) gene expression (Bush et al., 1997; Lee and Goldberg, 1998b; Stangl et al., 2002; Walcott and Heikkila, 2010; Young and Heikkila, 2010).

Heat shock proteins (HSPs) are a large group of molecular chaperones that are involved in many cellular processes including protein synthesis, folding/assembly, membrane translocation and degradation (Katschinski, 2004; Morimoto, 2008). HSPs also bind to denatured proteins in order to prevent stress-induced protein aggregation and assist in refolding them to their native state. HSPs have been documented in a wide variety of organisms from bacteria to humans. There are 6 different HSP gene families that have been characterized to date and are grouped based on size and include small HSPs (sHSPs), HSP40, HSP60, HSP70, HSP90 and HSP100. They can be constitutively expressed, strictly stress inducible or both inducible and constitutively regulated. HSP expression patterns vary between organisms, tissue types, and developmental stages (Lindquist, 1986; Feige et al., 1996; Heikkila, 2010). The sHSP family is made up of HSPs ranging in size from 16 to 42 kDa and are evolutionarily divergent with the exception of a conserved 80–100 amino acid α-crystallin domain (Arrigo, 1998; MacRae, 2000; Van Montfort et al., 2001; Heikkila, 2004). sHSPs form highly polymeric structures, which are necessary for their chaperone function in vivo. Various functions that have been suggested for sHSPs include resistance against apoptosis, acquisition of thermotolerance, actin capping/decapping activity,

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modulation of redox parameters, and cellular differentiation. Cytoplasmic HSP70 is a stress-inducible member of the HSP70 family that inhibits aggregation of unfolded proteins, assists in ATP-dependent protein folding, translocation of proteins across cell membranes and degradation of denatured and misfolded proteins (Katschinski, 2004; Daugaard et al., 2007). Stress-inducible *hsp* genes are regulated primarily at the transcriptional level via the interaction of heat shock factor 1 (HSF1) with the *cis*-acting heat shock element (HSE). The HSE consists of multiple inverted repeats of the pentamer sequence 5′-nGAAn-3′, which are generally present in the 5′ promoter of most *hsp* genes (Morimoto, 1998; Katschinski, 2004; Voellmy, 2004). Normally HSF1 is bound to HSP90 and exists as an inactive monomer in the cytoplasm (Voellmy, 2004). In response to an increase in the intracellular levels of denatured proteins, HSP90 is recruited to prevent their aggregation, thereby allowing HSF1 monomers to form hyperphosphorylated trimers. This trimerization results in the activation of the HSF1, subsequent localization to the nucleus, and binding to the HSE to initiate transcription of *hsp* genes by RNA polymerase II.

Previously, our laboratory has characterized the induction of *hsp* genes in *Xenopus laevis* embryos and cultured cells (Ovsenek and Heikkila, 1990; Krone et al., 1992; Heikkila et al., 1997; Ohan et al., 1998; Lang et al., 1999, 2000; Heikkila, 2004; Gellalchew and Heikkila, 2005; Manwell and Heikkila, 2007; Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009; Young et al., 2009; Heikkila, 2010). For example, various cellular stresses including heat shock, sodium arsenite, and cadmium chloride have all been shown to induce HSP30 and HSP70 accumulation in A6 kidney epithelial cells and/or embryos (Lang et al., 1999, 2000; Gellalchew and Heikkila, 2005; Woolfson and Heikkila, 2009; Young et al., 2009; Heikkila, 2010). This response was regulated, in part, at the transcriptional level, since KNK437, an inhibitor of HSF1–HSE binding activity, inhibited *hsp* gene expression (Manwell and Heikkila, 2007; Voyer and Heikkila, 2008; Woolfson and Heikkila, 2009; Young et al., 2009). Immunocytochemical studies revealed that HSP30 was localized primarily in the cytoplasm in a punctate pattern (Gellalchew and Heikkila, 2005; Manwell and Heikkila, 2007). It is likely that HSP30 functions as a molecular chaperone since it inhibited heat-induced target protein aggregation and maintained heat- or chemically-denatured luciferase in a folding competent state (Fernando and Heikkila, 2000; Fernando et al., 2002; Abdulle et al., 2002). Recently, we reported that A6 cells treated with known proteasomal inhibitors carbobenzoxy-leucyl-L-leucyl-L-leucinal (MG132), lactacystin, and celastrol inhibited the CT-like activity and induced HSP accumulation (Walcott and Heikkila, 2010; Young and Heikkila, 2010).

In the present study, we examined the effect of curcumin on proteasome activity and HSP accumulation in A6 cells. Curcumin is an active ingredient in the Indian spice, Turmeric, which is obtained from the rhizome of the plant *Curcuma longa* (Ammon and Wahl, 1991). Turmeric has been used widely in Ayurvedic Indian Medicine as an herbal remedy to treat common eye infections, mosquito bites, burns, fever and various other skin diseases. Curcumin has also been shown to possess various beneficial properties including anti-inflammatory, antioxidant, chemopreventive, and chemotherapeutic activities (Aggarwal et al., 2003; Duvoix et al., 2003; Campbell and Collett, 2005). Recently, curcumin was found to increase the relative levels of ubiquitinated proteins and inhibit the chymotrypsin-like activity of the 26S proteasome in SW480 and HCT-116 human colon cancer cells (Milacic et al., 2008). Additionally, curcumin was found to induce *hsp70* gene expression in HeLa cells, human colorectal carcinoma cells and human leukemia K562 cells (Dunsmore et al., 2001; Chen et al., 2001; Teiten et al., 2009). Compared to the extensive number of studies detailing the effect of curcumin on health and various disease states relatively little research has been carried out examining the effect of curcumin on proteasomal inhibition and *hsp* gene expression.

In the current study, we show in an amphibian model system that curcumin inhibited the UPS as indicated by inhibition of chymotrypsin-like activity and the enhanced accumulation of ubiquitinated proteins. Moreover, curcumin induced HSP30 and HSP70 accumulation in a time- and dose-dependent manner. This phenomenon was controlled, at least in part, at the transcriptional level since pre-treatment of cells with KNK437, a HSF1 inhibitor, repressed HSP accumulation. Additionally, curcumin acted synergistically with mild heat shock to enhance HSP accumulation. Immunocytochemical analysis revealed that curcumin-induced HSP30 was detectable in the cytoplasm in a punctate pattern with minimal detrimental effects on the actin cytoskeleton. Finally, prior exposure of cells to curcumin conferred a state of thermotolerance by protecting them against a subsequent thermal challenge.

2. Materials and methods

2.1. Maintenance and treatment of cells

X. laevis A6 cells were obtained from the American Type Culture Collection (CCL-102; American Type Culture Collection (ATCC), Rockville, MD, USA) and grown in 55% Leibovitz L-15 Media containing 10% (v/v) fetal bovine serum (100 U/mL) and 1% penicillin/streptomycin (100 μg/mL; all purchased through Sigma-Aldrich, Oakville, ON, Canada) at 22 °C in T75 cm^2 BD falcon culture flasks (BD Biosciences, Mississauga, ON, Canada). Flasks of cells subjected to heat stress were immersed in regulated water baths at 30 °C or 33 °C for 2 or 6 h followed by incubation at 22 °C for 2 h prior to harvest. Curcumin (Sigma-Aldrich) treatments of cells were performed at 22 °C or 30 °C using dilutions from a 100 mM curcumin stock solution dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) and stored at −20 °C. A6 cells were also treated with 30 μM MG132 (Sigma-Aldrich; stock solution of 21 mM was dissolved in DMSO and stored at −20 °C). Also, some cells were pre-treated with 100 μM KNK437 (Calbiochem, Gibbstown, NJ, USA; stock solution of 5 mg/mL was dissolved in DMSO) for 6 h before curcumin treatments to inhibit HSF1 activation. Cells were rinsed using 65% HBSS and removed via scraping in 1 mL of 100% HBSS. Cells were centrifuged at 21,900 *g* for 1 min and the resulting pellets were stored at −80 °C until protein isolation.

2.2. Detection of chymotrypsin-like activity

Flasks of A6 cells were treated with 30 μM curcumin at 22 °C for 14 or 24 h. Since MG132 was shown to inhibit proteasome activity including CT-like activity in A6 cells (Walcott and Heikkila, 2010), a 30 μM MG132 treatment was used in the present study for comparison purposes. After treatments, cells were trypsinized and resuspended in 5 mL of fresh 55% L-15 media. For each sample, the total number of cells per mL was determined using a Bright-Line haemocytometer (Hausser Scientific, Horsham, PA, USA). Aliquots of 15,000 cells were suspended in 100 μL of L-15 media for determination of chymotrypsin-like activity using the Promega Proteasome-Glo cell-based luminescent assay kit (Promega Corp., Madison, WI, USA) according to the manufacturer's recommendations. The luminescence of each sample was measured using the Victor^3 luminometer (Perkin Elmer Inc., Waltham, MA, USA) containing a filter set at 340/480 nm.

2.3. Protein isolation, quantification and immunoblot analysis

Total protein was isolated from cells as described previously by Young et al. (2009). Protein was quantified using a bicinchoninic acid (BCA) Protein Assay Kit by following manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Immunoblot analysis was performed using 20 μg of protein except for blots examining ubiquitinated protein for which 60 μg was employed. Polyacrylamide gels were electrophoresed on a BioRad Mini Protean III gel system (BioRad) and transferred to the nitrocellulose membrane with a Trans-Blot Semi-Dry Transfer Cell (BioRad) at 25 V for 20 min. Blots