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Effect of isothiocyanates, BITC and PEITC, on stress protein accumulation, protein aggregation and aggresome-like structure formation in *Xenopus* A6 kidney epithelial cells

Imran Khamis, John J. Heikkila*

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

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ABSTRACT

Numerous studies have elucidated the health benefits of organosulfur compounds, known as isothiocyanates (ITCs), derived from cruciferous vegetables. As electrophiles, ITCs have the ability to directly bind and modify thiol-containing compounds such as glutathione and cellular protein, including tubulin. While the biochemical effects of ITCs have been well characterized, less information is available regarding their effects on the accumulation of stress-inducible heme oxygenase-1 (HO-1), heat shock proteins (HSPs) and the possible formation of aggregated protein due to thiol modification. The present study has examined the effect of the ITCs, benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC), on the accumulation of HO-1, HSP70 and HSP30 in *Xenopus laevis* A6 kidney epithelial cells. Immunoblot analysis revealed that both BITC and PEITC induced the accumulation of HO-1 and HSP70 whereas HSP30 levels were enhanced only in cells treated with BITC. Immunocytochemistry determined that ITC treatment induced F-actin disorganization and membrane ruffling and enhanced accumulation of HO-1 in the cytoplasm. Additionally, BITC induced enhanced levels of ubiquitinated protein, aggregated protein, and the collapse and fragmentation of microtubules. In comparison, treatment of cells with the proteasomal inhibitor, MG132, induced the accumulation of all three stress proteins, aggregated protein and aggresome-like structures. Finally, cells pretreated with BITC inhibited the formation of MG132-induced aggresome-like structures in the perinuclear region. This latter finding suggests that BITC-induced microtubule fragmentation may impede the movement of aggregated protein via microtubules and their subsequent coalescence into aggresome-like structures in the perinuclear region.

1. Introduction

Organosulfur compounds known as isothiocyanates (ITCs) occur naturally as glucosinolates in cruciferous vegetables (Molina-Vargas, 2013). Treatment of plants with physical stress such as biting or chewing, activates myrosinase, an enzyme which hydrolyzes glucosinolates into ITCs (Zhang et al., 2006). Since ITCs are electrophilic, they can readily form conjugates with thiols as well as covalently bind glutathione and cellular protein including tubulin (Li et al., 2012). Numerous studies have reported human health promoting properties of ITCs such as their chemopreventive efficacy and possible prevention of neurodegenerative diseases (Zhang et al., 2006; Mi et al., 2007, 2008, 2009, 2010; Li et al., 2012; Mi et al., 2011; Sarkars et al., 2013; Giacoppo et al., 2015). Two of the most extensively studied ITCs are benzyl isothiocyanate (BITC) and phenethyl isothiocyanate (PEITC).

Given the importance and abundance of tubulin within the cell, any chemical modification of the protein by ITCs could result in a significant conformational change. In fact, tubulin modification was suggested as being the main mechanism through which BITC and PEITC induced cell cycle arrest and apoptosis leading to the suppression of mammalian oncogenic cells (Zhang et al., 2006; Sarkars et al., 2013). A previous study found that thiol side chains of cysteine residues of α- and β-tubulin were prone to modification by BITC and to a lesser extent PEITC (Mi et al., 2008). Additionally, it was shown that BITC had a more pronounced effect on inducing proteasomal inhibition than PEITC. Since BITC is a smaller compound, containing a methyl group extending from its aromatic hydrocarbon compared to an ethyl group for PEITC, it has greater accessibility to cysteine residues buried within the tubulin molecule thus increasing its affinity for the protein (Mi et al., 2008). Nevertheless, both ITCs inhibited proteasome protease activities by direct covalent interaction with thiol groups within the catalytic active site of proteasome subunits.

An increase in our knowledge of the effects of ITCs is important given their potential role as therapeutic compounds in the treatment of

* Corresponding author.
E-mail address: heikkila@uwaterloo.ca (J.J. Heikkila).

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cancer, neurodegenerative diseases and bacterial and viral infections (Dufour et al., 2013; Morris and Dave, 2014; Giacoppo et al., 2015). While studies in mammalian systems have characterized the ability of ITCs to directly bind and modify thiol-containing proteins including α-tubulin, less is known regarding their ability to induce the accumulation of stress proteins such as heme oxygenase (HO-1) and heat shock proteins (HSPs) or their impact on the F-actin and microtubule cytoskeleton. Stress-inducible HO-1, also known as HSP32, catalyzes the catabolism of heme to iron, carbon monoxide and biliverdin (Tenhunen et al., 1968; Ryter et al., 1999; Gozzelino et al., 2010; Correa-Costa et al., 2012). HO-1, which is found in every cell, is essential for human health since its deficiency is characterized by growth retardation, hemolytic anemia, endothelial damage, iron deposition and increased vulnerability to oxidative stress-related injury (Yachie et al., 1999; Chau, 2015). In mammalian systems, a variety of inducers including curcumin, MG132, sodium arsenite, and cadmium were reported to induce *ho-1* gene expression (Alam et al., 2000; Wu et al., 2004; Yamamoto et al., 2010; Wang et al., 2013). Stress-induced *ho-1* gene expression is controlled primarily at the transcriptional level (Ryter et al., 2006). The primary transcription factor responsible for expression of the *ho-1* gene is the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2; Choi et al., 2014). Regarded as a member of the leucine zipper transcription factors, Nrf2 is supressed under basal conditions as it is bound to Kelch-like ECH-associated protein 1 (Keap1). Stress-induced modification of cysteine residues results in Keap1 dissociating from Nrf2, which then translocates into the nucleus. Subsequently, Nrf2 forms a heterodimer with Maf protein before binding to the antioxidant response element to induce *ho-1* gene expression (Choi et al., 2014).

In mammalian cells, both BITC and PEITC were found to induce the accumulation of the heat shock protein, HSP70 (Naidu et al., 2016). The heat shock response is a universal cellular cytoprotective mechanism that results in the accumulation of HSPs including HSP90, HSP70 and small HSPs, which are molecular chaperones that confer resistance against proteotoxic stress induced by elevated temperatures, heavy metals, oxidants or disease states (Verghese et al., 2012). Stress-induced accumulation of unfolded protein triggers the activation and binding of heat shock factor 1 (HSF1) to the heat shock element (HSE), an enhancer situated in the upstream promoter region of *hsp* genes (Morimoto, 1998, 2008; Xie et al., 2014). The primary role of HSPs is to bind unfolded protein and prevent the formation of toxic protein aggregates. However, some stressors, such as sodium arsenite in high concentrations, can cause a large increase in the amount of unfolded or misfolded proteins and overwhelm the molecular chaperone system leading to the formation of toxic protein aggregates (Del Razo et al., 2001; Liu et al., 2001; Samuel et al., 2005; Brunt et al., 2012; Khan et al., 2015). Furthermore, an impairment of the ubiquitin proteasome system, which is the primary system for the degradation of damaged protein, can further exacerbate this situation. In order to isolate and minimize the effect of toxic protein aggregates, the cell sequesters these aggregates into aggresome-like structures encased within a vimentin cage (An and Statsyuk, 2015). Studies with proteasomal inhibitors such as MG132 have demonstrated that this process occurs through a microtubule-dependent process, which involves the transport of aggregated protein along microtubule networks using dynein motors to the perinuclear region and their subsequent proteolysis by autophagy (Uversky and Fink, 2007; Zaarur et al., 2008; Bolhuis and Richter-Landsberg, 2010; Xiong et al., 2013; Khan and Heikkila, 2014).

Xenopus laevis adults, embryos and cultured cells have been extensively used as model systems in cell and molecular biology research. Much of the information obtained from studies with *Xenopus* is applicable to humans. A6 kidney epithelial cells, derived from *Xenopus laevis* proximal renal tubules, was used to study the expression of various stress proteins including the accumulation of HO-1 in response to cadmium, arsenite and proteasomal inhibitors (Music et al., 2014; Shirriff and Heikkila, 2017). This cell line has been vital in the analysis of *Xenopus* HSP70 and HSP30 accumulation after treatment with

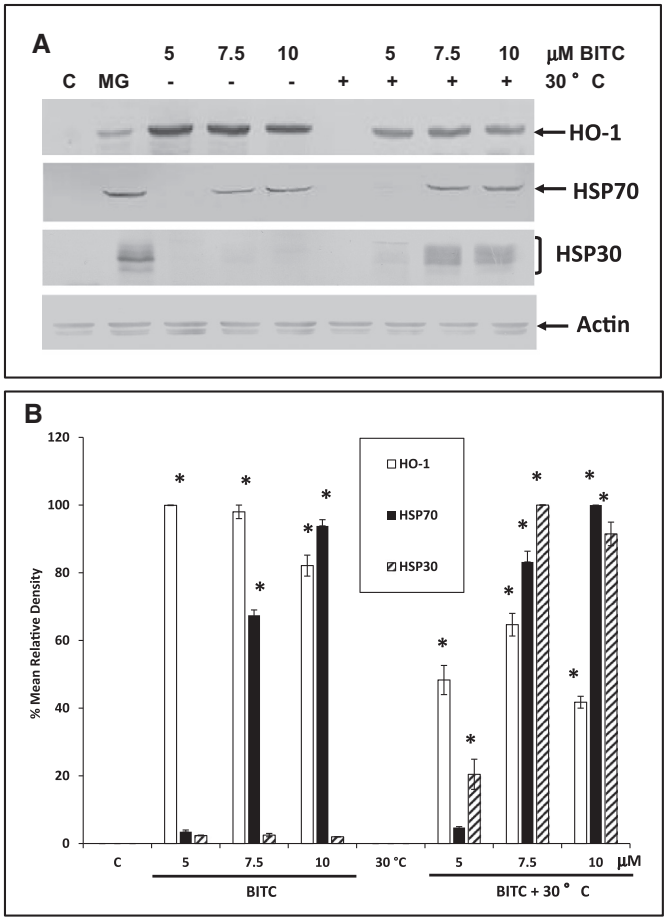

Fig. 1. Relative levels of HO-1, HSP70 and HSP30 in cells subjected to BITC treatment at 22 or 30 °C. A6 cells were maintained at 22 °C (C) or exposed to either 5, 7.5 or 10 µM BITC at 22 or 30 °C for 12 h. Additionally, cells were exposed to a temperature of 30 °C for 12 h or treated with 30 µM MG132 (MG; inducer of HO-1, HSP70 and HSP30 accumulation in A6 cells) for 12 h at 22 °C. A) Total protein was isolated and subjected to immunoblot analysis using an anti-HO-1, anti-HSP70, anti-HSP30 or anti-actin antibody as described in Materials and methods. This panel displays a representative immunoblot. B) ImageJ software was used to perform densitometric analysis of signals obtained for HO-1, HSP70 and HSP30 as described in Materials and methods. The data were expressed as a percentage of the maximum band density obtained for each protein (5 µM BITC at 22 °C for HO-1; 10 µM at 30 °C for HSP70; 7.5 µM at 30 °C for HSP30), while standard errors were indicated with vertical bars. A one-way ANOVA with a Tukey's Multiple Comparisons post-test was used to determine significance. Significant differences between the control cells and treated cells are indicated with an asterisk ($p < 0.05$). These data are representative of 4 separate experiments.

various environmental stressors including heat shock, sodium arsenite and cadmium chloride (Ohan et al., 1998; Gellalchew and Heikkila, 2005; Woolfson and Heikkila, 2009; Heikkila, 2010; Brunt et al., 2012; Khamis and Heikkila, 2013). *Xenopus* HSP70 is a stress-inducible molecular chaperone that inhibits aggregation of unfolded protein and can refold protein in an ATP-dependent manner (Heikkila, 2010). HSP30 is the major stress-inducible sHSP in *Xenopus* but has counterparts in other frogs as well as fish and birds but not mammals (Helbing et al., 1996; Norris et al., 1997; Katoh et al., 2004; Elicker and Hutson, 2007; Mulligan-Tuttle and Heikkila, 2007; Heikkila, 2017). As a molecular chaperone, HSP30 can bind to stress-induced unfolded protein, inhibit their aggregation and maintain them in a folding competent state (Fernando and Heikkila, 2000; Abdulle et al., 2002; Fernando et al., 2002, 2003). Also, studies with *Xenopus* A6 cells revealed that that HSP30 co-localized with aggresome-like structures in response to certain stressors such as MG132, arsenite and cadmium as well as recovery from heat shock (Khan and Heikkila, 2014; Khan et al., 2015; Shirriff and Heikkila, 2017).