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L-THREONINE INDUCES HEAT SHOCK PROTEIN EXPRESSION AND DECREASES APOPTOSIS IN HEAT STRESSED INTESTINAL EPITHELIAL CELLS

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Abstract

Objective—Osmotically acting amino acids can be cytoprotective following injury. As threonine (THR) induces osmotic cell-swelling, our aim was to investigate the potential for THR to induce cellular protection in intestinal epithelial cells and evaluate possible mechanisms of protection.

Methods—Cells treated with a range of THR doses were evaluated following heat stress (HS) injury. Alpha-aminoisobutyric acid (AIB), a non-metabolizable amino acid analog, was used as an osmotic control. MTS assays were used to assess cell survival. Heat shock protein (HSP) expression and cleaved caspase-3 (CC3) were evaluated via western blot. Cell morphology and cell size analyzed via microscopy.

Results—Following HS, THR treatment increased cell viability versus CT in a dose-dependent fashion from 5 to 20 mM. The non-metabolized amino acid analogue, Alpha-aminoisobutyric acid (AIB) also increased cell survival in HS cells versus HS-CT. HSP70 and HSP25 expression increased with THR and AIB treatment versus HS-CT. THR also increased HSP25 in non-stressed cells. Microscopic evaluation revealed both THR and AIB preserved structural integrity of the actin cytoskeleton in HS-cells versus HS-CT. THR, but not AIB, enhanced nuclear translocation

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of HSP25 during HS. This nuclear translocation, was associated with a 60% decrease in apoptosis in HS cells with THR. No anti-apoptotic effect was observed with AIB.

Conclusions—This is the first demonstration THR increases HSP70 and HSP 25 and protects cells from HS. THR's mechanism of protection may involve cytoskeletal stabilization, HSP up-regulation and nuclear translocation, and decreased apoptosis. THR's protection appears to involve both cell swelling-dependent and independent processes.

Keywords

Heat shock protein 25; IEC-18; cell survival; Alpha-aminoisobutyric acid; cell swelling; heat shock protein 70

INTRODUCTION

Defects in intestinal epithelial barrier function have been proposed to play a role in a range of diseases such as the systemic inflammatory response syndrome (SIRS), sepsis, inflammatory bowel disease, asthma, allergies, type 1 diabetes, cardiovascular disease and even autism[1–5]. Intestinal health-targeted therapeutic agents able to preserve intestinal barrier function have significant clinical implications in both prevention and treatment of these disease states.

Osmotically acting amino acids, such as glutamine, can be cytoprotective following injury *in vitro* and *in vivo*. Like glutamine, the majority of threonine (THR) enters the cell via a sodium dependent transporter, which causes an influx of water to induce cell-swelling. Such osmoregulatory processes are important for the survival of living organisms. The ability of the cell to adapt to environmental changes in osmolarity and ionic strength is essential to maintenance of proper intracellular osmotic pressure and chemical potentials of metabolites[6]. Since osmotic changes are a major physical stress that all cells may experience during their lifetime, cell signaling pathways related to osmotic stress play key roles in the activation of specific genes that are capable of inducing cellular protection [6–8].

One specific cell protection pathway that cellular osmotic changes have been linked to is activation of the heat shock protein (HSP) pathway[9]. Heat shock proteins are one of the most basic mechanisms of cellular protection and can induce significant “stress tolerance” in cells subjected to heat or oxidant injury[10, 11]. HSP70 is associated with the preservation of intracellular proteins that would otherwise be denatured by lethal stressors[10, 11]. Both hyper- or hypotonicity can cause heat shock factor 1 (HSF-1) trimerization, migration to the nucleus, and subsequent transcriptional activation of the HSP70 gene [8, 9, 12]. The heat shock response to hypertonicity is even more rapid than the response to elevated temperatures [13]. In 1997, Caruccio et. al found HSF-binding activity could be induced prominently under both hypo-osmotic and hyperosmotic conditions[9]. Another key member of the HSP family, HSP25/27 is involved in thermotolerance, cytoprotection and enhanced cell survival under stress conditions[11]. Phosphorylation of HSP25/27 enhances F-actin polymerization and stabilizes the F-actin network in response to heat and other stresses [14–17]. It also preserves the focal contacts fixed at the cell membrane by preventing F-actin

fragmentation [17]. Furthermore, HSP25 nuclear translocation has been shown to decrease apoptosis by preventing DNA fragmentation and nuclear breakdown [18].

THR's ability to protect the intestinal epithelia, potentially via an osmotic effect, in vitro is currently unknown. Thus, the aim of this study was to investigate the cell protective potential of THR and to investigate the mechanistic pathways by which THR may act to induce cell protection following injury. Alpha-aminoisobutyric acid (AIB), a non-metabolizable amino acid analog, is known to induce cell swelling as it is taken up by similar transport systems as THR[19]. Thus, AIB can be utilized to determine the contribution of cell swelling to HSP induction and cellular protection, independent from potential metabolism-related effects of amino acid uptake, in our injury model. We hypothesized THR would protect cells by increasing cell size, via activation of the cell swelling pathway, and enhancing HSP25 and HSP70 expression. To investigate this idea, we conducted a series of experiments on heat stressed cells with the following objectives: 1. Determine if THR and AIB can protect cells from lethal heat stress, 2. Determine if THR and AIB treatments cause cell swelling in our model, and 3. Determine if heat shock protein expression, the cellular localization of HSP25, and threonine metabolism (versus cell swelling alone) are associated in this mechanism of protection.

EXPERIMENTAL PROCEDURES

All chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO) unless otherwise specified.

Cell Culture

Intestinal Epithelial-18 (IEC-18) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), (Cellgro Mediatech Inc, Herndon, VA) supplemented with 10% fetal bovine serum (FBS) (Cellgro Mediatech Inc, Herndon, VA) 2mM L-GLN (Sigma, St Louis, MO), 10 ml/L of antibiotic solution containing penicillin G (10,000U/mL) and streptomycin (10,000 µg/mL) (Cellgro Mediatech Inc., Herndon, VA). and 0.1% insulin (Sigma, St Louis, MO). All cells were maintained in a humidified 37°C incubator with 5% CO₂.

Heat Stress Injury

A heat stress model of injury was used for all experiments. Prior to heat stress, media was replaced with DMEM either with or without THR or AIB for 15 minutes. For cell viability, 96-well plates were submerged in a 44°C water bath for 50 min (lethal heat stress) and allowed to recover at 37°C for 24 hours. For western blots, 10 cm dishes were heat stressed at 43°C for 45 minutes (non-lethal heat stress) and cells were allowed to recover at 37°C for 30 min, or 2.5 hours before harvesting. For cell size microscopy experiments; cells were treated with either a 15 or 30 min non-lethal HS and were immediately fixed and stained for visualization.

Cell Viability

Cells were seeded in 96 well plates (7000 cells per well), and allowed to grow for 24 hours in full media. Cells were then treated and subjected to lethal HS (as specified above). Cell

viability was evaluated via a soluble tetrazolium salt (MTS) assay (Promega, Madison, WI) as per manufacturer's instructions 24 hours later. Briefly, 1 part PMS was added to 20 parts MTS immediately before the solution was diluted 1:5 in phenol red-free DMEM and was then added to phosphate buffered saline (PBS) washed cells. MTS was bio-reduced by cells into a colored, soluble formazan product. Absorbance values were read after 3 hours at 490 nm using an ELISA plate reader (Thermo Electro Corporation, San Jose, CA); references included readings at 650 nm and no-cell blank wells. Higher absorbance values reflect greater cell proliferation/viability. The values for the same six to twelve wells for each treatment group were averaged per experiment and each experiment was repeated a minimum of 3 times. All heat stressed groups were normalized to their individual non-heat stressed controls to account for possible differences in cell growth, or chemical toxicity.

Heat Shock Protein Expression Measurement

IEC-18 cells were seeded in 10 cm dishes and allowed to grow for 24 hrs, treated with and without THR or AIB and subjected to heat stress (as previously described). Cells were washed in ice-cold PBS, harvested and lysed in MPER (mammalian protein extraction reagent) (Thermo Scientific, Waltham, MA). Western blot analysis was used to evaluate changes in HSP25 and HSP70 levels. For changes in HSP25 cellular localization, cells were harvested and separated into nuclear and cytoplasmic fractions using the Pierce Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER) (Thermo Scientific, Waltham, MA) plus protease inhibitors (Roche, Indianapolis, IN). Protein was determined with BCA protein assay (Pierce, Rockford, IL). 20 µg of each sample was added to a 2× treatment buffer (0.125 M Tris pH 6.8, 4% sodium dodecylsulfate, 20% glycerol, and 10% β-mercaptoethanol), boiled for 3 minutes, and then loaded into a NuPAGE 4%–12% Bis-TrisGel (Invitrogen, Carlsbad, CA). Proteins were electrophoretically separated with a mini-gel system and transferred to PVDF membranes (Millipore, Billerica, MA), using the mini-transblot cell transfer system (Biorad, Hercules, CA). Membranes were blocked with 5% nonfat milk in PBS-Tween for 1.5 hours at room temperature. Primary antibodies against HSP25 (Cat# SPA801), HSP70 (Cat#SPA810) (StressGen, Victoria, BC, Canada), or β-actin (Cat# A5316) (Sigma-Aldrich, St. Louis, MO), were added to antibody buffer (blocking solution) and incubated overnight at 4 degrees C. After washing three times with PBS-Tween over 30 min, secondary antibodies (peroxidase-conjugated goat anti-mouse IgG, (Pierce Rockford, IL) or donkey anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA)) were applied at a 1:3000 dilution for 1.5 hours. Blots were washed three times with PBS-Tween over 30 min, incubated in commercial enhanced chemiluminescence reagents (Pierce, Rockford, IL), and exposed utilizing a UVP chemiluminescent darkroom system (UVP, Upland, CA). Densitometry was normalized against β-actin. Each experiment was repeated four times (using different cell passages) for total HSP70 and HSP25 expression, and three times for HSP25 cellular localization. Results from each run were averaged together.

Evaluation of Cellular Apoptosis

Caspase 3 is an inactive pro enzyme that is cleaved and activated by upstream proteases in cells that are undergoing apoptosis. Presence of the cleaved “active caspase 3” (CC3) can indicate apoptotic cell populations. To determine if THR or AIB treatment protects via preventing apoptosis, western blot analysis was used to determine CC3 levels for each

treatment group. Cells were seeded, treated, and harvested (as above) and CC3 levels were analyzed in whole cell lysates with polyclonal antibody (Cat# 9664) (Cell Signaling Danvers, MA) (1:1000) via western blot (as specified above). The experiment was repeated 6 times (n=6) and all groups were normalized against β -actin.

Cell Size and F-Actin Assessment

Cell size, morphology and the F-actin cytoskeleton were visualized using immunocytochemistry. Cells were treated with 0mM THR, 20mM THR, or 10mM AIB for 15 min with or without subsequent non-lethal heat stress injury (as above) for 15 or 30 minutes. Cells were fixed in a 4% paraformaldehyde PBS solution for 15 minutes at room temperature. Cells were washed three times (10 min each) with PBS. Cell membranes were permeabilized in ice-cold acetone methanol (70%, 30% respectively) for 10 min and then allowed to air dry. Slides were blocked in 10% normal donkey serum (in PBS) for 1 hour at room temperature. Blocking solution was removed and slides were stained with Alexa-Fluor 488 phalloidin (for F-actin), Alexa 594 wheat germ agglutinin (for golgi) (both from Molecular Probes/Invitrogen, Carlsbad, CA), and bisbenzimidazole Hoechst 33258 (for nuclei) (Sigma-Aldrich, St. Louis, MO), in an antibody buffer containing 1% BSA/PBS for 1 hour at room temperature. Slides were again washed three times in PBS, mounted in anti-quenching media and examined for F-actin cytoskeletal disruption, cell morphology and cell size. Images were acquired using a Leica DRM mechanized fluorescence microscope equipped with a movable stage (Leica Microsystems, Exton, PA), three epifluorescence cubes (Cy-3, FITC, and AMCA) with dichroic filters (Chroma Technology, Brattleboro, VT) and a cooled charge-coupled device (CCD) camera (Cooke, Tonawanda, NY). Cell size was determined by masking a minimum of 50 cells (per group) using Slidebook software (Intelligent Imaging Innovations, Denver, CO) and determining the average area per cell (μm^2) for each group.

Statistics

A two-tailed, type 2 student's t-test analysis was done between groups for survival, protein expression, and microscopy experiments to determine statistical significance. A "p" value of <0.05 was considered significant.

RESULTS

THR treatment increases cell survival in heat stressed IEC-18 cells in a dose dependent manner

Cells were treated with increasing doses of THR (from 0mM to 20mM), heat stressed, and allowed to recover for 24 hours at 37°C. MTS assay results indicate cells subjected to lethal heat stress were protected by THR in a dose dependent manner. Figure 1 shows cells treated with 5mM, 10mM, and 20mM THR showed a 1.2 fold, 1.6 fold, and 2.2 fold increase (respectively) in survival during lethal heat stress (*p<0.04 vs. HS CT, n=3), whereas an iso-osmotic control amino acid mixture (containing 20mM total of valine, alanine and phenylalanine) failed to provide protection from lethal heat stress. Results were normalized to non-HS controls for each experimental group to account for differences in proliferation.

AIB treatment increase cell survival in heat stressed IEC-18 cells

To determine if AIB treatment could protect intestinal cells from lethal HS in vitro, cells were treated with or without 10mM AIB, subjected to lethal HS, and allowed to recover for 24 hours at 37°C. Figure 2 shows 10mM AIB treatment increased cell survival 1.75 fold in HS cells (n=9, *p=0.02 vs. HS CT) via MTS assay. Other doses of AIB were previously investigated with 10mM showing maximal protection, consistent with dosing information found in recent literature. A 2mM and 5mM dose failed to provide protection in these cells (data not shown). All heat shocked groups were normalized to their non-HS controls to specifically observe the effects of the heat stress.

THR and AIB stabilize the F-actin cytoskeleton in HS cells

Cells were fixed and stained for F-actin (FITC shown in green), golgi (cy3 shown in red), and nuclei (DAPI shown in blue) for visualization of cell size, morphology and the cytoskeleton. Rigid, sharp F-actin filaments can be visualized in non-stressed control cells (Fig. 3A). The golgi and nuclei appear structurally normal. F-actin disruption clearly occurs following heat stress, as rigid actin fibers are no longer present (Fig 3A, top right image). The golgi and nuclei also appear compromised as their shape and size seem irregular and aggregated. Both THR and AIB treatment preserved the F-actin cytoskeleton as these cells appear similar to non-stressed controls with sharp, rigid actin fibers. The golgi and nuclear structures also appear to be preserved in these cells after HS with THR and AIB.

THR and AIB treatment increases cell size in stressed and non-stressed cells

Cells grown on 4 well cell chamber slides increased in cell size following THR treatment in both control and HS cells. Slidebook software was used to measure cell size based on average cell area (μm^2) for each treatment. Mask statistics from 50 cells per experimental group showed heat stress decreased cell size by 27% (p<0.0001 vs. non-stressed CTs) (Fig. 3B). THR increased size in non-stressed cells by 45% (*p=0.0003 vs. CT). In cells subjected to HS, THR increased cell size by 53% at 15min, and 75% at 30min (#p=0.0003, and **p=0.00004 respectively vs. HS controls). AIB increased cell size in HS cells by 40% at 15 min and 47% at 30 min (p=0.049 vs. HS controls). Interestingly, THR treatment was slightly more potent at affecting cell size and inducing cell swelling, than AIB.

THR and AIB enhance HSP25 and HSP70 expression in IEC18 cells following HS

Western blots show dramatic increases in HSPs with both THR and AIB treatments. As shown in Figure 4(A), THR treatment increased HSP25 and HSP70 levels more than 5 fold in HS cells (p<0.02 vs. HS alone) with only a 15 min treatment prior to injury. Figure 4(B) reveals AIB increased HSP70 nine-fold and HSP25 six-fold following HS vs. HS CT (p<0.03, and p<0.001, respectively). Relative densitometry is shown, (normalized to β -actin) for averages from 4 separate experiments.

THR increases cytoplasmic HSP25 in control cells

Cell lysates separated into nuclear and cytoplasmic fractions shows HSP25 was predominantly cytoplasmic in control cells. Figure 5(A) shows prior to HS, threonine can increase cytoplasmic HSP25 expression. 2mM threonine treatment increased cytoplasmic

HSP25 content by 50% ($p=0.04$ vs. CT cells, not shown), and at a 20mM concentration cytoplasmic HSP25 increased by 225% ($p<0.001$ vs. controls $n=3$). HS cells also showed a clear trend of increased HSP25 in the cytoplasm with THR treatment. All bands were normalized to β -actin. These are the first data showing threonine treatment can rapidly increase cytoplasmic HSP25 expression in non-stressed cells.

THR enhances nuclear content of HSP25 in HS cells

Since enhanced nuclear translocation of HSP25 has been shown to be associated with attenuated cellular apoptosis [18], we investigated HSP25 cellular localization in THR treated cells. Figure 5(B) shows heat stress causes nuclear translocation of HSP25 ($p=0.04$ vs. non-HS CT). THR treatment enhances this effect causing a significant increase in HSP25 nuclear content ($P<0.03$ vs. HS CT, $n=3$). All groups were normalized to β -actin.

AIB increases cytoplasmic HSP25 in control cells

Like THR, AIB treatment also increased cytoplasmic HSP25 in IEC-18 cells. Fig. 6(A) shows AIB increases HSP25 in non-stressed cells by 57% ($*p<0.05$ vs. CT cells). There is also a trend of AIB increasing cytoplasmic HSP25 in HS cells.

AIB does not increase nuclear HSP25 in HS cells

Interestingly, AIB treatment did not lead to an increase in nuclear HSP25 content as THR did during HS (Fig. 6B). Nuclear HSP25 was present in AIB treated cells during HS, but the quantity was not greater than HS cells without AIB.

THR, but not AIB, treatment decreases apoptosis in IEC-18 cells subjected to lethal heat stress

To further assess the mechanism of cellular protection afforded by THR, apoptosis was evaluated via active caspase 3 (CC3) in cells treated with and without THR. Cellular apoptosis, as measured by CC3 increased dramatically in cells subjected to heat stress. Fig. 7(A) shows THR decreased CC3 by 54% vs. HS CT ($*p<0.001$). Again, consistent with the lack of an effect to increase nuclear HSP25 levels, Fig. 7(B) shows AIB did not decrease CC3 expression in HS cells. All groups were normalized against β -actin.

DISCUSSION

To our knowledge, this is the first demonstration that the amino acid THR can enhance the expression of HSPs and prevent cellular injury and apoptosis. Currently, data on the potential therapeutic effects of THR administration are limited. Restriction of dietary threonine in rat models has been shown to significantly and specifically impair intestinal mucin synthesis which can compromise gut barrier function [20]. However no previous specific experiments have been performed to establish the potential for THR to protect against cellular injury.

Previous data has indicated osmotically acting amino acids can be cytoprotective following injury *in vitro* and *in vivo* [21], and our data suggest that THR may, at least in part, work through an osmotic-related mechanism. Cell size and F-actin stabilization were both affected

by THR treatment, suggesting THR has the ability to affect the cellular osmosensing pathway. THR also enhanced protective HSP70 expression in HS cells, and led to enhanced HSP25 expression, even in the absence of stress. This pre-injury induction of HSP25 may potentially “precondition” the cell and enable it to survive future stressors. In support of an osmotic-effect of amino acids such as THR on HSP expression and cell protection, the non-metabolizable amino acid analog AIB, demonstrated a similar effect to THR on HSP70 expression, cytoplasmic HSP25, and cellular protection via MTS assay.

A unique effect of THR from the non-metabolized amino acid, AIB, is that THR enhanced nuclear translocation of HSP25 in heat stressed cells which has been shown to be associated with decreased apoptosis [18]. Consistent with THR’s ability to increase nuclear HSP25, THR decreased cellular apoptosis, as measured by CC3. Although both AIB and THR’s induced cellular HSP expression, AIB demonstrated a somewhat smaller protective as measured via the MTS assay (1.75 fold increase in survival vs. 2.2 fold increase in THR treated cells). We conclude, THR protection appears mediated in part via it’s effect on cell swelling, as AIB, a non-metabolizable amino acid also enhanced HSP25, HSP70 and increased cell survival significantly as measured by the MTS assay. Treatment with AIB did not attenuate cellular apoptosis, as measured by CC3, or increase nuclear HSP25 levels, suggesting this protective effect of THR could be dependent on cellular metabolism of THR. The amino acid glutamine, another osmotically acting amino acid, protects cells via multiple pathways (ie. acting as a metabolic fuel, HSP inducer, precursor of the protective antioxidant, glutathione, etc.). It is possible that THR’s mechanism of protection is also multifaceted and involves activation of multiple cell swelling and osmotic pathways, including exerting a unique effect on the cellular localization of HSP25. Future research on the specific protective effect of THR versus AIB on necrotic versus apoptotic cell death would be useful to further understand potential mechanisms of THR-induced cellular protection. Additionally, as CC3 is only one intermediate in the apoptosis cascade, study of other apoptotic markers would assist in further defining if THR’s anti-apoptotic effect is related to metabolism versus osmotic mechanisms.

In conclusion, these are the first data showing that the amino acid, THR, can prevent cellular apoptosis and enhance cellular heat shock protein expression in an *in vitro* model of heat stress. THR’s ability to induce cell swelling and enhance HSP25 and HSP70 may be an integral component of THR’s mechanism of cellular protection. Further, THR ability to induce nuclear HSP25 translocation, and specifically reduce cellular apoptosis, may depend on the cellular metabolism of THR.

Although the concentrations of THR utilized in this trial are above those commonly found in plasma, concentrations of other osmotically acting amino acids (glutamine) in the 2–10 mM range been found to be easily attainable in an *in vivo* model without adverse consequences to the organism[22]. Further, local gut concentrations in the 10–20 mM range are easily attainable via local administration, such as with an enema delivery. Thus, it is possible that THR could be employed clinically as protective agent against intestinal injury. THR treatment could potentially have significant clinical implications in both prevention and therapeutic intervention in a range of acute and chronic inflammatory diseases.

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Figure 1. THR treatment increases cell survival in heat stressed IEC-18 cells in a dose dependent manner

Cells subjected to lethal heat stress were protected by increasing doses of THR via MTS assay. Cell survival increased 1.2 fold, 1.6 fold and 2.2 fold with 5mM, 10mM and 20mM THR (respectively)(* $p < 0.04$ vs. HS CT, $n = 3$). An iso-osmotic control amino acid cocktail (20mM valine, alanine and phenylalanine) failed to provide protection from lethal heat stress. Results were normalized to non-HS controls for each experimental group.

Figure 2. AIB treatment increases cell survival in heat stressed IEC-18

Cells subjected to lethal heat stress were also protected by the non-metabolizable amino acid analog, AIB. 10mM AIB increased cell survival 1.75 fold (* $p < 0.05$ vs. HS CT, $n=3$).

Results were normalized to non-stressed controls for each experimental group.

Figure 3. Both THR and AIB stabilize the F-actin cytoskeleton and increases cell size in HS cells IEC18 cells were grown on 4 well cell chamber slides and subjected to a non-lethal heat stress (43° C) for either 15 or 30 minutes. Cells were fixed and stained with Alexa Fluor 488 phalloidin for F-actin (green), Cy3-conjugated wheat germ agglutinin (WGA) for golgi (red), and bis-benzimide for nuclei (blue). Morphology and organelle structures were analyzed (A). Cell shrinkage and F-actin disruption clearly occur with heat stress as cells appear smaller and less rigid. The distribution of the golgi apparatus appears to be compromised as the WGA (shown in red) is aggregated and no longer evenly distributed around the nuclei. The nuclei appear smaller and are “encapsulated” but the shrunk golgi. Cells that were treated 15 minutes prior to HS with either THR or AIB appear larger and the F-actin fibers look functional and rigid. The integrity of the golgi and nuclei also appear to be preserved in these cells. For cell size (B), cells were masked, and the average cell area (μ m²) was determined for each group using Slidebook imaging software. Threonine increased cell size by 45.2% in control cells even in the absence of HS (*p=0.0003 vs. CT). In HS cells, threonine treatment increased cell size by 52.6% at 15min, and 75.2% at 30min (#p=0.0003, and **p=0.00004 respectively vs. non-HS controls). AIB, increased cell size in HS cells by 40% at 15 min and 47% at 30 min. (p<0.05 vs. HS controls). Cell area was measured for a minimum of 50 cells per experimental group.

Figure 4. THR and AIB increase HSP25 and HSP70 IEC18 cells

(A) Heat stressed cells treated with 20mM THR increased HSP25 and HSP70 expression more than 5 fold via western blot (* $p < 0.02$ vs. HS CT cells). (B) AIB also had the ability to enhance HSP25 and HSP70 expression after HS in these cells (* $p < 0.03$ vs. HS CT). Relative densitometry is shown from an average of 4 experimental runs (normalized to β -actin).

Figure 5. THR increases cytoplasmic HSP25 in stressed and non-stressed cells

Cell lysates separated into nuclear and cytoplasmic fractions shows HSP25 was predominantly cytoplasmic in control cells. Prior to HS, THR can increase cytoplasmic HSP25 expression (A). 20mM threonine treatment increased cytoplasmic HSP25 content by 225% ($p < 0.001$ vs. controls). In HS cells, THR treatment trended towards an increase in cytoplasmic HSP25. During HS (B), HSP25 translocated to the nucleus and THR treatment enhanced this effect (* $P < 0.03$ vs. HS CT, $n = 3$). All bands were normalized to β -actin. (Representative blot from 3 separate experiments shown).

Figure 6. AIB increases cytoplasmic HSP25 but does not enhance it's nuclear translocation
 (A) AIB increased cytoplasmic HSP25 by 57% in non-stressed IEC-18 cells (* $p < 0.05$ vs. CT cells). Like THR, we observed a trend of increased cytoplasmic HSP25 during HS. However, AIB did not have the same effect on nuclear translocation of HSP25 during HS (B) as nuclear HSP25 content was equal in HS and HS+AIB groups. All groups were normalized to β -actin. (Representative blot from 3 separate experiments shown).

Figure 7. THR but not AIB treatment decreases apoptosis in IEC-18 cells subjected to lethal heat stress

Western blot analysis was used to determine cleaved caspase-3 (CC3) levels, as this enzyme can identify apoptotic cell populations. HS activates the apoptotic pathway in IEC18 cells and THR treatment decreased this effect by more than 50% ($p < 0.04$ vs. HS CT, $n = 3$).

Consistent with HSP25 nuclear translocation, cells treated with AIB did not show a decrease in CC3 expression during lethal HS. All groups were normalized against β -actin.

(Representative blot from 3 separate experiments shown).