Published in final edited form as:

Gastroenterology. 2007 December ; 133(6): 1893–1904.

Translational inhibition of colonic epithelial heat shock proteins by interferon-gamma and TNF-alpha in intestinal inflammation

Shien Hu¹, Mae J. Ciancio^{1,*}, Maor Lahav², Mikihiro Fujiya^{1,3}, Lev Lichtenstein^{1,4}, Shrikant Anant⁵, Mark W. Musch¹, and Eugene B. Chang¹

¹ The Martin Boyer Laboratories, Department of Medicine, University of Chicago IBD Research Center, Chicago, IL

² Department of Gastroenterology, Chaim Sheba Medical Center, Affiliated to the Tel-Aviv University, Tel-Hashomer, Israel

³ Third Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan

⁴ Department of Gastroenterology, Soroka University Medical Center, Beer-Sheva, Israel

⁵ Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK

Abstract

Background & Aims—Inducible heat shock proteins (iHsp), Hsp25/27 and Hsp70, play essential roles in protecting cells against stress and, in intestinal mucosal inflammation, potentially lessening the extent and severity of injury. We examined the expression and regulation of iHsp in human and experimental inflammatory bowel diseases (IBD) and *in vitro*.

Methods—iHsp expression and regulation were assessed in normal and IBD colonic biopsies, IL-10^{-/-} mice, and YAMC colonic epithelial cells by immunohistochemistry, Western blot and real-time PCR. Phosphorylation of double-stranded RNA-dependent protein kinase (PKR) and eukaryotic initiation factor- α (eIF-2 α) was determined by Western blot.

Results—Hsp25/27 and Hsp70 levels were selectively reduced in areas of active mucosal inflammation associated with human IBD and IL-10^{-/-} mice with colitis. Wild-type mice treated *in vivo* with IFN- γ +TNF- α also demonstrated reduced colonic Hsp25/27 and Hsp70. In YAMCs, IFN- γ +TNF- α inhibited heat induction of Hsp25/27 and Hsp70, an effect not associated with changes in iHsp mRNA or protein half-lives, but caused by suppressed *de novo* iHsp synthesis. IFN- γ +TNF- α co-treatment activated PKR, resulting in phosphorylation and inactivation of eIF-2 α , an essential factor in protein translation. These effects were not due to induced apoptosis and could be negated by PKR-inhibitor and siRNA to PKR. Increased phosphorylation of PKR and eIF-2 α were also observed in active IBD tissues.

Conclusions—Mucosal inflammation is associated with iHsp down-regulation, an effect that appears mediated by translational down-regulation by pro-inflammatory cytokines. In the context of IBD, we propose that this mechanism contributes to the severity, extent, and persistence of inflammation-induced mucosal injury.

Correspondence: Eugene B. Chang, M. D., Martin Boyer Professor of Medicine, The University of Chicago, 5841 S. Maryland Ave. Room G705, MC 6084, Chicago, IL, 60637, Phone: 773-702-6458 Fax: 773-702-2281, Email: echang@medicine.bsd.uchicago.edu.

*Co-first author – had equal contribution in primary execution of the studies.

Disclosure information: None of the authors have any conflict of interest to disclose.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

The extent and severity of mucosal lesions associated with IBD are dependent on the balance between aggressive, cell-destructive processes and counteracting cytoprotective and wound reparative mechanisms. In the context of IBD, investigations of the latter are few and strategies to promote cellular protection of inflamed mucosa have been inadequately explored. In this regard, inducible heat shock proteins (iHsp) warrant further investigation because of their known cytoprotective properties, especially Hsp25 and Hsp70. In humans, Hsp27 is the homolog of Hsp25. Inducible Hsps are part of a highly conserved class of proteins that act to protect cellular structure and function.^{1–6} In normal intestinal mucosa, Hsp25/27 and Hsp70 are expressed “constitutively” by surface epithelium in the colon and stomach, areas that are continuously subjected to inducing signals from enteric flora and short chain fatty acids.^{7–9} Maintained expression of Hsp25/27 and Hsp70 contributes to colonic epithelial resistance to bacterial toxins and inflammation-associated stress.^{4, 10, 11}

We report here that colonic epithelial expression of Hsp25/27 and Hsp70 are significantly and selectively reduced in actively inflamed mucosa from both human IBD biopsies and IL-10^{-/-} mice with spontaneous colitis. A significant part of this effect is mediated by the pro-inflammatory cytokines, IFN- γ and TNF- α . We demonstrate that IFN- γ and TNF- α co-treatment down-regulates colonic epithelial Hsp25/27 and Hsp70 translation without altering mRNA levels or accelerating protein degradation. We hypothesize that IFN- γ and TNF- α co-treatment promotes tissue inflammation by stimulating the phosphorylation of the RNA-dependent protein kinase (PKR), which phosphorylates eukaryotic initiation factor- α (eIF-2 α). This results in reduced protein translation under conditions of stress, as observed with IBD and experimental colitis. However, inhibited translation of iHsp is particularly pronounced and out of proportion to that of other proteins, raising the possibility of additional translation regulatory mechanisms that are set into play in the context of mucosal inflammation. The down-regulated expression of these key cytoprotective proteins would render the intestinal mucosa more vulnerable to inflammation-associated stress and injury.

Materials and Methods

Human biopsies

Colonic pinch biopsies were obtained from normal, Crohn's Disease (CD) and Ulcerative Colitis (UC) patients undergoing colonoscopy at the University of Chicago Medical Center under a protocol approved by the Institutional Review Board. Informed consent was granted prior to obtaining tissues. After removal, biopsies were placed immediately on ice and rinsed in ice-cold phosphate buffered saline (PBS) prior to cell lysis for RNA and protein extraction, as detailed below. Tissues from the same patients were also fixed in neutral buffered formalin for hematoxylin/eosin or immunohistochemistry staining. Hematoxylin/eosin-stained sections were evaluated pathologically to distinguish actively inflamed tissue from uninvolved in a blinded manner using a scoring system as previously described.¹²

Mouse colitis and in vivo cytokine studies

Male wild-type or IL-10^{-/-} gene-deficient mice on the C57Bl/6 background (Jackson Laboratories, Bar Harbor, ME) were bred under conventional conditions and allowed free access to food and water. Mice were sacrificed at 20 weeks.¹³

For treatment with cytokines, adult C57Bl/6 male mice (20–25gm) were given one intraperitoneal injection of IFN- γ (2500U; PeproTech, Rocky Hill, NJ), TNF- α (1000ng; PeproTech) or a combination of IFN- γ and TNF- α 48 hours prior to sacrifice.

For animal studies, colonic mucosa was scraped and homogenized for RNA and protein extraction as described below. Tissues from the same samples were also fixed in formalin for hematoxylin/eosin staining. States of inflammation were assessed as above.¹²

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Cell culture

Conditionally-immortalized young adult mouse colonic epithelial cells (YAMC) were used, which was a generous gift from Dr. Robert Whitehead, Vanderbilt University, Nashville, TN. YAMCs were grown at 33°C as previously described.¹⁴ Cells were fed IFN- γ -free medium and switched to the non-permissive conditions of 37°C for 16–24 hours. During this time, SV40 large T antigen is no longer produced and any remaining protein misfolds because of a temperature-sensitive mutation at amino acid 58 (tsA). During this period, cells were treated with the cytokines murine IFN- γ (200U/ml), murine TNF- α (100ng/ml) or a combination of IFN- γ and TNF- α for 8 hours prior to heat shock and harvest. For *in vitro* assessment of iHsp production, cells were heated to 42°C for 23 minutes and returned to 37°C for 120 minutes, providing a maximal iHsp induction.¹⁵ To inhibit PKR and eIF-2 α phosphorylation, the PKR inhibitor (PKR-I, 30nM, Calbiochem, San Diego, CA) was added 30 minutes before heat induction. PKR expression was also inhibited by a Stealth silencing oligonucleotide (NM_011163, 5'-GGACCUCCACAUGACAGAAGGUUUA-3') designed by the RNAi Designer software (Invitrogen, Carlsbad, CA). As a negative control, an oligonucleotide with the same base composition but different sequence was used. Oligonucleotides were diluted with Optimem (Invitrogen) to a final concentration of 20nM. Silentfect reagent (BioRad, Hercules, CA) was diluted to 2 μ l/mL separately with Optimem, mixed with diluted oligos, allowed to complex for 30 minutes at room temperature, and added to the cells for 24 hours before harvest. Decreased PKR expression was confirmed by Western blotting.

In all experiments for harvest, cells were rinsed 2 times and scraped into ice-cold PBS. Cells were pelleted (14,000g $\times$ 20 sec) and lysed for RNA and protein extraction as described below.

Immunohistochemical staining for iHsp

Sections of human biopsies and mouse tissues were fixed in 10% (vol/vol) neutral buffered formalin, embedded in paraffin, and 4 μ m cross-sections cut. Sections were stained for human Hsp27, mouse Hsp25 or Hsp70 using the Vecastain Elite ABC kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions modified to use citrate-microwave antigen recapture as previously described using antibodies described below.^{8,16}

Western blot analysis

Human biopsies, mouse mucosal samples, or YAMCs were homogenized in 10mM Tris pH 7.4, 5mM MgCl₂, complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), 50U/ml DNase (Amersham, Piscataway, NJ) and 50U/ml RNase (Ambion Inc, Austin, TX). Ten μ l was removed for protein analysis using the bicinchoninic acid method. To the remainder, 3X Laemmli stop solution was added and heated to 65 °C for 10 minutes.

Twenty μ g protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes in 25 mM Tris, pH 8.8; 192 mM glycine; 15% vol/vol methanol. Membranes were blocked with 5% wt/vol nonfat dry milk in tween-tris buffered saline (TTBS). Primary antibodies, for Hsp25 (SPA801; Stressgen), Hsp27 (SPA800; Stressgen), Hsp70 (SPA810; Stressgen), Hsc70 (SPA815; Stressgen), β -actin (AAN01; Cytoskeleton, Denver, CO), villin (610359; BD Biosciences, San Jose, CA), eIF-2 α (9722; Cell Signaling, Beverly, MA), phosphorylated eIF-2 α (Ser51) (9721; Cell Signaling), PKR (07-151; Upstate,

Charlottesville, VA) or phosphorylated PKR (Thr446) (07-532; Upstate), were added and incubated overnight at 4°C. Membranes were washed with TTBS, incubated with horseradish peroxidase-conjugated species-appropriate secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, and developed using an enhanced chemiluminescence system (Supersignal; Pierce, Rockford, IL).

Quantification of images was done by scanning densitometry using NIH Image J 1.54 software (National Institutes of Health, Bethesda, Maryland).

Analysis of apoptosis

To determine whether apoptosis occurred in YAMCs, analysis of the cleavage products of poly-ADP ribose polymerase (PARP) and caspase 3 were determined.^{17, 18} YAMCs were treated with IFN- γ and TNF- α for 8 hours and heat shocked as described previously. Cells treated with staurosporine (1 μ M) for 3 hours were analyzed as a positive control for apoptosis. The cleaved caspase 3 and cleaved PARP protein was detected by Western blotting using antibodies specific to caspase3 (Cell Signaling #9662, Beverly, MA) and PARP (Cell Signaling #9532).

Real-time PCR for Hsp mRNA

Total RNA was extracted from human colonic biopsies, mouse intestinal mucosal samples, or pelleted YAMCs by Trizol (Invitrogen, Grand Island, NY) according to the manufacturer's instruction. Complementary DNA was synthesized using SuperScript II (Invitrogen) and random hexonucleotide primer. The mouse Hsp25 (L07577), mouse Hsp70 (NM_010478), human Hsp27 (NM_001540) and human Hsp70 (NM_005346) sequences were downloaded from GenBank. The sense and antisense primers for mouse Hsp25 are: 5'-GAAGAAAGGCAGGACGAACA-3' and 5'-CCTCAGGGGATAGGGAAGAG-3'; for mouse Hsp70: 5'-GGCTGATCGGCCGCAAGTT-3' and 5'-GGAAGGGCCAGTGCTTCAT-3'; for mouse GAPDH: 5'-GGCAAATTC AACGGCACAGT-3' and 5'-AGATGGTGATGGGCTTCCC-3'; for human Hsp27: 5'-CGTGTCCTGGATGTCAACC-3' and 5'-TCTCCACCACGCCATCCT-3'; for human Hsp70 5'-ACTGCCCTGATCAAGCGC-3' and 5'-CGGGTTGGTTGTCGGAGTAG-3'. Real-time PCR was performed in an iCycler (Bio-Rad) using iQSYBR Green PCR supermix (Bio-Rad). The two-step quantification cycling protocol was used. The Ct value is defined as the cycle number at which the fluorescence crosses a fixed threshold above the baseline. As a relative quantitation, fold changes were measured using the $\Delta\Delta$ Ct method. For each sample, the Ct value of iHsp mRNA was measured and compared to the GAPDH endogenous control as Δ Ct, (Δ Ct = Ct_{Hsp} - Ct_{GAPDH}). The fold change of iHsp mRNA in the unknown sample relative to control sample was determined by $2^{-\Delta\Delta$ Ct}, where $\Delta\Delta$ Ct = Δ Ct_{Unknown} - Δ Ct_{Control}.¹⁹

Incorporation of ³⁵S-methionine or ³H-leucine

To measure translation, ³⁵S-methionine or ³H-leucine labeled amino acid incorporation was used. After cytokine treatment and heat induction, YAMCs were returned to 37°C 5% CO₂ incubator, incubated in either serum-free methionine-free RPMI (Biosource International, Rockville, MD) with 50 μ Ci/ml of ³⁵S-methionine (Express protein labeling mix, NEG072007MC, Perkin Elmer, the mixture contains 73% L-³⁵S-methionine and 22% L-³⁵S-cysteine) or serum-free leucine-free RPMI with 70 μ Ci/ml of ³H-leucine (L-4,5-³H-leucine, MT672, Movarek Biochemicals, Brea, CA) for 60 minutes immediately before harvest. Cells were harvested and pelleted as previously described at 60, 90, and 120 minutes after heat induction. Pelleted cells were lysed in RIPA buffer (50mM Tris pH8.0, 150mM NaCl, 0.1% SDS, 1% Triton-X, 0.5% Na deoxycholate, 1mM DTT, protease inhibitors, 100U/ml RNASIN).

As a measure of total protein synthesis, ^{35}S -methionine or ^3H -leucine incorporation into trichloroacetic acid (TCA)-precipitated material was determined. Twenty μl cell lysate was incubated in 2ml 20% wt/vol TCA for 30 minutes. TCA precipitates were filtered onto HA filters (Millipore Corp, Bedford, MA) and washed twice with 10% TCA. Filters were solubilized and radioactivity quantified by liquid scintillation counting.

To immunoprecipitate Hsp25, Hsp70 or β -actin, lysates were incubated overnight at 4°C with anti-Hsp25, anti-Hsp70 or anti- β -actin antibody (same as used in Western blot) coupled to Protein A-Sepharose (Sigma). Immunoprecipitates were pelleted by centrifugation, washed four times with RIPA buffer, boiled for 5 minutes in 2X Laemmli buffer, and separated on SDS-PAGE. To measure incorporation of ^{35}S into immunoprecipitated Hsp70 or β -actin, gels were treated for fluorography (soaking in 10% TCA for 15 minutes followed by 1M sodium salicylate for 15 minutes). Gels were dried and exposed to film at -80°C for 6–12 hours. Image quantification of Hsp70 and β -actin was done as described above.¹⁵ To measure incorporation of ^3H into immunoprecipitated Hsp25, gels were sliced around 25kDa and solubilized in 0.5ml TS-2 tissue solubilizer (RPI Corp., Mt. Prospect, IL) overnight. The ^3H in each sample was quantified by liquid scintillation counting.

Pulse-chase to determine Hsp70 degradation

To measure the rates of Hsp70 degradation, Hsp70 was pulse labeled with ^{35}S -methionine in methionine free medium for 60 minutes after heat induction, and chased with complete medium including all amino acids non-radiolabeled. Cells were treated with cytokines for up to 8 hours and harvested at 2, 4 and 8 hours after pulse-labeling. Hsp70 was immunoprecipitated and detected by fluorographs of SDS-PAGE as described above.

Statistical Analysis

Results are presented as the means $\pm$ SEM for the indicated number of experiments. The results of multiple experiments were analyzed by using paired Student's *t* test or analysis of variance using a Bonferroni correction for multiple comparisons.

Results

Colonic expression of Hsp27 and Hsp70 is down-regulated in Crohn's Disease and Ulcerative colitis

Colonic tissues from patients with no history of IBD (Control) demonstrated significant Hsp27 and Hsp70 expression in surface, but not crypt epithelial cells (Figure 1A). In contrast, specimens from areas actively inflamed from CD showed significantly reduced Hsp27 and Hsp70 staining in the surface epithelial cells (Figure 1A). Specimens from active UC patients demonstrated a comparable reduction in Hsp27 and Hsp70 compared to control areas (data not shown).

Western blot analyses were performed on mucosal biopsies to provide a more quantitative assessment of Hsp27 and Hsp70 expression. Hsp27 and Hsp70 levels were lower in the actively inflamed (IN) specimens compared to uninvolved (UN) areas obtained from the same patients (Figure 1B). In contrast, Hsc70, β -actin and the epithelial cell marker, villin, were expressed at the same level in control and colitis samples.

Inducible Hsp mRNA abundance was measured by real-time PCR (Figure 1C). No significant differences in Hsp27 and Hsp70 mRNA levels were detected, suggesting that decreased iHsp expression associated with inflamed mucosa was not due to inhibited gene transcription, but rather to a post-transcriptional mechanism.

Colonic expression of Hsp25 and Hsp70 is significantly and selectively down-regulated in IL-10^{-/-} colitis

We next determined if inflammation-associated iHsp down-regulation is unique to human IBD or whether similar responses could be observed in a murine model of chronic colitis. IL-10^{-/-} mice develop colitis spontaneously. The mucosal inflammation is discontinuous, T-cell dependent, and transmural, all features that resemble human CD.¹³ In our animal facility, approximately 20% of IL-10^{-/-} mice develop inflammation and injury, as demonstrated by histological grading, weight loss, and appearance of diarrhea at 20 weeks. In mice with intestinal inflammation (IN), colonic Hsp25 and Hsp70 were significantly and selectively down-regulated compared to wild-type controls (WT) and IL-10^{-/-} mice without intestinal inflammation (UN) (Figure 2A). Expression of Hsc70, β -actin and villin were unchanged in wild-type and IL-10^{-/-} mice. No significant decreases in Hsp25 or Hsp70 mRNA levels were detected in IL-10^{-/-} mouse colonic mucosa (Figure 2B), indicating that post-transcriptional regulation might be involved.

Pro-inflammatory cytokines down-regulate colonic Hsp25 and Hsp70 in vivo

To determine if cytokines can contribute to the changes in Hsp25 and Hsp70 expression, C57Bl/6 mice were given an intraperitoneal injection of either IFN- γ (2500U), TNF- α (1000ng), or both IFN- γ and TNF- α (IFN- γ +TNF- α). Only IFN- γ +TNF- α co-treatment significantly and selectively reduced Hsp25 and Hsp70 levels in colonic mucosa without altering Hsc70, β -actin and villin (Figure 3A). No significant differences in Hsp25 and Hsp70 mRNA levels were detected, which correlated with findings observed in human biopsies (Figure 1C) and IL-10^{-/-} mice (Figure 2B).

Pro-inflammatory cytokines down-regulate colonic epithelial Hsp25 and Hsp70 expression in vitro

To investigate mechanisms responsible for the inflammation-induced reduction in colonic iHsp expression, YAMCs were used to examine the direct cellular effects of specific pro-inflammatory cytokines on Hsp25 and Hsp70 production.^{11, 16} YAMC cells were pre-treated with either IFN- γ (200U/ml), TNF- α (100ng/ml), or IFN- γ +TNF- α co-treatment for 8 hours prior to inducing iHsp production by heat shock. YAMCs pre-treated with IFN- γ +TNF- α demonstrated a significant reduction in Hsp25 and Hsp70 relative to cells that were only heat shocked (HS-control) (Figure 4A). IFN- γ and TNF- α alone did not affect heat induced iHsp production.

To determine if cytokine pre-treatment reduced Hsp25 and Hsp70 transcription, mRNA levels were measured by real-time PCR (Figure 4B). Heat shock rapidly induced iHsp mRNAs, reaching maximal level in 90 minutes and returning to near baseline levels within 4 hours. The transience of the increased iHsp mRNA contrasts to the prolonged expression of iHsp protein and likely results from a feedback transcriptional down-regulation, as previously described.²⁰ As Hsp70 protein expression increases, it binds the transcription factor, heat shock factor-1, preventing transcription of heat inducible genes.²⁰ Treatment with IFN- γ +TNF- α did not alter Hsp25 and Hsp70 mRNA responses induced by heat shock. Thus, the reduction in iHsp production subsequent to IFN- γ +TNF- α co-treatment arises from regulation at the translational or post-translational level.

Pro-inflammatory cytokines do not stimulate apoptosis in vitro within the time frame of the experimental design

To determine if the effects of IFN- γ +TNF- α were due to induction of apoptosis in the YAMCs, the cleavage and activation of two apoptotic enzymes were determined (same samples analyzed in Figure 4A). Caspase 3 is an asparagine protease that mediates numerous apoptotic signals,

including poly ADP ribose polymerase (PARP) cleavage. PARP is involved in cellular disassembly and serves as a marker of apoptotic cells.^{17, 18} Heat shock (HS) causes a small degree of caspase 3 and PARP activation within the time frame of the experiments (Figure 4C). However, IFN- γ +TNF- α pretreatment for 8 hours did not enhance activation of either caspase 3 or PARP. The positive control, staurosporine (1 μ M $\times$ 3 hours, (+)), resulted in both PARP and caspase 3 cleavage.

Pro-inflammatory cytokines selectively inhibit *de novo* protein synthesis of Hsp25 and Hsp70 in YAMCs

Radiolabeled amino acid incorporation into Hsp25 and Hsp70 was used to determine the influence of cytokine treatment on *de novo* protein synthesis.¹⁶ ³⁵S-methionine or ³H-leucine was added to cells at various time points post-heat shock. Cells were harvested 60 minutes later to determine ³⁵S or ³H incorporation. Under basal conditions, IFN- γ +TNF- α co-treatment for 8 hours did not alter the rate of incorporation of radiolabeled amino acids into total protein (Figure 5D & 5F). Heat shock caused a large, but transient decrease in radiolabeled amino acid incorporation, with significant recovery seen 60–120 min after heat shock. Pretreatment with IFN- γ +TNF- α did not alter the heat shock induced decrease in ³⁵S or ³H amino acid incorporation, however, the rate of return back to basal level of incorporation was slowed by the cytokine pretreatment (see 60–120 min interval, Figures. 5D and 5F).

To determine cytokine effects on Hsp70 synthesis, Hsp70 was immunoprecipitated, run on SDS-PAGE and analyzed by fluorography. Little or no synthesis of ³⁵S-labeled Hsp70 was observed prior to heat shock (Figure 5A). Within the first 60 minutes after heat shock, ³⁵S-labeled Hsp70 was greater in control cells compared to cells pre-treated with IFN- γ +TNF- α . Decreased ³⁵S-labeled Hsp70 in IFN- γ +TNF- α treated cells continued to be observed for the next 2 hours compared to controls (Figures 5A, 5B & 5C). With IFN- γ +TNF- α co-treatment, incorporation of ³⁵S-amino acids into Hsp70 was substantially less than that of β -actin (Figure. 5B).

Since Hsp25 has only one methionine, incorporation of ³H-leucine was used to measure Hsp25 synthesis. Immunoprecipitated Hsp25 with ³H-leucine incorporation was separated on SDS-PAGE and measured by scintigraphy. In cells pretreated with IFN- γ +TNF- α , ³H-leucine incorporation for both time intervals was significantly less than that of control cells (Figure 5E). These data, along with the lack of changes in Hsp25 and Hsp70 mRNA, suggest that IFN- γ +TNF- α inhibit iHsp synthesis at the level of protein translation.

Hsp70 degradation is not affected by pro-inflammatory cytokines

To determine if cytokines reduce Hsp70 protein expression by accelerating its degradation, Hsp70 was pulse-labeled for 1 hour after heat induction using ³⁵S-methionine and chased with medium with non-radioactive amino acids. Cells were treated with IFN- γ +TNF- α immediately after pulse-labeling and harvested at 2, 4 or 8 hours (Figure 6A). Labeled Hsp70 decreased by 20% by 8 hours, which was not altered in cytokine treated cells, suggesting no effect on degradation (Figure 6B&C).

Pro-inflammatory cytokines activate PKR and inhibit eIF-2 α -dependent protein translation

The above studies suggest a cytokine-induced inhibition of mRNA translation underlying reduced Hsp25 and Hsp70. To explore this further, phosphorylation of the translational regulators PKR and eIF-2 α were assessed. Under various stresses, PKR is activated by phosphorylation at Thr446 & Thr451, which then phosphorylates eIF-2 α at Ser51 to regulate protein translation. Phosphorylation of eIF-2 α prevents its ability to load the starting tRNA-Met into the elongation complex.²¹ IFN- γ +TNF- α co-treatment followed by HS enhanced phosphorylation of PKR and eIF-2 α (p-PKR and p-eIF-2 α) in YAMCs without altering the

levels of either protein (Figure 7A). To assess the relevance of p-PKR on cytokine-induced eIF-2 α phosphorylation and iHsp reduction, cells were pretreated with an inhibitor of PKR.²² The PKR inhibitor (PKR-I) treatment blocked IFN- γ +TNF- α induced phosphorylation of PKR and subsequent eIF-2 α phosphorylation in HS-treated conditions. By reducing p-eIF-2 α , the inhibition of heat induced iHsp was partially reversed. These findings support the concept that IFN- γ +TNF- α pre-treatment activates PKR to stimulate eIF-2 α phosphorylation in response to HS, which further inhibits iHsp mRNA translation.

As further support for the role of PKR in mediating IFN- γ +TNF- α down-regulation of iHsp, PKR expression was inhibited using silencing RNA (Figure 7B). PKR expression was decreased by over 80% in cells where a specific siRNA (si-PKR) was used, but not a control oligonucleotide with the same base composition but different sequence (si-C). In cells with reduced PKR expression, IFN- γ +TNF- α did not stimulate phosphorylation of eIF-2 α , and were unable to down-regulate heat induction of iHsp. In contrast, treatment with control oligonucleotide had no effect on IFN- γ +TNF- α actions.

Increased phosphorylation of PKR and eIF-2 α in human and experimental colitis

To determine if PKR activation occurs *in vivo*, phosphorylation of PKR and eIF-2 α were measured in the same colonic mucosa samples of IBD patients and IL-10^{-/-} mice as previously analyzed in Figures 1 and 2. Increased phosphorylation of PKR and eIF-2 α were observed in actively inflamed samples (inflamed, IN) compared to the uninvolved samples (uninflamed, UN) from both CD and UC patients and control biopsies from patients with no history of IBD (Control) (Figure 8A). Phosphorylation of PKR and eIF-2 α were also increased in mucosa from IL-10^{-/-} mice with colitis (inflamed, IN) compared to the samples from wild-type mice (WT) and IL-10^{-/-} mice without colitis (uninflamed, UN), while levels of total PKR and eIF-2 α were not changed (Figure 8B).

Discussion

Hsp25/27 and Hsp70 are constitutively expressed by surface epithelial cells of the colon that have direct contact with potentially harmful enteric microorganisms.^{8, 9} Several reports have demonstrated the importance of iHsp in protecting intestinal epithelial cells from colitogenic and infectious agents both *in vivo* and *in vitro*.^{7, 15} In light of their pivotal role in maintaining intestinal homeostasis, our findings of reduced surface Hsp25/27 and Hsp70 expression in areas of mucosal inflammation in human IBD (Figure 1) and experimental colitis (Figure 2) were unanticipated. A previous report had suggested that in UC, but not CD, that areas of mucosa without inflammation as well as areas with inflammation demonstrated increased expression of Hsp70.²³ The antibodies used by these investigators were different than those used in this study and their quantification relied on immunohistochemical technology. Because the finding of decreased iHsp expression in both UC and CD biopsies was unexpected, we further evaluated this relationship in the IL10^{-/-} model of intestinal inflammation. These data confirmed those in IBD, showing decreased colonic mucosal Hsp25 and Hsp70 expression. Decreased iHsp expression could render the mucosa more susceptible to inflammatory stress. We were also intrigued with the finding that there were no measurable changes in the respective mRNA levels of iHsp in tissues from actively involved areas of human IBD and in experimental colitis scrapings, despite significant decreases in iHsp protein (Figures 1C and 2B). These results suggested that the observed selective down-regulation of iHsp occurs at a post-transcriptional level.

As increased levels of pro-inflammatory cytokines are present during human IBD and experimental colitis^{24, 25}, we examined the effects of two potent pro-inflammatory agents, IFN- γ and TNF- α , on iHsp protein and mRNA levels both *in vivo* and *in vitro*. Systemic administration of IFN- γ and TNF- α together, but not alone, down-regulated colonic mucosal

Hsp25 and Hsp70 production without measurable decreases in mRNA levels (Figure 3) in a manner reminiscent of events observed in inflamed mucosa (Figure 1 & 2). This cytokine-induced inhibitory effect was relatively specific, as it was not observed with other cytokines, such as IL-10, IL-2 and IL-6 (data not shown). Similar cytokine effects on iHsp expression were recapitulated in an *in vitro* approach using the YAMC colonic epithelial cell line (Figure 4A). IFN- γ and TNF- α co-treatment had no effect on HSF-1 binding to HSE in response to thermal stress (data not shown), nor on Hsp25 or Hsp70 mRNA abundance (Figure 4B), or protein degradation (Figure 6). However, the cytokine combination decreased *de novo* Hsp25 and Hsp70 protein synthesis, consistent with translational inhibition of protein synthesis (Figure 5).

Translational regulation is important under cellular stress conditions, because it permits an immediate and rapid response to changes in physiological condition. A variety of stimuli, including ultraviolet irradiation, viral infection and oxidative stress, affect protein translation by activating distinct protein kinases, including PKR, GCN2, heme-regulated eIF-2 α kinase (HRI) and plasmic reticulum-resident kinase (PERK).^{26, 27} These proteins modulate eIF-2 α activity through phosphorylation. Phosphorylation of eIF-2 α reduces eIF-2-GTP formation from eIF-2-GDP by competitive inhibition, thus interfering with the formation of the initial ribosomal complex.^{27, 28, 29}

We found that IFN- γ +TNF- α co-treatment stimulated phosphorylation of PKR and eIF-2 α , an effect that was blocked by either PKR-I, a specific PKR inhibitor, or siRNA specific to PKR (Figure 7). PKR-I can also partially rescue iHsp induction inhibited by IFN- γ +TNF- α (Figure 7A). Using a more selective and potent approach with siRNA silencing of PKR, we found almost complete rescue of the cytokine inhibition of iHsp (Figure 7B).

Consistent with the *in vitro* findings, the activation of PKR and following increased eIF-2 α phosphorylation were detected in the colonic biopsies from active CD and UC patients (Figure 8A), and in mucosa from IL-10^{-/-} colitis (Figure 8B) compared to those from non-inflamed areas and normal controls. Thus, in a pro-inflammatory cytokine milieu, such as that reported during IBD^{24, 25}, PKR is activated in inflammation-stressed intestinal epithelial cells, eIF-2 α is hyperphosphorylated and ribosomal complex formation is inhibited.

Similar findings involving translational regulation are likely to be found with other cellular proteins. For example, the synthesis of β -actin was also decreased after this treatment (Figure 5B), but to a much lesser extent than the iHsp. GCN4 expression has also been reported to be selectively regulated following phosphorylation of eIF-2 α .²⁶ The basis for this selectively larger decrease in iHsp mRNA translation is not entirely clear, because the activation of the PKR-eIF-2 α pathway does not impart any specificity. However, a number of mechanisms may also be involved. The rate of mRNA translation, for example, can be regulated by microRNAs (miRNAs) or RNA binding proteins, both of which may bind the 5' or 3' untranslated regions (UTR) of mRNAs.³⁰⁻³⁴ MicroRNAs are short RNAs from non-coding regions of the genome that either degrade or disrupt translation of mRNA transcripts post-transcriptionally by base-pairing with their mRNA targets.^{30, 31} It is possible that the binding of miRNAs or RNA binding proteins to iHsp mRNA is activated or the levels of miRNAs or RNA binding proteins are increased by the treatment of IFN- γ and TNF- α . The binding of RNA binding proteins may also be important in relation to the recent identification of granules within cells which appear to sequester certain mRNA.²⁷ These stress granules appear to form only under certain conditions and may regulate the ability of certain mRNA to be translated, which cannot occur while the mRNA reside in the granules. A hypothetical model for inflammation-associated translational down-regulation of iHsp is shown in supporting document. Further investigations beyond the scope of this study are therefore needed to define more precisely the basis of the selective down-regulation of iHsp. It should also be noted that the observed changes in iHsp

expression by IFN- γ and TNF- α , within the time frame of the experiments, were not caused by apoptosis (Figure 4C).

In the context of chronic inflammation associated with IBD, the down-regulation of critical cytoprotective proteins, such as iHsp, through translational inhibition could profound negative consequences.³⁵ Their compromised expression renders the mucosa highly susceptible to inflammatory and immune processes, and to potentially otherwise harmless enteric flora.^{7, 11} In addition, therapeutic agents known to enhance mucosal Hsp expression, including glucocorticoids, mesalamine, short chain fatty acids, IL-10 and probiotics, would be less effective in inducing *de novo* synthesis of iHsp because of the cytokine-inhibition of the polyribosomal machinery.³⁶ In this instance, therapy should logically be directed at inhibiting pro-inflammatory cytokines, in an attempt to restore a milieu more conducive to cytoprotection and wound healing. On the other hand, therapeutic strategies to stimulate production of intestinal epithelial iHsp might be useful in the maintenance of remission, i.e. to sustain the expression of epithelial iHsp and thus counteract or prevent the initiation of inflammation-induced apoptosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Grant Support: This work was supported by NIH grants DK-47722 (EBC), DK-38510 (EBC), NIH supplement award DK-47722-11S1 (MJC), the Digestive Disease Research Core Center DK-42086 of the University of Chicago and Research Training Awards (SH) from the Crohn's and Colitis Foundation of America, Moshe Goldgraber Advanced IBD Fellowship (LL) and the Gastrointestinal Research Foundation of Chicago (MJC).

We are grateful to Stephen B. Hanauer and Laura Harrell for providing clinical biopsies and to Ken Drabik for cell culture.

References

1. Musch MW, Kaplan B, Chang EB. Role of increased basal expression of heat shock protein 72 in colonic epithelial Caco2_{BBE} adenocarcinoma cells. *Cell Growth & Differentiation* 2001;12(8):419–26. [PubMed: 11504707]
2. Ropeleski MJ, Tang J, Walsh-Reitz MM, Musch MW, Chang EB. Interleukin-11-induced heat shock protein 25 confers intestinal epithelial-specific cytoprotection from oxidant stress. *Gastroenterology* 2003;124(5):1358–68. [PubMed: 12730876]
3. Ropeleski MJ, Riehm J, Baer KA, Musch MW, Chang EB. Anti-apoptotic effects of L-glutamine-mediated transcriptional modulation of the heat shock protein 72 during heat shock. *Gastroenterology* 2005;129(1):170–84. [PubMed: 16012946]
4. Liu TS, Musch MW, Sugi K, Walsh-Reitz MM, Ropeleski MJ, Hendrickson BA, Pothoulakis C, Lamont JT, Chang EB. Protective role of HSP72 against *Clostridium difficile* toxin A-induced intestinal epithelial cell dysfunction. *American Journal of Physiology - Cell Physiology* 2003;284(4):C1073–82. [PubMed: 12490434]
5. Sussman CR, Renfro JL. Heat shock-induced protection and enhancement of Na⁺-glucose cotransport by LLC-PK1 monolayers. *American Journal of Physiology* 1997;273:F530–7. [PubMed: 9362330]
6. Musch MW, Sugi K, Straus D, Chang EB. Heat-shock protein 72 protects against oxidant-induced injury of barrier function of human colonic epithelial Caco2/bbe cells. *Gastroenterology* 1999;117:115–22. [PubMed: 10381917]
7. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell* 2004;118:229–241. [PubMed: 15260992]

8. Kojima K, Musch MW, Ren H, Boone DL, Hendrickson BA, Ma A, Chang EB. Enteric flora and lymphocyte-derived cytokines determine expression of heat shock proteins in mouse colonic epithelial cells. *Gastroenterology* 2003;124:1395–1407. [PubMed: 12730879]
9. Arvans DL, Vavricka SR, Ren H, Musch MW, Kang L, Rocha FG, Lucioni A, Turner JR, Alverdy J, Chang EB. Luminal bacterial flora determines physiological expression of intestinal epithelial cytoprotective heat shock proteins 25 and 72. *American Journal of Physiology* 2005;288:G696–704. [PubMed: 15528251]
10. Fujiya M, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y, Schneewind O, Jabri B, Chang EB. The *Bacillus subtilis* quorum-sensing molecule CSF contributes to intestinal homeostasis via OCRN2, a host cell membrane transporter. *Cell Host and Microbe* 2007;1:299–308. [PubMed: 18005709]
11. Ren H, Musch MW, Kojima K, Boone D, Ma A, Chang EB. Short-chain fatty acids induce intestinal epithelial heat shock protein 25 expression in rats and IEC 18 cells. *Gastroenterology* 2001;121:631–639. [PubMed: 11522747]
12. Dieleman LA, Palmen MJ, Akol H, Bloemena E, Pena AS, Meuwissen SGM, van Rees EP. Chronic experimental colitis induced by dextran sulphate sodium (DSS) is characterized by Th1 and Th2 cytokines. *Clinical & Experimental Immunology* 1998;114:385–391. [PubMed: 9844047]
13. Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263–74. [PubMed: 8402911]
14. Whitehead RH, Van Eeden PE, Noble MD, Ataliotis P, Jat PS. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *Proceedings of the National Academy of Sciences* 1993;90:587–591.
15. Musch MW, Ciancio MJ, Sarge K, Chang EB. Induction of heat shock protein 70 protects intestinal epithelial IEC-18 cells from oxidant and heat injury. *American Journal of Physiology* 1996;270:C429–C436. [PubMed: 8779904]
16. Kojima K, Musch MW, Ropeleski MJ, Boone DL, Ma A, Chang EB. *Escherichia coli* LPS induces heat shock protein 25 in intestinal epithelial cells through MAP kinase activation. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2004;286:G645–652. [PubMed: 14630641]
17. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earshaw WC. Cleavage of poly (ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 1994;371:346–7. [PubMed: 8090205]
18. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 1995;376:37–43. [PubMed: 7596430]
19. Schmittgen TD, Zakrajsek BA, Mills AG, Gorn V, Singer MJ, Reed MW. Quantitative reverse transcription polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Analytical Biochemistry* 2000;285:194–204. [PubMed: 11017702]
20. Malago JJ, Koninkx FJG, van Dijk JE. The heat shock response and cytoprotection of the intestinal epithelium. *Cell stress & chaperones* 2002;7:191–199. [PubMed: 12380687]
21. Srivastava SP, Kumar KU, Kaufman RJ. Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *The Journal of Biological Chemistry* 1998;273:2416–2423. [PubMed: 9442091]
22. Jammi NV, Whitby LR, Beal PA. Small molecule inhibitors of the RNA-dependent protein kinase. *Biochemical and Biophysical Research Communications* 2003;308:50–57. [PubMed: 12890478]
23. Ludwig D, Stahl M, Ibrahim ET, Wenzel BE, Drabicki D, Wecke A, Fellermann K, Stange EF. Enhanced intestinal expression of heat shock protein 70 in patients with inflammatory bowel diseases. *Digestive Diseases & Sciences* 1999;44:1440–1447. [PubMed: 10489932]
24. Papadakis KA, Targan SR. Role of cytokines in the pathogenesis of inflammatory bowel disease. *Annual Review of Medicine* 2000;51:289–298.
25. Fuss IJ. Cytokine network in inflammatory bowel disease. *Current Drug Targets - Inflammation & Allergy* 2003;2:101–112. [PubMed: 14561161]
26. Holcik M, Sonenberg N. Translational control in stress and apoptosis. *Nature Reviews Molecular Cell Biology* 2005;6:318–327.
27. Anderson P, Kedersha N. Visible stressed: the role of eIF2, TIA-1, and stress granules in protein translation. *Cell Stress & Chaperones* 2002;7:213–221. [PubMed: 12380690]

28. Rhoads RE, Joshi B, Minich WB. Participation of initiation factors in the recruitment of mRNA to ribosomes. *Biochimie* 1994;76:831–838. [PubMed: 7880899]
29. Kedersha N, Anderson P. Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochemical Society Transactions* 2002;30:963–969. [PubMed: 12440955]
30. Jing Q, Huang S, Guth S, Zarubin T, Motoyama A, Chen J, Di Padova F, Lin SC, Gram H, Han J. Involvement of microRNA in AU-rich element-mediated mRNA instability. *Cell* 2005;120(5):623–34. [PubMed: 15766526]
31. Wickens M, Goldstrohm A. A place to die, a place to sleep. *Science* 2003;300:753–755. [PubMed: 12730589]
32. Moore MJ. From birth to death: the complex lives of eukaryotic mRNAs. *Science* 2005;309:1514–1518. [PubMed: 16141059]
33. Rubtsova MP, Sizova DV, Dmitriev SE, Ivanov DS, Prassolov VS, Shatsky IN. Distinctive properties of the 5'-untranslated region of human hsp70 mRNA. *Journal of Biological Chemistry* 2003;278(25):22350–6. [PubMed: 12682055]
34. Mukhopadhyay D, Houchen CW, Kennedy S, Dieckgraefe BK, Anant S. Coupled mRNA stabilization and translational silencing of cyclooxygenase-2 by a novel RNA binding protein, CUGBP2. *Molecular Cell* 2003;11:113–126. [PubMed: 12535526]
35. Ruemmele FM, Seidman EG, Lentze MJ. Regulation of intestinal epithelial cell apoptosis and the pathogenesis of inflammatory bowel disorders. *Journal of Pediatric Gastroenterology & Nutrition* 2002;34:254–260. [PubMed: 11964947]
36. Petrof EO, Ciancio MJ, Chang EB. Role and regulation of intestinal epithelial heat shock proteins in health and disease. *Chinese Journal of Digestive Diseases* 2004;5:45–50. [PubMed: 15612656]

Abbreviations

IBD	inflammatory bowel diseases
IL	interleukin
PCR	polymerase chain reaction
TNF-α	tumor necrosis factor alpha

Figure 1. iHsp protein, but not mRNA, expression is significantly and selectively down-regulated in human IBD

A) Immunohistochemical staining for Hsp27 (human homolog to murine Hsp25) and Hsp70 in sections from colon of normal and active colitis. Image shown is representative of six individual experiments. **B)** Western blots of Hsp27, Hsp70, Hsc70, villin and β-actin in colonic biopsies from normal control, uninvolved (UN) and actively inflamed (IN) areas of CD and UC patients. **C)** Real-time PCR analysis of mRNA abundance of Hsp27 and Hsp70 in colonic biopsies from normal control, uninvolved (UN) and actively inflamed (IN) mucosa of CD and UC patients. Changes in iHsp mRNA relative to GAPDH were determined as fold change over

control, as described in the methods. Results for panel **B** and **C** are means $\pm$ SEM, n=6. *p<0.05 compared with Control by analysis of variance using a Bonferroni correction.

Figure 2. Colonic Hsp25 and Hsp70 protein, but not mRNA, expression is decreased in colon of IL-10^{-/-}-gene-deficient mice with inflammation

IL-10^{-/-}-gene-deficient mice on the C57Bl/6 background were sacrificed at the age of 20 weeks. Wild-type mice were sacrificed as control. **A)** Western blot of Hsp25, Hsp70, Hsc70, villin and β-actin; and **B)** Real-time PCR analysis of Hsp25 and Hsp70 mRNA abundance in colonic mucosa from wild-type mice (WT), IL-10^{-/-}-gene-deficient mice without colitis (UN), and IL-10^{-/-}-gene-deficient mice with colitis (IN). Results are means ± SEM, n=4. *p<0.05 compared with WT by analysis of variance using a Bonferroni correction.

Figure 3. IFN- γ +TNF- α co-treatment of mice down-regulates colonic Hsp25 and Hsp70 protein expression *in vivo*, without changing mRNA expression

Mice were given an intraperitoneal dose of IFN- γ (2500U), TNF- α (1000ng) or a combination of IFN- γ +TNF- α at 48 hours before sacrifice. Mice without cytokine injection were sacrificed as control. **A)** Western blot of Hsp25, Hsp70, Hsc70, villin and β -actin; **B)** Real-time PCR analysis of Hsp25 and Hsp70 mRNA abundance in colonic mucosa. Results are means $\pm$ SEM, n=4. *p<0.05 compared with Control by analysis of variance using a Bonferroni correction.

Figure 4. IFN- γ +TNF- α co-treatment to YAMCs down-regulates Hsp25 and Hsp70 protein, but not mRNA, expression

YAMCs were treated with IFN- γ (200U/ml), TNF- α (100ng/ml), or both IFN- γ +TNF- α for 8 hours and then stimulated with heat shock (HS, 42°C for 23 minutes). **A)** Cells were harvested for total protein at 120 minutes after HS. Cells without cytokine treatment and HS were harvested as basal, and cells with HS only were harvested as HS-control. Hsp25, Hsp70, Hsc70, villin and β -actin were analyzed by Western blot. **B)** Cells were harvested for total RNA at 0, 15, 30, 60, 90, 120, 150 and 210 minutes after HS. Cells without HS were harvested as basal and cells without IFN- γ and TNF- α treatment were harvested as control. Hsp25 and Hsp70 mRNA abundance was analyzed using real-time PCR. **C)** PARP and caspase 3 in YAMCs were analyzed on Western blots under basal, HS control, IFN- γ and/or TNF- α treated conditions using the same samples as in Figure 4A. Cells treated with staurosporine (1 μ M) for 3 hours were analyzed as positive control (+) for apoptosis. Image shown representative of four individual experiments. Results are means $\pm$ SEM, n=4. For panel A, *p<0.05 compared with HS-control by analysis of variance using a Bonferroni correction. For panel B, statistical

comparisons were made by paired Student's t-test between Control and IFN- γ +TNF- α treated samples at each time point. No significant differences were observed.

Figure 5. IFN- γ +TNF- α inhibits *de novo* Hsp70 protein synthesis in YAMCs

YAMCs were treated with 200U/ml IFN- γ and 100ng/ml TNF- α (+) for 8 hours, then subjected to HS at 42°C for 23 minutes and pulsed with ^{35}S -methionine or ^3H -leucine for 60 minutes at varying times. Cells without HS were harvested as basal, and cells without cytokine treatment were harvested as control (-). **A**) Fluorograph of ^{35}S -methionine-labeled Hsp70 protein for 60 minutes under non-stress condition (Basal) and HS for determination of protein synthesis. **B**) Fluorograph of ^{35}S -methionine-labeled Hsp70 and β -actin determination of protein synthesized for 120 minutes with or without IFN- γ +TNF- α co-treatment. **C**) Quantification of fold changes in *de novo* Hsp70 synthesis over basal, with and without IFN- γ +TNF- α co-treatment. **D**) TCA precipitable ^{35}S -methionine-labeled protein. **E**) Incorporation of ^3H -

leucine in Hsp25 protein for 60 minutes under non-stress condition (Basal) and HS for determination of protein synthesis. **F)** TCA precipitable ^3H -leucine-labeled protein. Results are means $\pm$ SEM, n=4. For panel B, C, D, E and F, *p<0.05 comparing Control and IFN- γ +TNF- α treated samples by paired Student's t-test at each time point. For panel B, #p<0.05 comparing Hsp70 and β -actin in IFN- γ +TNF- α treated samples by paired Student's t-test.

Figure 6. IFN- γ +TNF- α co-treatment has no effect on protein degradation of Hsp70 in YAMCs Hsp70 protein degradation was determined using pulse chase experiment. YAMCs were incubated with ^{35}S -methionine for 1 hour to pulse label *de novo* proteins. IFN- γ (200U/ml) and TNF- α (100ng/ml) were added to cells after pulse label. Cells were harvested for protein analysis at 0, 2, 4 and 8 hours after cytokine treatment. Cells without cytokine treatment were harvested as control. **A**) Schematic of time course for cell treatment. **B**) Fluorograph of immuno-precipitated Hsp70 in cells with (+) and without (-) cytokine treatment. Image is representative of four individual experiments. **C**) Quantification of fold changes of Hsp70 over basal. Results are means $\pm$ SEM, $n = 4$. For panel C, statistical comparisons were made by paired Student's t-test between Control and IFN- γ +TNF- α treated samples at each time point. No significant differences were observed.

Figure 7. IFN- γ +TNF- α inhibit iHsp production via activation of PKR and phosphorylation of eIF-2 α in YAMCs

A) YAMCs were treated with IFN- γ (200U/ml) and TNF- α (100ng/ml) for 8 hours, PKR-I (30nM) for 30 minutes and then subjected to HS. Hsp25, Hsp70, Hsc70, phospho-PKR (p-PKR), PKR, phospho- eIF-2 α (p-eIF-2 α) and eIF-2 α were analyzed by Western blot under basal (-), HS (+), IFN- γ +TNF- α (+) and/or PKR-I (+) treated conditions. **B)** YAMCs were treated with silencing RNA to PKR (si-PKR) and control silencing RNA (si-C) for 16 hours prior to IFN- γ +TNF- α and HS treatment. Hsp25, Hsp70, Hsc70, p-PKR, PKR, p-eIF-2 α and eIF-2 α were analyzed by Western blot. Image shown is representative of four individual

experiments. Results are means $\pm$ SEM, n=4. For panel B, *p<0.05 compared with HS control (HS) by analysis of variance using a Bonferroni correction.

Figure 8. Phosphorylation of PKR and eIF-2 α is up-regulated in inflamed mucosa *in vivo*

A) Phospho-PKR (p-PKR) and phospho-eIF-2 α (p-eIF-2 α) were analyzed by Western blot from colonic pinch biopsies from control, uninvolved (UN) and actively inflamed (IN) areas of CD and UC patients using the same samples as in Figure 1B. **B)** PKR, p-PKR, eIF-2 α and p-eIF-2 α were analyzed on Western blot for colonic mucosa from wild-type mice (WT), IL-10^{-/-} mice without colitis (UN) and IL-10^{-/-} mice with colitis (IN) using the same samples as in Figure 2. Results are means $\pm$ SEM. For panel A, *p<0.05 compared with Control analyzed on same blot by analysis of variance using a Bonferroni correction, n=6. For panel B, *p<0.05 compared with WT by analysis of variance using a Bonferroni correction, n=4.