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Inhibition of heat shock protein expression by *Helicobacter pylori*

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Abstract

Heat shock proteins (HSPs) are primarily known as molecular chaperones that are induced by cell stress and prevent protein aggregation and facilitate folding. Recent evidence suggests that exposure of cells to microbial pathogens can also induce HSPs, which then modulate both innate and adaptive immune responses. Paradoxically, *Helicobacter pylori* has been found to decrease expression of HSPs. We sought to investigate this phenomenon further and to examine the role of different *H. pylori* strains and recognized virulence factors in cell culture and in the mouse model. Co-culture of *H. pylori* with two gastric carcinoma cell lines reduced expression of HSP70 and, to a lesser extent, HSP60. Down modulation of HSPs was not dependent on the presence of the vacuolating cytotoxin (VacA) or the *cag* pathogenicity island (*cag* PAI). C57BL/6 mice infected with a human *H. pylori* strain also demonstrated reduced expression of HSP70, HSP8, and heat shock factor 1 (HSF-1), a transcriptional activator of HSP70. In contrast, the bacterial pathogen, *S. Typhimurium* up-regulated HSP expression. Since HSPs are thought to function as danger signals during microbial infection, *H. pylori* down-regulation of HSPs may be a mechanism of immune evasion that promotes chronic infection.

Keywords

Helicobacter pylori; Heat shock proteins

1. Introduction

Helicobacter pylori is a gram-negative spiral bacterium that infects the gastric mucosa of approximately half the world's population and causes chronic gastritis [1]. About 10% of those infected go on to develop peptic ulcer disease or gastric adenocarcinoma, which is the second most common cause of cancer death [2]. Since *H. pylori* infection causes clinical illness in only a minority of those infected, there is considerable interest in bacterial and host factors that may be associated with disease. One such factor is the cytotoxin associated gene pathogenicity island (*cag* PAI), a 40 kb cluster of approximately 27 genes that encodes a

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type IV secretion system and is more often found in *H. pylori* strains recovered from patients with peptic ulcer or gastric cancer than in those with asymptomatic gastritis [3]. The type IV secretion apparatus forms a syringe-like pilus that penetrates gastric epithelial cells and facilitates the translocation of CagA, peptidoglycan, and possibly other bacterial components into host epithelial cells [2]. Certain alleles of the vacuolating cytotoxin (VacA), which causes vacuolization in gastric epithelial cells *in vitro* [4], are also associated with disease.

Heat shock proteins (HSP) make up approximately 5–10% of the total protein content of cells under conditions of normal, healthy growth [5]. Under these conditions, HSPs are known to function as intracellular molecular chaperones that facilitate protein transport, prevent protein aggregation during folding, and protect newly synthesized polypeptide chains [6,7]. Categorized into several families based on their approximate molecular weight, HSPs may be expressed constitutively (HSP60, HSP90) or induced by stress (HSP27, HSP70, HSP40). Inducible HSPs are activated upon exposure not only to high temperature, but also oxidative stress, nutritional deficiencies, ultraviolet radiation, chemicals, ethanol, and infection [8]. In addition, HSPs appear to be regulated at the transcriptional level by heat shock factors (HSF), transcription factors that interact with heat shock elements in the promoter regions of heat shock genes [5,9]. It has been proposed that during infection, HSPs serve as an early warning “danger” signal during their extracellular release, which activates proinflammatory innate immunity [10–12]. For example, extracellular HSP70 has been shown to bind to Toll-like receptors (TLR) 2 and 4 on the surface of antigen-presenting cells, similar to lipopolysaccharide, inducing the production of the proinflammatory cytokines IL-6, IL-1 β , and TNF- α , as well as nitric oxide, a product with prominent antimicrobial activity [13–15].

Although bacterial infection is generally associated with increased HSP expression, recent evidence suggests that *H. pylori* infection is associated with decreased expression of HSPs [16–18]. To better understand the role of *H. pylori* on host HSP expression, we examined the heat shock response using both *in vitro* cell culture and an *in vivo* mouse model. Since genetic diversity and bacterial components such as the *cag* PAI and *vacA* contribute to virulence [2], we also examined isogenic mutants to determine whether these factors may play a role in decreasing the epithelial cell HSP expression.

2. Results

2.1. Effect of *H. pylori* strains J166, J99, and 26695 on HSP expression

To determine the effect of *H. pylori* infection on HSP expression, we co-cultured *H. pylori* with gastric epithelial cells for 6–48 h, and performed Western blots with antibodies specific for HSP70, HSP27, and HSP60. *H. pylori* strain J166 reduced the expression of HSP70 in AGS and Kato III cells (Fig. 1A) at all time points, but had no effect on HSP27 (data not shown). *H. pylori* J166 also decreased the expression of HSP60 in AGS (but not Kato III) cells, though the effect was not as prominent and was seen only after 6 and 12 h of co-culture. Since *H. pylori* strains are highly diverse [19], we next asked if the effect of *H. pylori* on HSP expression was strain specific. We cultivated *H. pylori* strains J166, J99 and 26695 with AGS cells, and examined expression of HSP70 and HSP60. All three *H. pylori*

strains markedly reduced the expression of HSP70 after 24 h of co-culture. *H. pylori* J99 and 26695 also induced modest decreases in HSP60 after 24 h of co-culture (Fig. 1B). Previous studies have shown that *Salmonella* induces the expression of HSP70 and HSP90 in enterocyte-like Caco-2 cells [20]. Therefore, to determine if the decreased HSP expression we observed was specific to *H. pylori*, we examined the effect of *S. Typhimurium* on the expression of HSP70, HSP60, and HSP27 in AGS cells using Western blots. The results demonstrated that, in contrast to *H. pylori*, culturing AGS cells with *S. Typhimurium* induced the expression of HSP70 and HSP60 at 6 h (Fig. 1C), with no effect on the level of HSP27 expression (data not shown). As a positive control, the level of β -actin expression was also monitored over time, and remained stable. These results suggest that *H. pylori* specifically down-modulates expression of HSP70 and HSP60.

2.2. Role of *cagA*, *vacA*, and the *cag* PAI on HSP expression in AGS cells

To determine the effect of key *H. pylori* virulence factors on the *H. pylori*-dependent down-regulation of HSP expression, we constructed isogenic mutants in *vacA*, the entire *cag* PAI, and *cagA*, which encodes the only known protein effector secreted by the *H. pylori* Type IV secretions system. The effect of wild type J166 and each knockout strain on HSP expression in AGS cells was examined by Western blot as before. Both wild-type J166 and all knockout strains down-regulated HSP70, but not HSP60 expression, in comparison to uninfected cells and β -actin controls (Fig. 1B), suggesting that none of these bacterial virulence factors are required for *H. pylori*-induced down modulation of HSPs.

2.3. Analysis of HSP expression in J166-infected mice

Mice ($N = 8$ per group) were inoculated with *H. pylori* or sham inoculated and sacrificed 1 or 12 weeks later. Gastric tissue was harvested at each time point for total RNA isolation and quantitative *H. pylori* culture. At 1 week, eight J166-infected mice were successfully colonized with a mean of 8.8×10^5 CFU/g of stomach tissue. At 12 weeks, the eight infected mice had a mean bacterial load of 8.0×10^4 CFU/g (Fig. 2). At both time points, the sham-inoculated controls were culture negative for *H. pylori*. To quantify HSP expression in *H. pylori*-infected mice, TaqMan PCR was performed on cDNA from stomach tissue isolated at 1 and 12 weeks post-inoculation. Mean level of HSP70, HSP8, and HSF-1 expression normalized to β -actin was determined in *H. pylori*-infected and uninfected mice at both time points. Decreased expression of HSP70 and HSP8 was observed at both time points, but reached statistical significance only at 12 weeks post-inoculation ($p < 0.05$). HSF-1 expression was reduced at both time points but did not reach statistical significance.

3. Discussion and conclusions

The surprising finding that *H. pylori* down regulates HSP expression was first reported by Konturek and colleagues [16,18], who asked whether HSP70 might be involved in the *H. pylori*-induced impairment of adaptation to administration of aspirin [21]. Here we confirmed these findings in the *H. pylori* mouse model, and extended the results to different gastric cancer cell lines, and different mouse and *H. pylori* strains. Furthermore, we demonstrated for the first time using defined isogenic knockout strains that the key virulence determinants in *H. pylori* pathogenesis, *VacA*, *CagA*, and the *cag* PAI, are not required for

down modulation of HSPs. Although there is one report that addition of exogenous CagA increases the inhibitory effect of *H. pylori* on HSP70 [18], the physiological relevance of this is unclear. The effect in cell culture was most prominent with HSP70 (Fig. 1A,B), but in mice (Fig. 3) we also found *H. pylori*-induced down modulation of HSP8, a member of the HSP70 protein family [22], and HSF-1, a transcriptional activator of HSP70. Proteomic [17] and microarray studies in epithelial cell cultures [23–25] and in animal models [26], including our own studies in rhesus macaques [27], have also found a decrease in HSP expression with *H. pylori* infection. Thus, reduced HSP expression with *H. pylori* infection is a robust phenomenon that can be demonstrated across different cell lines, animal models, and *H. pylori* strains, and appears to be independent of recognized virulence factors.

Although best known as intracellular chaperones, it is increasingly recognized that HSPs also have pleomorphic effects on both innate and adaptive immunity. For example, release of HSPs from damaged tissue acts as a danger signal that may be recognized by toll like receptors (TLR), much like bacterial pathogen associated molecular patterns, and activate the innate immune system [28]. HSPs have also been demonstrated to provide adjuvant-like signals that stimulate dendritic cells and, together with antigen, trigger autoimmunity [29]. Bacterial products, including LPS and GroEL, the bacterial orthologue of Hsp60, can also directly stimulate host expression of HSP60 and HSP70 [30]. Viewed in this way, *H. pylori* down-modulation of HSPs may be seen as a mechanism of immune evasion. There are in fact multiple strategies used by *H. pylori* to suppress host immunity, which presumably contribute to bacterial persistence in the face of both a cellular and humoral immune response. Examples include inhibition of B and T cell proliferation, poor recognition of pathogen associated molecular patterns such as flagellin and LPS, and induction of regulatory T cells (reviewed in ref. [31]). Down modulation of HSPs may be another such mechanism. On the other hand, *H. pylori* also has multiple mechanisms to promote inflammation, most notably the *cag* PAI. Furthermore, induction of HSP70 may have anti-inflammatory activity, by inhibiting the expression of IL-1 β , IL-8, and TNF- α -induced NF- κ B expression [32,33]. The presence of HSP70 has also been associated with elevated concentrations of the anti-inflammatory cytokines IL-10 and IL-1ra [34,35]. Thus, HSPs may dampen the host's ability to trigger an inflammatory response. This complexity only serves to reinforce the idea that for *H. pylori*, and probably many other bacterial pathogens, inflammation is neither good nor bad, but is rather a highly regulated and intrinsic part of chronic infection.

4. Materials and methods

4.1. Bacterial strains and culture

H. pylori strain J166 contains a functional *cag* PAI and the s1m1 allele of the *vacA* cytotoxin [36]. *H. pylori* J99 and 26695 are also *cag* PAI+ and *vacA*+ and their genomes have been fully sequenced [37]. Wild-type *H. pylori* strains were cultivated on brucella agar or brucella broth (Difco Laboratories, Detroit, MI) containing 5% bovine calf serum (GibcoBRL, Gaithersburg, MD) supplemented with antibiotics (trimethoprim, 5 mg/l; vancomycin, 10 mg/l; polymixin B, 2.5 IU/l; amphotericin B, 4 mg/l; all from Sigma, St. Louis, MO) and incubated at 37 °C with an atmosphere that contained 5% CO₂. *H. pylori vacA*–, *cagA*–, and

cag PAI– derivatives of J166 were constructed using allelic exchange methods similar to those previously described [38]. Briefly, a non-polar kanamycin or chloramphenicol resistance cassette [39] and 1.5–2.1 kb fragments of the genes directly flanking the gene of interest were PCR amplified with compatible restriction sites at the 5' and 3' ends. All three fragments were restricted with the appropriate enzymes and ligated into pBluescript SK+/- (Stratagene, La Jolla, CA), creating a shuttle plasmid that was used to transform *E. coli* Top 10 (Invitrogen, Carlsbad, CA). Deletions of *vacA*, *cagA*, and the *cag* PAI were confirmed by a PCR reaction using primers specific for an area upstream of the gene of interest and within the antibiotic resistance cassette (not shown). Plasmids were transferred into *H. pylori* J166 using natural transformation and selection for kanamycin (*cagA*, *vacA*) or chloramphenicol-resistant clones (*cag*PAI).

S. enterica serovar Typhimurium strain IR715 is a virulent, nalidixic acid-resistant derivative of ATCC strain 14028 [40]. IR715 was cultured aerobically at 37 °C in Luria–Bertani broth (10 g/l Tryptone, 5 g/lyeast extract, 5 g/l NaCl). For cell culture infection, IR715 was grown overnight at 37 °C in 4 ml of LB broth in a roller. A volume of 0.04 ml of this overnight culture was used for inoculation of 4 ml of LB broth, and bacteria were grown at 37 °C for 3 h in a roller.

4.2. Gastric cell culture

AGS or Kato III human gastric carcinoma cells (ATCC) were grown in RPMI 1640 (Gibco BRL, Grand Island, NY) with 10% fetal bovine serum, or IMDM medium (Gibco BRL) with 20% fetal bovine serum, respectively, supplemented with 100 units/ml each of penicillin and streptomycin (Gibco BRL). Approximately 2.5×10^5 AGS or Kato III cells were seeded in 25 cm² T-25 flasks, and grown in medium without antibiotic additives in a humidified 5% CO₂ atmosphere for 24 h. *H. pylori* from plate culture diluted in brucella broth to an OD of 1.0 (5.0×10^8 CFU/ml) was co-cultured with AGS or Kato III cells at an MOI of 100:1, and harvested after 6, 12, 24, or 48 h.

S. Typhimurium was used to inoculate AGS cells that were seeded as described above and infected with approximately 1 ml of 10^7 CFU/ml at an MOI of 10:1. AGS cells were co-cultured with *S. Typhimurium* for 1 h at 37 °C in 5% CO₂ to allow intracellular invasion. Each flask was washed five times with sterile phosphate-buffered saline (PBS) (2.7 mM KCl, 1.8 mM KH₂PO₄, 140 mM NaCl, 10 mM Na₂HPO₄, pH 7.4) to remove extracellular bacteria, and medium containing gentamicin (0.1 mg/l) was added for 90 min at 37 °C in 5% CO₂. After three washes with PBS, the cells were lysed for protein as described below.

4.3. Immunoblots

Cells were washed once with 5 ml of cold RPMI 1640 supplemented with 100 mM sodium orthovanadate, and scraped into a 1.5 ml microcentrifuge tube. Cells were pelleted, resuspended, and lysed in 100 µl of cold NENT (1% NP-40; 5 mM EDTA; 250 mM NaCl; 25 mM Tris–HCl, pH 8.0; 1 mM PMSF; 1 mM Na₃VO₄). Protein concentration was determined by the Bradford Assay (Bio–Rad, Hercules, CA). Samples were stored at –80 °C prior to analysis. Protein samples were boiled with 2× protein dye (62.5 mM Tris–HCl, pH 6.8; 4% SDS; 25% glycerol; 0.05% bromophenol blue, w/v), and 10 µg was loaded and

electrophoresed on 12% polyacrylamide gels using the Mini-PROTEAN-II® Electrophoresis Cell (Bio–Rad, Hercules, CA) at 200 V for 35 min.

Transfer onto PVDF membranes (Amersham Biosciences, Uppsala, Sweden) was performed in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) using the Mini-Trans® Blot Electrophoretic Transfer Cell (Bio–Rad, Hercules, CA) at 100 V for 1 h. The PVDF membrane was blocked (5% non-fat dry milk in TTBS) for 1 h at room temperature, followed by a 1-h exposure to primary antibody specific for HSP27, HSP70, or HSP60 (Stressgen Bioreagents, Ann Arbor, MI) diluted 1:500 or for β -actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:1000, and secondary antibody diluted 1:20,000 (anti-mouse IgG-HRP, Sigma–Aldrich) in blocking buffer. The membrane was then washed once for 15 min, and three times for 5 min each in TTBS buffer (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20 v/v). Detection of membrane-bound proteins was performed using an ECL detection kit and the Typhoon 8600 Scanner (Amersham Biosciences, Uppsala, Sweden).

4.4. Animals and experimental infection

Specific pathogen (*Helicobacter*) free, female C57BL/6 mice were purchased from Jackson Lab (Bar Harbor, ME), housed in micro-isolator cages, and provided with autoclaved food, water, and bedding. At 8–12 weeks of age mice were inoculated three times by oral gavage every 48 h with brucella broth (sham controls, $N=16$) or with 1 ml containing approximately 2.5×10^9 CFU of *H. pylori* grown in liquid culture for 18–24 h ($N=16$). At 1 week (Group 1) and 12 weeks (Group 2) post-inoculation, mice (eight infected, eight uninfected) were euthanized with an overdose of Nembutal sodium solution (Abbott Laboratories, North Chicago, IL), followed by cervical dislocation. The stomach was dissected from the mice by sterile technique and cut in half along the cephalocaudal axis. Half of the stomach was plated onto brucella agar (Difco Laboratories, Detroit, MI) containing 5% bovine calf serum (GibcoBRL, Gaithersburg, MD) supplemented with antibiotics (amphotericin B, 125 μ g/l; bacitracin, 10 mg/l; nalidixic acid, 535 μ g/l; polymyxin B, 165 μ g/l; vancomycin, 5 mg/l; all from Sigma, St. Louis, MO) for quantitative culture. The other half of the stomach was washed in brucella broth, and the epithelial cell layer was scraped into a 1.5 ml microcentrifuge tube of 1 ml Trizol reagent using a razor blade. Tissue was vortexed for 30 s and homogenized using a glass pestle. Samples were stored in Trizol reagent at -80°C until analysis. All animal experiments were performed under protocols approved by ALAAC and the Institutional Animal Care and Use Committee of the University of California, Davis.

4.5. Quantitative RT-PCR

Total RNA was isolated from mouse stomach tissue using Trizol according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA was treated with DNase I (Roche Applied Sciences, Mannheim, Germany), purified using an RNeasy clean up kit (QIAGEN, Inc., Valencia, CA), and suspended in ultra pure water (GibcoBRL). First strand cDNA synthesis was performed using SuperScript™ III in 20 μ l reactions according to the manufacturer's instructions using oligo(dT)₂₀ primers (Invitrogen, Carlsbad, CA). Gene-specific primers and probes (Table 1) were designed using Primer Express 1.0 software

(Applied Biosystems, Foster City, CA) to amplify a product between 400 and 700 bp with a calculated melting temperature of 58–60 °C, and amplified products were between 400 and 700 bp. For standard curves, PCR amplified fragments (HSP8, HSF-1, β -actin) were cloned into the pDRIVE vector using the Qiagen PCR Cloning Kit (QIAGEN, Inc., Valencia, CA). Plasmid was transformed into Top-10 One-Shot® Competent *E. coli* cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA), and plated on LB supplemented with 25 mg/l of kanamycin and 160 mg/ml X-GAL. White colonies were selected and propagated in 5 ml LB with 25 mg/l kanamycin, and plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN, Inc., Valencia, CA). Correct plasmid insert sequence was verified by DNA sequence analysis. Copy count of the plasmid was determined quantitatively based on plasmid size and concentration. Cloned mouse HSP70 (Catalog No. 10699031) was obtained from ATCC as a plasmid in *E. coli* and cultured using the manufacturer's instructions. Standard curves were generated to represent log of the copy number vs. the cycle threshold (C_t) value.

One hundred nanograms of mouse cDNA in 5- μ l aliquots were used for each TaqMan reaction. TaqMan reactions were carried out in a total volume of 20 μ l containing 2 $\times$ Universal TaqMan PCR Master Mix (Applied Biosystems), 300 nM forward primer, 300 nM reverse primer, and 200 nM 6FAM/TAMRA TaqMan probe. An Applied Biosystems Prism 7700 Sequence Detection System was used with the default thermal cycling program (95 °C for 10 min followed by 40 cycles of 95 °C, 15 s, 60 °C, 1 min). The threshold was set at 0.05 units of normalized fluorescence; the threshold cycle (C_t) was measured for each well. Mean copy number for each gene normalized to β -actin was determined for each group, and statistical differences ($p < 0.05$) were determined using the Student's *t*-test.

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Fig. 1.

Effects of *H. pylori* infection on HSP70, HSP60, and β -actin expression. (A) Western blots of *H. pylori* strain J166-infected Kato III and AGS gastric carcinoma cells. (B) Western blot of *S. Typhimurium* infected AGS gastric carcinoma cells. (C) Western blots of AGS cells co-cultured with *H. pylori* strains J166, J99, 26695, and J166 with isogenic deletions of VacA, CagA and the *cag* PAI.

Fig. 2.
Quantitative cultures of *H. pylori* J166 from infected C57BL/6 mice 1 and 12 weeks post-inoculation.

Fig. 3. mRNA expression levels normalized to β -actin mRNA copy number was determined using TaqMan PCR. Mean ($\pm$ standard error) mRNA copy number for uninfected (Control) and *H. pylori*-infected mice at 1 and 12 weeks post-inoculation were normalized to mRNA copy number for β -actin. Differences between groups were determined using the Student's *t*-test (*, $p < 0.05$). Results are shown for (A) HSP8, (B) HSP70, and (C) HSF-1.

Table 1

DNA primers and probes used for real time quantitative RT-PCR.

Gene	GenBank accession	Sense (5'–3')	Antisense (5'–3')	Probe (5'–3') (6FAM-TAMRA)
HSP70	BC054782	CCAAGGTGCAGGTGAACTACAA	CAGCACCATGGACGAGATCTC	AGAGCCGGTCGTTCTTCCCGGA
HSP8	NM_031165	CTCGATTGAGGAGTTGAATGCT	GCATCTCGAAGGGCCTTCT	TTCCGTGGCACA CTGGACCCTGTA
HSF-1	NM_008296	GTGTCCCCCTGAAGAGTG	CCTTCATCAGCTGCACATCTG	AATACGCCAGGACAGTGTACCCGG
β-actin	M12481	CCTAAGGCCAACCGTGAAAA	GTCACCGGAGTCCATCACAAT	TCAACACCCCAGCCATGTAC