Insulin Signaling and the Heat Shock Response Modulate Protein Homeostasis in the Caenorhabditis elegans Intestine during Infection*

Akiko Mohri-Shiomi and Danielle A. Garsin

From the Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Texas 77030

During infection, damage can occur to the host as an outcome of both pathogen virulence mechanisms and host defense strategies. Using aggregation of a model polyglutamine-containing protein as an indicator in Caenorhabditis elegans, we show that protein damage occurs specifically at the site of the host-pathogen interaction, the intestine, in response to various bacterial pathogens. We demonstrate that the insulin signaling pathway and the heat shock transcription factor (HSF-1) influence the amount of aggregation that occurs, in addition to heat shock proteins and oxidative stress enzymes. We also show that addition of the antioxidants epigallocatechin gallate and α-lipoic acid reduces polyglutamine aggregation. The influence of oxidative stress enzymes and exogenous antioxidants on protein aggregation suggests that reactive oxygen species produced by the host are a source of protein damage during infection. We propose a model in which heat shock proteins and oxidative stress enzymes regulated by insulin signaling and HSF-1 are required for tissue protection during infection, to minimize the effects of protein damage occurring as a result of host-pathogen interactions.

Innate immunity is comprised of strategies that allow an organism to immediately defend itself against an invading pathogen. In addition to mechanisms that actively destroy the infecting agent, the organism must protect itself from damage. Damage to the host can occur from virulence mechanisms of the pathogen, but also as a side effect of the immune response of the host. One pathway that we have shown contributes greatly to pathogen resistance in the model host Caenorhabditis elegans is insulin signaling (1). In addition to increased pathogen resistance, loss of insulin signaling in C. elegans results in several cytoprotective phenotypes such as stress resistance (oxidative stress, heat stress) and long lifespan. The insulin signaling pathway consists of a receptor, DAF-2, that when stimulated activates a phosphatidylinositol 3-kinase signaling cascade that culminates in the phosphorylation and down-regulation of the transcription factor DAF-16. A mutation in DAF-2, or any of the other upstream signaling components prevents inhibition of DAF-16, causing greater transcriptional activity (reviewed in Refs. 2–5).

It was previously shown that pathogen resistance mediated by increased DAF-16 activity is dependent, at least in part, on the heat shock transcription factor HSF-1. The loss of hsf-1 in a daf-2 mutant, or a mutant that overexpresses daf-16, causes a reduction in pathogen resistance and the overexpression of hsf-1 increases pathogen resistance (6). HSF-1 likely mediates these effects by controlling the expression of genes encoding heat shock proteins (HSPs). HSPs are protein chaperones that bind to unfolded or damaged proteins and prevent aggregation until they can be refolded or recycled (7–11). HSPs regulated by HSF-1 were demonstrated to be protective against bacterial pathogens (6) and we showed that ROS, a possible source of cellular damage, is generated by the pathogen-exposed worm (12). Based on these data, we postulate that protein quality control is perturbed at the site of infection.

Protein homeostasis has been studied in the worm by fusing aggregation-prone polyglutamine proteins to fluorescent proteins such as yellow fluorescent protein (YFP), which has the significant advantage of allowing one to measure aggregation under various conditions in live animals using a simple visual assay (13, 14). The amount of aggregation has been documented to correlate with the global balance of protein quality, and is highly sensitive to perturbations in protein synthesis, trafficking, folding, or degradation (15, 16). In one study, Huntington-like polyglutamine (poly[Q]40) repeat protein fused to YFP was expressed in the body wall muscle cells of C. elegans. The amount of poly[Q]40::YFP aggregation increased as the worms aged indicating a loss of protein quality control (17).

However, the amount of aggregation at a given age was found to be modulated by the insulin signaling pathway and HSF-1 (17, 18). Additionally, reducing the expression of certain small HSPs (but not oxidative stress enzymes) increased aggregation (18). From this work it was concluded that insulin signaling and HSF-1 can delay aging, at least in part, by increasing small HSP expression (18).

Herein we introduce a system to study protein homeostasis in the context of infection. Because we demonstrate that the protective effects of HSF-1 arise from its expression specifically in the intestine, the site of infection, we developed transgenic worms that express poly(Q)::YFP at this location. By assaying...
polyglutamine aggregation in the intestine we show that protein quality control is perturbed during the disease process. Aggregation occurs in the intestine, but not the muscle cells, suggesting that protein homeostasis problems are only occurring at the site of infection and not throughout the worm. We show that insulin signaling and HSF-1, as well as HSPs and oxidative stress enzymes, modulate the amount of polyglutamine aggregation. Additionally, the presence of exogenous antioxidants decreases aggregation, suggesting that ROS is a significant source of protein damage during infection. We propose that a mechanism of insulin signaling and protection against bacterial pathogens by HSF-1 is through expression of genes that encode factors to prevent and ameliorate protein damage. We conclude that maintaining protein homeostasis during infection is a crucial component of the innate immune response.

**EXPERIMENTAL PROCEDURES**

*C. elegans and Bacterial Stains—* C. elegans strains were grown and maintained as previously described (19). The following bacterial strains were used in this study: Escherichia coli OP50 (20), Enterococcus faecalis OG1RF (21), Pseudomonas aeruginosa PA14 (22), Staphylococcus aureus NCTC8325 (45), and Salmonella enterica SL1344 (23). The following C. elegans strains were used in this study: wild-type N2, GF12 hsf-1(sy441), and AM141: N2rmls133 (P[unc-54] Q40::YFP) (16). All C. elegans strains used were obtained from the Caenorhabditis Genetics Center.

**DNA Cloning—hsf-1 expression constructs were generated by subcloning the full-length hsf-1 wild-type cDNA into pPD30.38 (unc-54 promoter) (gift from A. Fire, Stanford University) (24), vectors containing the F25B3.3 promoter (gift from D. Pilgrim, University of Alberta) (25), or the vha-6 promoter (gift from R. Morimoto, Northwestern University) (26). YFP-tagged polyglutamine (poly(Q)) constructs were generated by subcloning the various lengths of poly(Q)-encoding sequences into vectors containing the vha-6 promoter. YFP-tagged poly(Q) constructs cloned into pPD30.38 (Q0, Q13, Q20, Q40, Q60, and Q82) were obtained from R. Morimoto (Northwestern University) (27). Additional details of oligonucleotide sequences and restriction sites used are available on request. Successful construction was confirmed by DNA sequencing.

**Generation of Transgenic Lines—** For generation of transgenic animals, hsf-1 or poly(Q) expressing plasmids were mixed at 2 or 10 ng/μl, respectively, with the pRF4 rol-6(su1006) plasmid at 80 ng/μl and pBlueScript II for a total DNA concentration of 130 ng/μl (28). Mixtures were microinjected into the gonads of adult N2 or hsf-1(sy441) hermaphrodites, respectively, using standard methods (29). Transgenic F1 progeny were selected on the basis of roller phenotype. Individual transgenic F2 animals were picked to establish independent lines. At least two independent lines for each transgene were examined.

**C. elegans Killing Assays—** Killing assays were performed as previously described (30). 60 worms were used in each experiment. The data were analyzed using GraphPad Prism 3.0. Survival was plotted by the Kaplan-Meier method and the curves compared using the log-rank test, which generates a p value testing the null hypothesis that the survival curves are identical. p values of 0.05 or less were considered significantly different from the null hypothesis.

**Fluorescence Microscopy—** Animals were viewed with an Olympus BX60 upright epifluorescence microscope for epifluorescence, and the number of poly(Q) aggregates was counted. Aggregates were defined as discrete structures with boundaries distinguishable from surrounding fluorescence on all sides. All photographs represent what was typically seen upon examination and were taken at the same exposure and the levels manipulated identically in Adobe Photoshop CS. 20 worms were viewed for each condition, averaged, and the standard error calculated using GraphPad Prism 3.0. The significance of differences between conditions was determined by an unpaired t test. p values of 0.05 or less were considered significantly different from the null hypothesis.

**RNAi—** RNAi exposure was performed with some modifications as previously described (6). Except for the hsf-1 and hsp-6 RNAi worms, adults were allowed to lay eggs on RNAi-expressing bacteria for 24 h. The eggs were allowed to develop into L4 larvae on RNAi or vector control plates at 20 °C. hsf-1 and hsp-6 RNAi was performed as follows. L2–L3 larvae on NGM plates were collected by washing with M9 buffer, and then they were exposed to hsf-1 or hsp-6 expressing bacteria for 24 h. L4 larvae were transferred to assay plates with pathogenic or non-pathogenic bacteria. Less than 5% of the L4 worms had aggregates (data not shown). Except for the daf-2, daf-21, and sod-3 RNAi constructs, all our RNAi constructs were obtained from GeneService Ltd. (Cambridge, UK) (31, 32). The daf-2 RNAI construct was the same as used in Chavez et al. (12), and the daf-21 and sod-3 RNAI constructs were generated as described below. The identity of the clones was confirmed by sequencing.

**Generation of the Vectors for daf-21 and sod-3 RNAi—** The daf-21 RNAI construct was created by PCR amplification from total genomic DNA by using gene-specific oligonucleotides, digestion with XbaI/NcoI, and ligation into appropriately digested plasmid L4440 (gift from A. Fire, Stanford University) (33). Oligonucleotide sequences used for amplification of trigger sequences were: daf-21 forward, 5’-GCTCTAGAGTGTC-CCAGAAGCGCCGAAAC-3’ and daf-21 reverse, 5’-CATGCGATGGTTAGCTGCCATCCTCATGCG-3’ resulting in a 2280-bp product. sod-3 RNAI construct was created by digesting sod-3 cDNA from a cDNA clone yk264q10 (gift from Y. Kohara, National Institute of Genetics) with EcoRI/XhoI, and ligation into appropriately digested plasmid L4440.

**Antioxidant Experiments—** Experiments were performed as previously described with some modifications (34). Epigallocatechin-3-gallate (EGCG), a polyphenol compound, and α-lipoic acid (LA) were purchased from Sigma. Stock solutions of EGCG or LA were made in 100% ethanol and used at a final concentration of 25 μM. The chemicals were added directly to the NGM or BHI plates. The final concentration for ethanol was less than 0.1% when dissolved in the plates. Adults were allowed to lay eggs on NGM plates with no chemical, EGCG or LA. The eggs were allowed to develop into L4 larvae on the plates at 20 °C. L4 larvae were exposed to *E. coli* or *E. faecalis* at 25 °C on NGM plates or BHI plates with no chemical, EGCG or LA.
respectively. After a 2-day exposure, the percentage of worms with aggregates was counted.

RESULTS

hsf-1 Is Required in the Intestine, but Not Muscles or Nerves, for Protection against Infection—An earlier study showed that HSF-1 is crucial to mediating resistance in the worm; loss of the gene encoding HSF-1 by mutation or RNAi caused increased susceptibility, whereas overexpression of the gene caused increased resistance (6). It was shown in our previous work that E. faecalis causes a persistent infection in the intestine of the worm (30). Therefore, we predicted that HSF-1 would mediate its protective effects in this tissue. As shown in Fig. 1, an hsf-1 mutant is more susceptible to E. faecalis than wild type as previously published (6). We complemented this mutant using tissue-specific promoters (24–26). Green fluorescent protein was also put under the control of these promoters and tissue-specific expression verified (data not shown). When under the control of a neuron-specific or muscle-specific promoter, the worm strains are just as susceptible as the hsf-1 mutant. However, when we put hsf-1 under the control of an intestinal promoter, resistance was restored to wild-type levels (Fig. 1). These results demonstrate that the protective effects of HSF-1 are mediated specifically by expression in the intestine. In contrast, lifespan assays testing a dominant negative HSF-1 mutant found that HSF-1 contributed to longevity when expressed in any one of these tissues (36).

Exposure to Pathogens Enhances Polyglutamine Aggregation Specifically in the Intestine—The requirement for HSF-1 in the intestine, the site of infection, suggests the possibility of cellular protein damage at this location. For more direct evidence of protein damage, we examined infected worms for changes in protein quality control using a poly(Q) repeat protein expressed in the intestine. Formerly, Huntington-like poly(Q) repeat proteins fused to YFP were expressed in the body wall muscle cells of C. elegans and increases in aggregation observed with increases in length (17). A length of 40 repeats was found to be the threshold length, resulting in a mixture of focal fluorescence (aggregated protein) and diffuse fluorescence (non-aggregated protein), which varied depending on factors that affect protein homeostasis (16, 17). We expressed different lengths of poly(Q) fused to YFP in the worm intestine to find the threshold length in this tissue, as shown in Fig. 2A. On day 2 of adulthood, worms containing constructs with less than 44 repeats had diffuse fluorescence throughout the intestine, whereas those with more than 44 repeats had focal fluorescence. Most of the worms with a construct containing 44 repeats had diffuse fluorescence, but some had a mixture of diffuse and focal fluorescence. The percentage of worms with aggregates increases with age. Percentage of worms with aggregates in transgenics containing Q33 (○), Q40 (△), Q44 (●), Q64 (▲), and Q82 (▲) over time. L4 worms (day 0 worms) were exposed to E. coli for 2, 4, 6, and 8 days. The error bars represent the mean ± S.E.

FIGURE 2. Length-dependent aggregation of poly(Q)-YFP fusion proteins. A, fluorescence and Nomarski micrographs of 2-day-old adults on E. coli expressing different lengths of poly(Q):YFP in the intestine. Most Q44 worms had diffuse fluorescence, but some had a mixture of diffuse and focal fluorescence. B, the percentage of worms with aggregates increases with age. Percentage of worms with aggregates in transgenics containing Q33 (○), Q40 (△), Q44 (●), Q64 (▲), Q82 (▲), and Q0 (●) over time. L4 worms (day 0 worms) were exposed to E. coli for 2, 4, 6, and 8 days. The error bars represent the mean ± S.E.
Poly(Q) aggregation in the intestine occurs in response to Gram-negative and Gram-positive pathogens. L4 Q44 worms were exposed to S. aureus (NCTC8325) for 54 h (p = 0.0008) (A), P. aeruginosa (PA14) for 48 h (p = 0.0027) (B), and S. enterica (SL1344) for 3 days (p = 0.0011) (C). The average was calculated from at least three independent experiments each with an n of 20 worms. The error bars represent the mean ± S.E.

Protein Homeostasis in C. elegans during Infection

lies would increase aggregation of the threshold length polyglutamine (Q44) in the intestine compared with exposure to E. coli. In Fig. 3, A and B, 83% of Q44 worms had detectable aggregates by day 3 of exposure to E. faecalis and over 92% by day 5. In contrast, 33% of worms on E. coli had aggregates on day 3, and only 41% by day 5. These data suggest that more damage occurs in the intestine of worms exposed to the pathogen E. faecalis than on its normal food source E. coli. To test if damage occurs in other tissues, we examined the effects of exposure to E. faecalis on poly(Q)44::YFP expressed in the body wall muscle cells (17). Because all the worms had some aggregates, we counted the number of poly(Q)44::YFP aggregates rather than the number of worms with aggregates. We observed an increase in the number of aggregates over time, as previously observed (17), but we found no increase in the number of aggregates in the body wall muscle cells of worms feeding on E. coli compared with those exposed to E. faecalis (Fig. 3C). We conclude from these experiments that during infection with E. faecalis protein damage occurs at the site of the host-pathogen interaction, the intestine, and not throughout the worm.

To investigate whether or not the observed polyglutamine aggregation is specific to E. faecalis or can be generalized to other pathogens, we tested Staphylococcus aureus, P. aeruginosa, and S. enterica, other major agents of human infection that have previously been characterized as killing the worm (30, 37–39). S. aureus and P. aeruginosa kill more quickly than E. faecalis and S. enterica with an LT50 of 2 days versus 4 and 5 days (30, 37–39). In correlation with the faster course of infection, we observed aggregation occurring earlier with 90% or more of the worms containing aggregates on both pathogens after 2 days of infection (Fig. 4, A and B). In contrast, we observed less than 80% of the worms on S. enterica experiencing aggregates by day 3 of infection (Fig. 4C). However, there were still more than twice as many aggregates on S. enterica as there were on E. coli (76 versus 35%).

daf-16 and hsf-1 RNAi Increases whereas daf-2 RNAi Reduces Pathogen-induced Aggregation in the Intestine—Because HSF-1 and insulin signaling affect susceptibility to pathogens and are involved in regulating HSPs, we predicted that the protein damage resulting from infection with E. faecalis would be influenced by these factors. To test our prediction, we used RNAi to reduce the expression of hsf-1, daf-16, and daf-2 in the intestinal poly(Q)44::YFP worms. We also exposed the worms to the vector alone as a control. The worms were exposed to E. coli strains expressing the RNA of the gene of interest during development, as described under “Experimental Procedures.” We modified our procedure for hsf-1 RNAi and only exposed the worms for 1 day due to the strong effects that hsf-1 RNAi had on aggregation, even before pathogen exposure. Following RNAi, the worms were then placed on either the pathogen E. faecalis or the control strain, E. coli OP50. For hsf-1 and daf-16 RNAi, the percentage of worms with aggregates was observed after 1 day, whereas for daf-2 the percentage was recorded after 4 days. Because the loss of hsf-1 and genes in the insulin signaling pathway was previously observed to change the number of aggregates in aging assays conducted while feeding on E. coli (17, 18), we subtracted the number of aggregates under non-pathogen feeding conditions to control for aggregation occurring as a result of the aging process.
Protein Homeostasis in C. elegans during Infection

As shown in Fig. 5, A and B, and Table 1, a dramatic, statistically significant increase in the percentage containing aggregates was observed for the hsf-1 and daf-16 RNAi worms compared with those exposed to the vector control when feeding on *E. faecalis*. Only 6–7% of the worms had aggregates when exposed to the vector control, with 27 and 23% of the worms exposed to hsf-1 and daf-16 RNAi, respectively. As shown in Fig. 5C and Table 1, daf-2 RNAi (which increases DAF-16 activity) greatly decreased the amount of protein aggregation that occurs in response to pathogens. Even after 4 days of feeding on *E. faecalis*, only 10% of the daf-2 RNAi worms had aggregates compared with 28% of the vector control worms. These data suggest that HSF-1 and DAF-16 are protective against infection-induced protein aggregation.

The Loss of HSPs and Oxidative Stress Enzymes Increases Pathogen-induced Aggregation in the Intestine—DAF-16 has previously been characterized as regulating the expression of genes encoding oxidative stress enzymes and both DAF-16 and HSF-1 have been implicated in the expression of genes encoding HSPs (18, 40). Some HSPs and some oxidative stress enzymes were found to be protective during infection (6, 12). Additionally, HSPs were characterized as protective against aggregation of polyglutamine in the muscle cells of aging worms (18). We therefore postulated that DAF-16 and HSF-1 ameliorate protein damage during infection by one or both classes of these proteins. Heat shock proteins would be expected to prevent protein aggregates by refolding or recycling damaged proteins. Oxidative stress enzymes might prevent protein damage from occurring in the first place by neutralizing ROS.

To test if HSPs prevent protein damage during infection, we reduced the expression of the encoding genes by RNAi and exposed to *E. faecalis* for 1 day as described in the previous section. As before, the percentage of worms with aggregates occurring in *C. elegans* exposed to their normal laboratory food source (*E. coli* OP50) was subtracted out to control for non-infection-related processes. As shown in Table 1, reduction of the expression of daf-21, hsp-12.6, hsp-16.41, F08H9.3, and F08H9.4 genes by RNAi resulted in a statistically significant increase in the number of aggregates caused by infection. These data suggest that these HSPs protect against protein damage specific to that occurring during the infection.

**TABLE 1**

Infection-induced poly(Q) aggregation of RNAi worms after exposure to *E. faecalis* for 24 h (mean ± S.E.)

| Gene name (Cosmid No.) | Gene description | RNAi clone name | Aggregates | n | p value* |
|------------------------|-----------------|----------------|------------|---|---------|
| Vector                 |                 |                |            | 18|         |
| Vector*                |                 |                | 6.09 ± 1.88| 7 | 0.0024  |
| Vector**               |                 |                | 7.78 ± 2.22| 9 |         |
| hsf-1 (Y53C10A.12)*    | Transcription factor | JA:Y53C10A.12 | 26.56 ± 5.51| 7 | 0.0089  |
| daf-16 (R13H8.1)       | Insulin/IGF receptor | JA:R13H8.1    | 22.72 ± 3.46| 5 |         |
| daf-2 (Y55D3A.5)*      | HSP90           | pAMS14         | 10.00 ± 5.00| 3 | 0.0254  |
| daf-21 (C47E8.5)       |                 | pAMS71         | 21.67 ± 3.33| 3 | 0.0194  |
| hsp-12.6 (F38E11.2)    | Small heat shock protein | JA:F38E11.2 | 39.00 ± 4.85| 5 | 0.0013  |
| hsp-16.41 (Y46H3A.2)   | Small heat shock protein | JA:Y46H3A.2  | 32.00 ± 4.06| 5 | 0.0035  |
| (F08H9.3)              |                 | JA:F08H9.3    | 29.00 ± 4.58| 5 |         |
| (F08H9.4)              |                 | JA:F08H9.4    | 26.24 ± 5.98| 7 | 0.0204  |
| hsp-16.1 (T27E4.8)     | Small heat shock protein | JA:T27E4.8 | 12.00 ± 6.25| 5 | 0.7875  |
| sip-1 (F43D9.4)        | Small heat shock protein | JA:F43D9.4  | 3.00 ± 2.55 | 5 | 0.1470  |
| hsp-70 (C12C8.1)       | HSP70           | JA:C12C8.1    | 14.00 ± 6.00| 5 | 0.5815  |
| (C30C11.4)             |                 | JA:C30C11.4   | 7.00 ± 5.61 | 5 | 0.6631  |
| hsp-70 (C37H5.8)*      | HSP70           | JA:C37H5.8    | 1.76 ± 3.95 | 5 | 0.1587  |
| (F44E5.4)              |                 | JA:F44E5.4    | 14.00 ± 8.72| 5 | 0.6819  |
| (F44E5.5)              |                 | JA:F44E5.5    | 21.00 ± 6.60| 5 | 0.1798  |
| hsp-6 (C37H5.8)*       | HSP70           | JA:C37H5.8    | 1.76 ± 3.95 | 5 | 0.1587  |
| ciz-1, -2, -3 (Y54G11A.6, 5, 13) | Catalases | JA:Y54G11A.6 | 26.67 ± 8.03| 6 | 0.0332  |
| ciz-2 (Y54G11A.5)      | Catalase        | JA:Y54G11A.5  | 21.67 ± 6.41| 6 | 0.0472  |
| sod-1 (C15F1.7)        | Superoxide dismutase | JA:C15F1.7 | 20.00 ± 2.65 | 6 | 0.0191  |
| sod-2 (F10D11.1)       | Superoxide dismutase | JA:F10D11.1 | 15.00 ± 4.66 | 6 | 0.1190  |
| sod-3 (C08A9.1)        | Superoxide dismutase | VA:CH1     | 18.33 ± 4.22 | 6 | 0.0412  |
| sod-4 (F35H2.1)        | Superoxide dismutase | JA:F35H2.1 | 29.17 ± 6.51 | 6 | 0.0095  |
| sod-5 (ZK430.6)        | Superoxide dismutase | JA:ZK430.6 | 18.33 ± 8.43 | 6 | 0.1653  |

*Unpaired t test. p values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time. p < 0.05 was considered significantly different from the null hypothesis.

*Exposed to RNAi bacteria for 24 h.

*Exposed to OP58 or OG1RF for 4 days.
In contrast to the HSPs, loss of oxidative stress enzymes did not cause increased aggregation in the muscle cells of aging worms (18). However, we had shown that ROS is generated by the worm in response to pathogens and oxidative stress enzymes expressed in the intestine (12). Therefore, we predicted that oxidants are a significant source of protein damage and loss of oxidative stress enzymes would increase polyglutamine aggregation in the intestine. When we measured the effects of genes encoding oxidative stress enzymes on polyglutamine aggregation we found that loss of ctl-2, sod-1, sod-3, and sod-4 by RNAi caused a statistically significant increase in the percentage of worms with aggregates due to infection (Table 1). One RNAi clone (JA:Y54G11A.6) targets all three catalase genes and also caused a significant increase in the number of aggregates. These data support our hypothesis that oxidative stress enzymes protect against protein damage that occurs during infection.

It is important to note that because of homology between some of the examined genes, our RNAi constructs may have targeted more than one gene for reduced expression. The additional targets of each RNAi construct are noted in Worm Base (www.wormbase.org). Therefore, the phenotype caused by any one RNAi construct may have resulted from the combined reduction in the expression of a few genes, making it difficult to discern the importance of an individual gene in some cases. However, we can conclude that both heat shock proteins and oxidative stress enzymes, in general, play an important protective role in protein integrity during infection.

**Antioxidants Reduce the Number of Worms with Aggregates**—Our results suggest that ROS are a significant source of protein damage during infection because loss of some oxidative stress enzymes increased protein aggregation (Table 1). To examine the influence of ROS in a different way we added the natural antioxidants EGCG and α-lipoic acid to our assay plates. These compounds have free radical scavenging activity and were previously shown to reduce the levels of ROS in *C. elegans* and extend lifespan (34). Poly(Q)44::YFP worms were exposed to these compounds throughout their development and during the killing assay at a concentration of 25 μM, a value previously demonstrated to influence levels of ROS and lifespan (34). The number of worms with aggregates was counted on day 2 of exposure to *E. faecalis* and compared with worms not exposed to these compounds. The number of worms with aggregates occurring during non-pathogen (OP50) exposure was subtracted out to control for any changes in aggregation due to non-infection related processes. As shown in Fig. 6, the percentage of worms with infection-induced aggregation was on average 40% under control conditions when no antioxidants were added. In comparison, the addition of EGCG or α-lipoic acid to the plates reduced the average percentage of worms with infection-induced aggregation to 4 and 13%, respectively, in a statistically significant manner. These results support our proposition that ROS contribute to the protein damage occurring during infection.

**DISCUSSION**

In this work we demonstrate that protein damage, as assayed by polyglutamine aggregation, occurs during bacterial infection of the worm. Damage was evident in the intestine (the site of infection), but not in the body wall muscle cells, and is modulated by the insulin signaling pathway and HSF-1. Importantly, HSF-1 was shown to protect against infection when produced exclusively in the intestine, but had no influence on *C. elegans* killing when produced only in the body wall muscle or the neuronal tissue. The level of protein damage was also affected by some of the genes that insulin signaling and HSF-1 regulate, including those encoding protein chaperones and oxidative stress enzymes (18, 40). The damage appears to be at least in part caused by ROS production, as loss of oxidative stress enzymes increases the aggregation and addition of antioxidants ameliorates the damage. These data support our model that protein damage accumulates at the site of infection and insulin signaling and HSF-1 modulate killing, at least in part, by regulating the expression of genes with protective effects.

Previous studies in other systems demonstrated that synthesis of heat shock proteins increases during infection and a role for HSPs in various aspects of mammalian acquired immunity has been postulated. For example, several studies point to the involvement of HSPs in antigen processing and presentation; their chaperone activity promotes folding and assembly of major histocompatibility complex-peptide complexes. It has been also shown that HSPs frequently remain associated with the peptide after it has bound major histocompatibility complex. This HSP-peptide derivative is often more immunogenic than either component alone. The association of HSPs with antigen presenting cells also leads to the activation of non-antigen-associated immune mechanisms such as the release of cytokines and reactive radicals (reviewed by Refs. 41 and 42)). *C. elegans* lacks acquired immunity and designated immune cells, yet HSPs are also vital during *C. elegans* immune response. We
Protein Homeostasis in C. elegans during Infection

speculate that HSPs play a fundamental role in immune response in most organisms simply by their ability to protect against protein damage occurring as a result of “immunological stress” induced by the attacking pathogen and the defending host.

The influence of oxidative stress enzymes on infection-induced polyglutamine aggregation is in contrast to what was previously observed in the body wall muscle cells of aging worms. Hsu et al. (18) found that RNAi of ctl-1 or sod-3 did not increase the number of aggregates. However, reducing the expression of several heat shock proteins increased aggregation, in agreement with what we found in the intestine during infection (18). The difference in the influence of oxidative stress on protein aggregation could be due to the tissue, body wall muscle versus intestine, or to the type of stress, aging compared with infection. To distinguish between these two possibilities we assayed aggregation during aging in the intestinal poly(Q)::YFP worms exposed to RNAi of the oxidative stress enzymes listed in Table 1. We found no increase in aggregation in the worms exposed to RNAi encoding the oxidative stress enzymes as compared with the vector control (data not shown). Therefore we conclude that oxidative stress enzymes are not as important in maintaining protein homeostasis during aging in the intestine as compared with infection. We postulate that infection causing a concentrated release of ROS, as measured in our previous study (12), increases the relative importance of the oxidative stress enzymes in controlling protein damage in the context of infection compared with aging.

The insulin signaling pathway and HSF-1 has been shown to affect resistance to various forms of stress such as oxidizing agents and heat (2, 5, 18). The levels of DAF-16 and HSF-1 activity are also correlated with lifespan and pathogen resistance (1, 6, 18, 35, 43, 44). One commonality between these various phenotypes is that they all require resistance to damage. In this work we have shown that cellular protein damage occurs during infection of C. elegans and is possibly a major factor contributing to poor outcome. The importance of resisting and repairing damage in the context of innate immunity is therefore crucial to survival. Of interest is the fact that aging and infection cause similar types of cellular damage and are similarly, but not exactly, influenced by the same proteic signaling pathways and mechanisms. This may explain why age influences infection outcome and infection and disease appear to influence the rate of aging.

Acknowledgments—We thank R. Morimoto and S. Fox for hsf-1 cDNA, a vha-6 promoter, the polyglutamine constructs, and technical support; D. Pilgrim for a F25B3.3 promoter; A. Fire for pdP vectors; Z. Zhou for pdPD49.26; F. Ausubel for PA14 and SL1344; Y. Kohara for yk clones. We thank the Caenorhabditis Genetics Center for the strains used in this study, which is funded by the NIH National Center for Research Resources (NCRR); J. Ahringer and Geneservice Ltd. for RNAi strains; and addgene for pdPD30.38. We gratefully acknowledge Z. Zhou for use of the injection microscope and W. Margolin for use of the fluorescent microscopy facility. We also thank K. Morano and M. Lorenz for helpful comments on the manuscript.

REFERENCES

1. Garsin, D. A., Villanueva, J. M., Begun, J., Kim, D. H., Sifri, C. D., Calderwood, S. B., Ruvkun, G., and Ausubel, F. M. (2003) Science 300, 1921
2. Finch, C. E., and Ruvkun, G. (2001) Ann. Rev. Genomics Hum. Genet. 2, 435–462
3. Guarente, L., and Kenyon, C. (2000) Nature 408, 255–262
4. Nelson, D. W., and Padgett, R. W. (2003) Genes Dev. 17, 813–818
5. Tatár, M., Bartke, A., and Antebi, A. (2003) Science 299, 1346–1351
6. Singh, V., and Aballay, A. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 13092–13097
7. Buchner, J. (1996) FASEB J. 10, 10–19
8. Frydman, J. (2001) Annu. Rev. Biochem. 70, 603–647
9. Sakahira, H., Breuer, P., Hayer-Hartl, M. K., and Hartl, F. U. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16412–16418
10. Horvitz, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10449–10453
11. Van Montfort, R., Slingsby, C., and Vierling, E. (2001) Adv. Protein Chem. 59, 105–156
12. Chavez, V., Mohri-Shiomi, A., Madani, A., Vega, L. A., and Garsin, D. A. (2007) Genetics 176, 1567–1577
13. Brignull, H. R., Morley, J. F., Garcia, S. M., and Morimoto, R. I. (2006) Methods Enzymol. 412, 256–282
14. Brignull, H. R., Morley, J. F., and Morimoto, R. I. (2007) Adv. Exp. Med. Biol. 594, 167–189
15. Gidalevitz, T., Ben-Zvi, A., Ho, K. H., Brignull, H. R., and Morimoto, R. I. (2006) Science 311, 1471–1474
16. Nollen, E. A., Garcia, S. M., van Haastten, G., Kim, S., Chavez, A., Morimoto, R. I., and Plasterk, R. H. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 6403–6408
17. Morley, J. F., Brignull, H. R., Weyers, J. J., and Morimoto, R. I. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10417–10422
18. Hsu, A. L., Murphy, C. T., and Kenyon, C. (2003) Science 300, 1142–1145
19. Hope, I. A. (1999) in C. elegans, A Practical Approach (Hope, I. A., ed) pp. 51–67, Oxford University Press, Oxford
20. Brenner, S. (1974) Genetics 77, 71–94
21. Dunny, G. M., Brown, B. L., and Clewell, D. B. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 3479–3483
22. Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., and Ausubel, F. M. (1999) Cell 96, 47–56
23. Wray, C., and Sojka, W. J. (1978) Res. Vet. Sci. 25, 139–143
24. Kelly, W. G., Xu, S., Montgomery, M. K., and Fire, A. (1997) Genetics 146, 227–238
25. Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y., and Hobert, O. (2001) Development 128, 1951–1969
26. Wang, J., Tokarz, R., and Savage-Dunn, C. (2002) Development 129, 4989–4998
27. Satyal, S. H., Schmidt, E., Kitagawa, K., Sondheimer, N., Lindquist, S., Kramer, J. M., and Morimoto, R. I. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5750–5755
28. Kramer, J. M., French, R. P., Park, E. C., and Johnson, J. J. (1990) Mol. Cell. Biol. 10, 2081–2089
29. Mello, C. C., Kramer, J. M., Stinchcomb, D., and Ambros, V. (1991) EMBO J. 10, 3959–3970
30. Garsin, D. A., Sifri, C. D., Mylonakis, E., Qin, X., Singh, K. V., Murray, B. E., Calderwood, S. B., and Ausubel, F. M. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10892–10897
31. Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000) Nature 408, 325–330
32. Kamath, R. S., Fraser, A. G., Dong, Y., Poulin, G., Dubin, R., Gotta, M., Kanapin, A., Le Bot, N., Moreno, S., Sohrmann, M., Welchman, D. P., Zipperlen, P., and Ahringer, J. (2003) Nature 421, 231–237
33. Timmons, L., and Fire, A. (1998) Nature 395, 854
34. Brown, M. K., Evans, J. L., and Luo, Y. (2006) Pharmacol. Biochem. Behav. 85, 620–628
35. Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997) Nature 389, 994–999
36. Morley, J. F., and Morimoto, R. I. (2004) Mol. Biol. Cell 15, 657–664
37. Aballay, A., Yorgey, P., and Ausubel, F. M. (2000) Curr. Biol. 10,
1539–1542
38. Labrousse, A., Chauvet, S., Couillault, C., Kurz, C. L., and Ewbank, J. J. (2000) Curr. Biol. 10, 1543–1545
39. Tan, M. W., Mahajan-Miklos, S., and Ausubel, F. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 715–720
40. Murphy, C. T., McCarroll, S. A., Bargmann, C. I., Fraser, A., Kamath, R. S., Ahringer, J., Li, H., and Kenyon, C. (2003) Nature 424, 277–283
41. Srivastava, P. (2002) Nat. Rev. Immunol. 2, 185–194
42. Zugel, U., and Kaufmann, S. H. (1999) Clin. Microbiol. Rev. 12, 19–39
43. Garigan, D., Hsu, A. L., Fraser, A. G., Kamath, R. S., Ahringer, J., and Kenyon, C. (2002) Genetics 161, 1101–1112
44. Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993) Nature 366, 461–464
45. Iandolo, J. J. (2000) Gram-positive Pathogens (Fischetti, V. A., Novick, R. P., Ferretti, J. I., Portnoy, D. A., and Rood, J. A., eds) pp. 317–325, ASM Press, Washington, D.C.