Transcriptional targets of DAF-16 insulin signaling pathway protect *C. elegans* from extreme hypertonic stress

S. Todd Lamitina and Kevin Strange

*Department of Molecular Physiology and Biophysics and Department of
Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee*

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All cells adapt to hypertonic stress by regulating their volume after
shrinkage, by accumulating organic osmolytes, and by activating
mechanisms that protect against and repair hypertonicity-induced
damage. In mammals and nematodes, inhibition of signaling from the
DAF-2/IGF-1 insulin receptor activates the DAF-16/FOXO transcrip-
tion factor, resulting in increased life span and resistance to some
types of stress. We tested the hypothesis that inhibition of insulin
signaling in *Caenorhabditis elegans* also increases hypertonic stress
resistance. Genetic inhibition of DAF-2 or its downstream target, the
AGE-1 phosphatidylinositol 3-kinase, confers striking resistance to a
normally lethal hypertonic shock in a DAF-16-dependent manner.
However, insulin signaling is not inhibited by or required for adap-
tation to hypertonic conditions. Microarray studies have identified 263
genes that are transcriptionally upregulated by DAF-16 activation. We
identified 14 DAF-16-upregulated genes by RNA interference screen-
ing that are required for *age-1* hypertonic stress resistance. These
genes encode heat shock proteins, proteins of unknown function, and
trehalose synthesis enzymes. Trehalose levels were elevated approx-
imately twofold in *age-1* mutants, but this increase was insufficient to
prevent rapid hypertonic shrinkage. However, *age-1* animals unable to
synthesize trehalose survive poorly under hypertonic conditions. We
conclude that increased expression of proteins that protect eukaryotic
cells against environmental stress and/or repair stress-induced molec-
ular damage confers hypertonic stress resistance in *C. elegans daf-2/
age-1* mutants. Elevated levels of solutes such as trehalose may also
function in a cytoprotective manner. Our studies provide novel in-
sights into stress resistance in animal cells and a foundation for new
studies aimed at defining molecular mechanisms underlying these
essential processes.

hypertonicity; *daf-2*; *age-1*; RNA interference

THE ABILITY TO CONTROL osmotic balance and repair osmotic
stress-induced damage is essential for cellular life. Organisms
and cells that live and function in osmotically unstable envi-
ronments such as the soil, the intertidal zone, and the mam-
malian gut and renal medulla are exposed regularly to large
fluctuations in extracellular tonicity. When exposed to hyper-
tonic stress, cells lose water and shrink. Adaptation to hyper-
tonicity involves three distinct processes. In response to shrink-
age, regulatory volume increase (RVI) mechanisms are acti-
vated rapidly and cell volume is regained through membrane
transport-mediated accumulation of inorganic ions and osmot-
ically obliged water (29). Both cell shrinkage and RVI inor-
ganic ion accumulation increase intracellular ionic strength,

which can disrupt cellular structure and function. Thus, when
exposed chronically to hypertonic stress, cells activate mech-
anisms that mediate the accumulation of "nonperturbing" or-
ganic osmolytes (52). As organic osmolytes are accumulated,
inorganic ions are lost from the cytoplasm and ionic strength is
lowered. Cells exposed to hypertonic stress also activate mech-
anisms that detect, repair, and protect against hypertonicity-
induced damage (6, 12, 27).

The effector mechanisms that mediate inorganic ion and
organic osmolyte accumulation in animal cells are generally
well understood (4, 39). However, major gaps exist in our
understanding of the signals and signaling pathways by which
cells detect volume perturbations and activate regulatory mech-
anisms. In addition, relatively little is known about how cells
sense and repair damage induced by hypertonic stress.

We demonstrated recently (28) that the soil-dwelling nem-
atode *Caenorhabditis elegans* survives and readily adapts to
osmotically stressful environments. Nematodes provide many
powerful experimental advantages for defining the molecular
mechanisms that allow animal cells to adapt to osmotic stress
(47). These advantages include forward and reverse genetic
tractability, easy laboratory culture, a fully sequenced and
well-annotated genome, and the availability of numerous mo-
lecular reagents, bioinformatics resources, and mutant worm
strains.

Mutations that inhibit insulin/IGF-like signaling in *C. el-
egans* activate the DAF-16/FOXO transcription factor and
increase life span and resistance to thermal, oxidative, and
hypoxic stress (22, 36, 43). Recent microarray analyses have
identified 263 genes that are transcriptionally upregulated in
these mutants (37, 38). Here, we demonstrate that insulin
signaling mutants exhibit a striking increase in hypertonic
stress resistance. However, insulin signaling is not osmotically
regulated or required for adaptation to hypertonic environ-
ments. Instead, inhibition of insulin signaling activates genes
that protect against and/or function in the repair of hypertonic-
ity-induced damage. Our studies provide novel insights into
osmoadaptation and stress resistance in animal cells and pro-
vide a foundation for new studies aimed at defining the mo-
lecular mechanisms underlying these essential processes.

MATERIALS AND METHODS

C. elegans strains. Nematodes were cultured at 16–25°C on agar
plates by standard methods. The following strains were used: Bristol
strain N2 (wild type), *daf-2(e1370)*, *age-1(hx546)*, *daf-16(m26)*,
daf-2(e1370); daf-16(m26), age-1(m333); daf-16(m26), zIs356

(P_{DAF-16}::DAF-16-GFP;*rol-6*). All *daf-2*, *age-1*, and *daf-16* mutants used in this study harbor loss-of-function mutations in their respective genes.

Survival studies. Growth agar tonicity was increased by adding NaCl (28). Synchronized (31) late L4 larvae and young adults were transferred to hypertonic agar, and survival was determined after 24 h. Worms were considered to be dead if they did not respond to repeated prodding with a platinum wire.

Volume and osmolyte determinations. Worms were imaged with a dissecting microscope and a charge-coupled device video camera. Images were digitized and analyzed with Metamorph imaging software (Universal Imaging, Downingtown, PA). Total body volume was calculated by measuring length and width at the widest point of the animal and by assuming that the body shape approximates a cylinder.

Whole worm glycerol content was quantified as described previously (28). Trehalose content was quantified by similar methods. Briefly, neutralized perchloric acid worm extracts were exposed to either 10 μl of trehalase buffer (50% glycerol, 1% Triton X-100, and 25 mM KH_2PO_4) or 10 μl of buffer containing 2 mU/μl of porcine trehalase (Sigma, St. Louis, MO). Samples were incubated at 37°C for 6 h, and glucose liberated by trehalase digestion was quantified with a commercially available kit (R-Biopharm, Marshall, MI).

GFP localization. Adult hermaphrodites expressing DAF-16::GFP were anesthetized for 20 min in control or hypertonic M9 buffer containing 0.1% tricaine and 0.01% tetramisole. Anesthetized worms were transferred to control or 200 mM NaCl 2% agar pads and imaged with a Nikon TE2000 inverted microscope. Differential interference contrast and fluorescence Z sections were captured for each worm. The Z section giving the clearest image of intestinal cell nuclei was used for quantifying the nucleus-to-cytoplasm green fluorescent protein (GFP) fluorescence ratio. Mean pixel intensity in the nucleus and an adjacent area of the cytoplasm was determined with Metamorph imaging software.

RNA interference. RNA interference (RNAi) gene knockdown was induced by feeding worms *Escherichia coli* (strain HT115) producing double-stranded RNA (dsRNA) (49) with a commercially available library (MRC geneservice, Cambridge, UK). Each *E. coli* strain in the library expresses dsRNA homologous to a single gene in the *C. elegans* genome. The effect of gene knockdown on hypertonic survival of *age-1* mutants was determined by transferring F_1 young adult progeny of dsRNA-fed animals to RNAi feeding plates containing 450, 500, and 550 mM NaCl spotted with dsRNA-producing bacteria. Plates were maintained at 25°C, and survival was measured after 20–24 h.

The hypertonic stress resistance of worms under RNAi feeding conditions was highly variable between experiments. This was likely due in part to functional redundancy of certain genes and to the involvement of multiple DAF-16-regulated genes in the hypertonic stress resistance phenotype. In addition, we found that RNAi feeding frequently increased hypertonic stress resistance compared with worms fed bacteria not producing dsRNA. The slow growth characteristics of HT115 dsRNA-producing bacteria may induce mild starvation, which would activate DAF-16 (18) and increase hypertonic stress resistance.
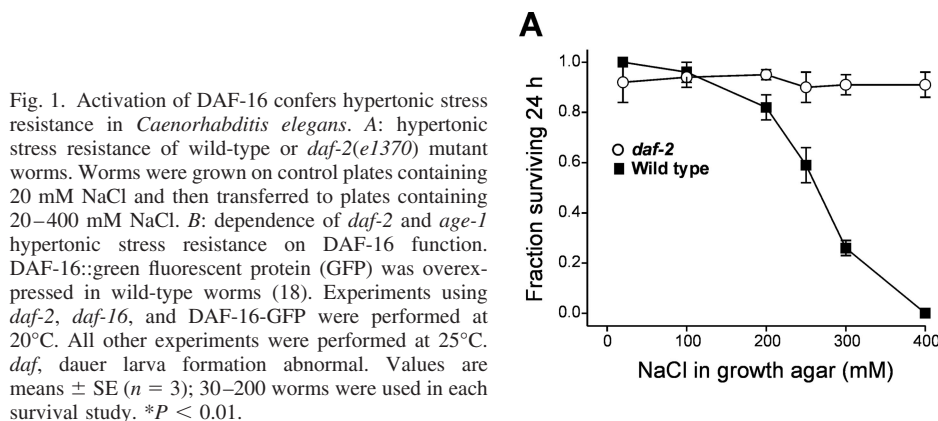
Given the variability in screening results, each target gene was initially screened two times. Genes that suppressed *age-1*-mediated hypertonic stress resistance when knocked down by RNAi in one or both trials were rescreened two to five times by exposing worms to RNAi plates containing 500 or 525 mM NaCl. Only genes that consistently exhibited RNAi-induced suppression of *age-1*-mediated hypertonic stress resistance are reported. Because of the interexperiment variability, we did not perform statistical analyses on these data and instead report the results of all individual experiments. Survival data were normalized to control worms fed bacteria producing GFP dsRNA. The sequences of all positive RNAi clones were confirmed by DNA sequencing.

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined with Student's two-tailed *t*-test for unpaired means. When comparing three or more groups, statistical significance was determined by one-way analysis of variance. *P* values of ≤0.05 were taken to indicate statistical significance.

RESULTS

Insulin/IGF-like signaling pathway mutants are osmotolerant. L2 stage *C. elegans* larvae can enter a "dauer" state under unfavorable environmental conditions of low food and high population density (17). Dauers are long-lived and stress tolerant (35). On return to more favorable conditions, dauers resume normal development. *daf* (dauer larva formation abnormal) mutants exhibit abnormal dauer formation. Several *daf* mutations affect genes that comprise an insulin/IGF-like signaling pathway (13, 26, 32, 33). Adult insulin signaling mutants are long-lived and exhibit increased resistance to thermal, oxidative, and hypoxic stress (3, 22, 36, 43).

We postulated that insulin signaling mutants also possess increased hypertonic stress resistance. As shown in Fig. 1*A*, wild-type worms survive well when exposed for 24 h to growth agar containing up to 200 mM NaCl. Survival is decreased with higher NaCl concentrations, and <10% of worms survive

Fig. 1. Activation of DAF-16 confers hypertonic stress resistance in *Caenorhabditis elegans*. *A*: hypertonic stress resistance of wild-type or *daf-2(e1370)* mutant worms. Worms were grown on control plates containing 20 mM NaCl and then transferred to plates containing 20–400 mM NaCl. *B*: dependence of *daf-2* and *age-1* hypertonic stress resistance on DAF-16 function. DAF-16::green fluorescent protein (GFP) was overexpressed in wild-type worms (18). Experiments using *daf-2*, *daf-16*, and DAF-16-GFP were performed at 20°C. All other experiments were performed at 25°C. *daf*, dauer larva formation abnormal. Values are means ± SE (*n* = 3); 30–200 worms were used in each survival study. **P* < 0.01.

a 24-h 400 mM NaCl exposure. Most of the worms surviving 400 mM NaCl appeared to be unhealthy and moved poorly.

daf-2 encodes the only insulin-like receptor gene in the *C. elegans* genome. A loss-of-function mutation in *daf-2* confers greatly increased hypertonic stress resistance (Fig. 1*A*). Over 80% of *daf-2* mutant worms survived a 24-h exposure to 400 mM NaCl growth agar (Fig. 1*A*). Surviving worms were healthy and showed activity levels similar to those of wild-type worms grown under control conditions.

To determine whether the increased hypertonic stress resistance was due to disruption of insulin signaling rather than mutation of *daf-2* per se, we characterized survival in *age-1* and *daf-16* loss-of-function mutants. *age-1* encodes a phosphatidylinositol 3-kinase (PI3-kinase) activated by ligand binding to DAF-2. As shown in Fig. 1*B*, *age-1* mutants also exhibit >80% survival on 400 mM NaCl. Therefore, inhibition of insulin signaling via loss-of-function mutations in *daf-2* or *age-1* results in increased hypertonic stress resistance in *C. elegans*.

Phenotypes arising from *daf-2* and *age-1* loss-of-function mutations require the FOXO-type transcription factor DAF-16 (Ref. 33; also see Fig. 6). We therefore examined hypertonic stress resistance in *daf-2* and *age-1* mutants carrying a loss-of-function mutation in *daf-16* (i.e., *daf-2;daf-16* and *age-1;daf-16* double mutants). As shown in Fig. 1*B*, loss-of-function mutation of *daf-16* suppressed the effect of *daf-2* and *age-1* loss-of-function mutations. Survival in the double mutants was similar to that of wild-type animals 24 h after exposure to 400 mM NaCl (Fig. 1*B*). In addition, overexpression of DAF-16 in wild-type animals, which extends life span and increases resistance to ultraviolet light and heat stress (18), also increases resistance to hypertonic stress (Fig. 1*B*). Together, these results demonstrate that DAF-16 is required for the increased hypertonic stress resistance of *daf-2* and *age-1* loss-of-function mutants.

Insulin signaling is not activated by hypertonic stress or required for osmoadaptation. Activation of the *age-1* PI3-kinase initiates a signaling cascade that leads to the phosphorylation of DAF-16 (34). Phosphorylated DAF-16 is thought to be inactive (19, 30) and is distributed equally between the cytoplasm and the nucleus (18, 34). Environmental stressors such as heat, oxidative stress, and starvation cause accumulation of a DAF-16-GFP fusion protein within the nucleus of *C. elegans* somatic cells, presumably due to inhibition of insulin signaling with subsequent dephosphorylation of DAF-16 (Ref. 18; also see Fig. 6). Therefore, the localization of DAF-16-GFP is thought to reflect the activity of the insulin signaling pathway.

Data shown in Fig. 1 suggest that insulin signaling might be inhibited by hypertonic stress. To assess this possibility, we examined DAF-16-GFP fusion protein localization in control worms, heat-shocked worms, and worms exposed to hypertonic growth agar. As shown in Fig. 2, DAF-16-GFP is distributed approximately equally between the nucleus and the cell cytoplasm in control worms. Exposure of worms to 37°C for 1 h induced a striking and significant ($P < 0.001$) translocation of the protein into the nucleus (Fig. 2, *A*, *B*, and *D*). However, exposure of worms to 200 mM NaCl for 1, 5, or 24 h had no significant ($P > 0.05$) effect on DAF-16-GFP distribution (Fig. 2, *A*, *C*, and *D*). Exposure to more extreme hypertonic stress (400 mM NaCl) also failed to induce DAF-16-GFP
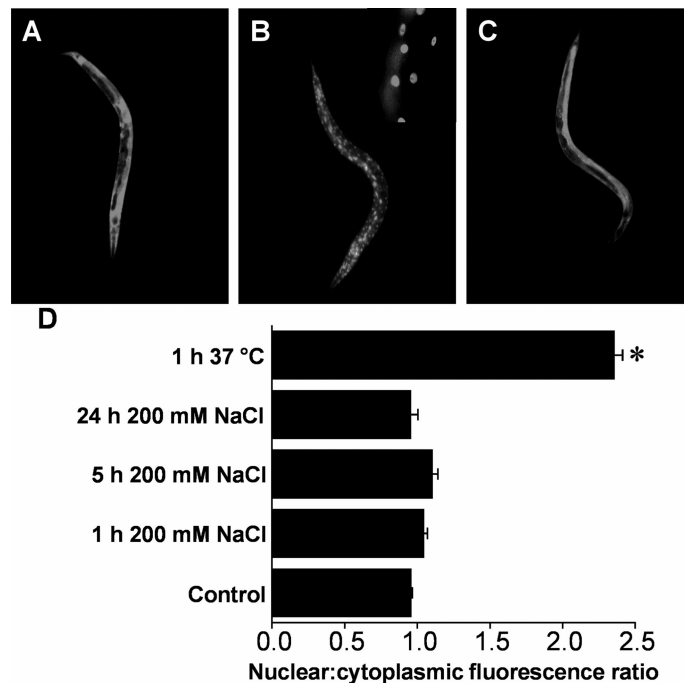

Fig. 2. Hypertonic stress does not activate DAF-16. *A–C*: cellular localization of DAF-16::GFP under different stress conditions. Synchronized young adults were exposed to 20 mM NaCl (*A*), 37°C for 60 min (*B*), or 300 mM NaCl for 4 h (*C*). *Inset* in *B* shows nuclei in intestinal cells. *D*: nucleus-to-cytoplasm ratio of GFP fluorescence in intestinal cells. The average pixel intensity of a region corresponding to the intestinal nucleus was divided by the average pixel intensity of a region within the intestinal cytoplasm. Values are means ± SE ($n = 3$). *$P < 0.001$ compared with control.

nuclear translocation (data not shown). These data suggest that hypertonic stress does not inhibit insulin signaling and induce subsequent translocation of DAF-16 into the nucleus.

As a further test for hypertonic stress-induced inhibition of insulin signaling, we assessed the ability of *daf-16* loss-of-function mutant worms to survive and adapt to hypertonic environments. Figure 3*A* demonstrates that the survival of wild-type worms and *daf-16* loss-of-function mutants is similar during acute exposure to hypertonic stress.

When grown on 200 mM NaCl, wild-type worms are able to survive well on growth agar containing 400 mM NaCl (28). If insulin signaling is inhibited by hypertonicity and is required for hypertonic stress resistance, then *daf-16* loss-of-function mutant worms should be unable to adapt to hypertonic conditions. As shown in Fig. 3*B*, unadapted *daf-16* worms survive poorly when exposed to 400 mM NaCl. However, when grown on 200 mM NaCl for 1–2 wk, survival on 400 mM NaCl is similar to that observed in adapted wild-type worms. Because inhibition of insulin signaling leads to DAF-16 nuclear localization and subsequent increase in stress resistance (18, 24), the results shown in Figs. 2 and 3 demonstrate that insulin signaling through DAF-16 is not altered by hypertonic stress or required for adaptation to hypertonic environments.

Resistance of age-1 loss-of-function mutants to osmotic stress is not mediated by enhanced glycerol accumulation. Organic osmolytes play essential roles in adaptation of cells and organisms to hypertonic environments (52). When exposed to high-NaCl growth conditions, *C. elegans* accumulates the organic osmolyte glycerol via synthesis from metabolic pre-

Fig. 3. DAF-16 is not required for acute hypertonic stress resistance or for adaptation to hypertonic conditions. *A*: hypertonic stress resistance of wild-type and *daf-16*(*m26*) loss-of-function mutants. Worms were grown on control plates containing 20 mM NaCl and then transferred to plates containing 20–400 mM NaCl. *B*: adaptation of wild-type and *daf-16*(*m26*) loss-of-function mutants to hypertonicity. Worms were grown on 20 mM NaCl or 200 mM NaCl for 1–2 wk and then transferred to plates containing 400 mM NaCl. Values are means ± SE (*n* = 3–4); 15–50 worms were used in each survival study. *$P < 0.01$.
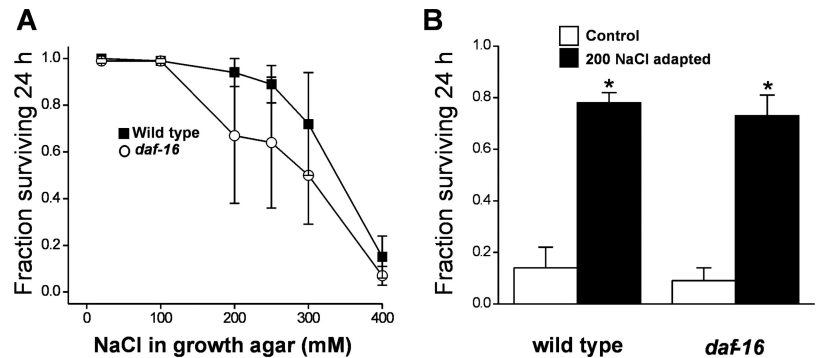
cursors (28). In yeast, glycerol 3-phosphate dehydrogenase (GPD) is rate limiting for glycerol synthesis and expression of GPD1 is upregulated during exposure to hypertonicity (1). Similarly, expression of the *C. elegans* GPD1 homolog F47G4.3 is upregulated when animals are exposed to high-NaCl agar (28).

Metabolism is substantially altered in dauer larvae, and transcriptional expression of F47G4.3 as well as another GPD homolog, K11H3.1, is upregulated severalfold in this developmental stage (51). This suggested that glycerol metabolism might be altered in insulin signaling mutants. An enhanced rate of glycerol accumulation or increased basal glycerol levels could account for increased resistance to hypertonic stress. To test this possibility, we measured glycerol levels in *age-1* loss-of-function mutant worms under control conditions and 6 h after exposure to 200 mM NaCl. Recently, we showed (28) that exposure of wild-type worms to 200 mM NaCl for 6 h increases glycerol levels approximately fivefold, from ~40 to ~210 nmol/mg protein. In contrast, glycerol levels in *age-1* worms exposed to 200 mM NaCl for 6 h increased only approximately twofold, from 73 ± 2 (*n* = 4) to 151 ± 14 (*n* = 3) nmol/mg protein. We conclude that an enhanced rate of glycerol accumulation is not responsible for increased hypertonic stress resistance in *age-1* loss-of-function mutants. However, as discussed in more detail below, the approximately twofold elevation of basal glycerol levels could contribute to hypertonic stress resistance in *age-1* loss-of-function mutants.

Insulin signaling through DAF-16-activated genes confers hypertonic stress resistance. The absence of hypertonicity-induced inhibition of insulin signaling or of a requirement for DAF-16 in acute or chronic osmoadaptation (Figs. 2 and 3) suggests that alterations in the expression of DAF-16 target genes must confer hypertonic stress resistance in insulin signaling mutants. Recent whole genome microarray analyses identified 263 genes transcriptionally upregulated during DAF-16 activation (14). These genes fall into two prominent classes: *1*) general stress response genes including small heat shock protein (HSP) genes and genes encoding proteins that protect cells from oxidative stress and *2*) antimicrobial protein genes.

DAF-16-upregulated HSPs play a role in conferring increased life span in worms where *daf-2* expression is disrupted by knockdown or loss-of-function mutations (38). In addition, HSPs have been shown to function in osmotic stress resistance in plants (48) and mammalian renal medullary cells (41, 42, 44). DAF-16 activation increases expression of several HSPs, including two HSP12 genes, four HSP16 genes, one HSP20

gene, one HSP70 gene, and two regulators of the HSP70/90 ATPase (37, 38). We tested the role of these HSPs in hypertonic stress resistance of *age-1* loss-of-function mutants with RNAi feeding methods. As shown in Fig. 4, knockdown of both DAF-16-upregulated HSP12 genes, an HSP70 gene, and both HSP70/90 regulators significantly reduced hypertonic stress resistance of *age-1* loss-of-function mutants. Knockdown of the other DAF-16-regulated HSP genes had no significant ($P > 0.05$) effect on hypertonic stress resistance of *age-1* mutant worms. Together, the data in Fig. 4 demonstrate that the hypertonic stress resistance phenotype of *age-1* loss-of-function mutant worms is mediated in part by DAF-16-regulated HSPs.

We also used RNAi knockdown to screen an additional 222 genes that are upregulated in response to inhibition of insulin signaling and subsequent DAF-16 activation (38). As shown in Table 1, knockdown of eight of these genes reduced hypertonic stress resistance in *age-1* loss-of-function mutants. These eight genes encode a diverse set of proteins including a putative homogentisate 1,2-dioxygenase, which is an enzyme that functions in phenylalanine and tyrosine metabolism, a C-type lectin, an esterase-like protein, an enzyme involved in polyun-
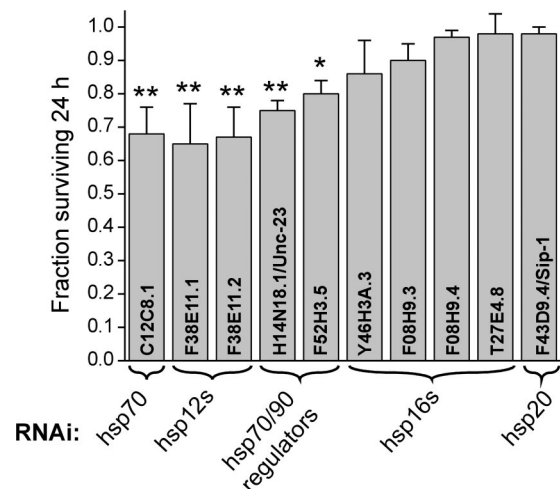
Fig. 4. Heat shock proteins (HSPs) contribute to the hypertonic stress-resistant phenotype of *age-1* animals. Adult offspring of *age-1* animals fed HSP double-stranded RNA were transferred to RNA interference (RNAi) feeding plates containing 450 mM NaCl. Survival was normalized to *age-1*; *GFP*(*RNAi*) animals, and values are means ± SE (*n* = 3–8) of normalized survival; 30–120 worms were used in each survival study. *$P < 0.05$, **$P < 0.01$ compared with *age-1*;*GFP*(*RNAi*) animals.

Table 1. *Effects of RNAi inhibition of DAF-16 upregulated genes on hypertonic stress resistance of age-1 mutant worms*

Sequence Name	Common Name	Biological Process*	Survival†			
W06D4.1	*hgo-1*	Putative homogentisate oxidase	0.96	0.81	0.85	
H16D19.1		Member of the C-type lectin protein family	0.93	0.65	0.75	
VZK8221.1	*fat-6*	Putative stearoyl-CoA delta-9 fatty acid desaturase involved in polyunsaturated fatty acid biosynthesis	0.62	0.00		
Y43F8A.3		Member of the esterase-like protein family	0.44	0.85		
T19B10.2		Protein of unknown function	0.04	0.06	0.00	
T25C12.2		Similar to *Caenothabditis elegans* T08A9.9, which is an antibacterial peptide	0.85	0.83		
W10G6.3	*ifa-2*	Member of the intermediate filament family; low similarity to lamin B1 (mouse Lmnb1); may be structural member of the nuclear lamina	0.45	0.29	0.28	
F19H8.1	*tps-2*	Member of the trehalose 6-phosphate synthase protein family	0.82	0.44	0.60	0.83
ZK54.2	*tps-1*	Member of the trehalose 6-phosphate synthase protein family	0.86	1.25	0.99	
F19H8.1 + ZK54.2	*tps-2 + tps-1*		0.13	0.09	0.48	

RNAi, RNA interference. *Annotations are from WormBase (**www.wormbase.org**). †Survival is normalized to *age-1;* green fluorescent protein (RNAi) control animals on 500 mM NaCl RNAi plates.

saturated fatty acid metabolism, trehalose 6-phosphate synthase (TPS), an unknown protein with homology to *C. elegans* antibacterial peptides, and an intermediate filament cytoskeletal protein. The strongest inhibition of hypertonic stress resistance in *age-1* mutants was observed with knockdown of T19B10.2, which encodes a protein containing an NH_2-terminal signal peptide and a coiled-coil protein interaction domain.

Two genes upregulated in insulin signaling loss-of-function mutants encode TPS1 and TPS2 (38), which catalyze the biosynthesis of trehalose, a disaccharide of glucose (40). Trehalose plays an important role as an organic osmolyte in numerous organisms including bacteria, yeast, and plants (15). The DAF-16-dependent upregulation of TPS1 and TPS2 suggested that *age-1* loss-of-function mutant animals may have increased trehalose levels relative to wild-type worms. To directly test this possibility, we compared whole worm trehalose content in wild-type and *age-1* mutants (Fig. 5). Under control conditions, *age-1* loss-of-function animals contain approximately twofold more trehalose than wild-type animals. Exposure of *age-1* mutants to 400 mM NaCl for 5 h further elevated trehalose content ~1.5-fold (Fig. 5).

RNAi knockdown of TPS2 consistently reduced the osmotic stress resistance of *age-1* mutants, whereas knockdown of TPS1 showed a more variable effect (Table 1). Simultaneous knockdown of both TPS1 and TPS2 strongly and consistently suppressed the hypertonic stress-resistant phenotype of *age-1* loss-of-function mutant animals (Table 1). Under low-NaCl control conditions knockdown of TPS1 and/or TPS2 had no effect on viability or the apparent health of *age-1* mutants, similar to what was observed previously for wild-type worms by Pellerone et al. (40). These investigators also showed that knockdown of both TPS1 and TPS2 together reduces trehalose content >90% (40). Together, these results suggest that elevated trehalose levels confer hypertonic stress resistance in *age-1* loss-of-function mutant worms.

Elevated trehalose and glycerol levels do not prevent acute hypertonicity-induced water loss and shrinkage. Recent studies demonstrated that loss-of-function mutations in *osr-1*, a gene with unknown function, induce a 30- to 40-fold increase in glycerol levels in nonosmotically stressed worms. Unlike wild-type animals, *osr-1* loss-of-function mutants do not undergo significant shrinkage when exposed acutely to 500 mM NaCl (45). Inhibition of shrinkage is most likely due to elevated glycerol levels, which are expected to reduce the osmotic gradient across the body wall when worms are exposed to hypertonic solutions. It was conceivable that the small elevation of trehalose and glycerol levels observed in *age-1* loss-of-function mutants conferred hypertonic stress resistance by preventing hypertonicity-induced water loss. We examined this possibility by measuring total body volume in worms transferred acutely to hypertonic growth agar. The *age-1* mutants and wild-type worms shrank 18 ± 2% and 22 ± 1%, respectively, after a 5-min exposure to agar containing 200 mM NaCl (means ± SE; $n = 3$ or 4). The extent of shrinkage was not significantly ($P > 0.2$) different, indicating that changes in the total osmotic content of *age-1* loss-of-function mutant worms are not sufficient to prevent osmotic water loss and acute shrinkage and therefore do not confer hypertonic stress resistance.

DISCUSSION

Mutations in genes that inhibit the activity of the DAF-2 insulin/IGF-like signaling pathway in *C. elegans* confer increased life span and resistance to thermal, oxidative, and hypoxic stress (3, 22, 36, 43). Microarray and RNAi studies have identified targets of the FOXO-type transcription factor DAF-16 that give rise to longevity (38). However, the mech-
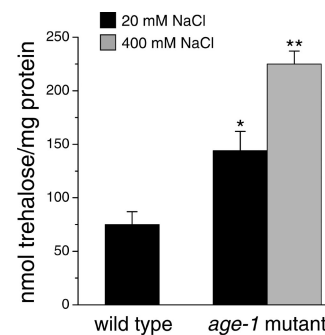

Fig. 5. Trehalose content is elevated in *age-1* loss-of-function mutant worms. Synchronized young adult animals were grown on 20 or 400 mM NaCl. Values are means ± SE ($n = 3$). *$P < 0.05$ compared with wild type; **$P < 0.05$ compared with *age-1* worms grown on 20 mM NaCl.

anisms by which insulin/IGF-like signaling mediates resistance to various types of stress were previously unknown.

We demonstrate here for the first time that inhibition of insulin signaling in *C. elegans* by loss-of-function mutations in either the DAF-2 insulin-like receptor or the PI3-kinase AGE-1 confers resistance to extreme hypertonic stress through activation of DAF-16 (Fig. 1). However, unlike thermal, oxidative, and starvation stress (18), DAF-16 is not activated by hypertonicity (Fig. 2). In addition, DAF-16 is not required for adaptation of *C. elegans* to hypertonic environments (Fig. 3). These results demonstrate that different signaling pathways regulate the expression of identical or functionally similar sets of genes that confer protection against hypertonic stress. Consistent with this notion, we have observed a significant overlap between genes transcriptionally activated by DAF-16 and those activated by hypertonicity (unpublished observations). These overlapping genes may represent functional homologs of the yeast core environmental stress response (CESR) genes, which are activated by most forms of stress (7).

To identify DAF-16-regulated genes required for hypertonic stress resistance and to begin defining their mechanisms of action, we carried out RNAi screens in *age-1* mutant worms. A major class of genes upregulated by DAF-16 activation is small HSPs (sHSPs). We identified two HSP12 genes, hsp12.3 and hsp12.6, that are functionally important for *age-1* hypertonic stress resistance (Fig. 4). Overexpression of sHSPs in mammalian glial cells has been shown to confer resistance to hypertonic stress (25). sHSPs are thought to oligomerize, bind, and stabilize partially unfolded proteins, preventing their aggregation until they can be refolded by larger ATPase-type chaperone complexes (50). Because increased intracellular ionic strength caused by hypertonic stress denatures proteins (46), upregulation of HSP12 gene expression by DAF-16 may allow *age-1* mutant worms to more rapidly stabilize partially denatured proteins and prevent their aggregation during the early stages of hypertonic stress.

We also examined the role of the HSP70/90 ATPase chaperone complex, several components of which are upregulated by DAF-16 (37, 38), in the hypertonic stress resistance-phenotype *age-1* loss-of-function mutants. RNAi knockdown of HSP70 and two regulators of the HSP70/90 ATPase reduces hypertonic stress resistance in *age-1* mutants (Fig. 4). Previous studies suggested an important role for HSP70 in hypertonic stress resistance. For example, loss of HSP70 in mice leads to increased apoptosis of kidney cells under hypertonic stress (44). HSP70 is induced in kidney cells exposed to gradual increases in extracellular osmolality (5). Interestingly, it has been shown that denatured proteins bound to sHSPs can be refolded through their interaction with HSP70 (14). Increased HSP70 activity in *age-1* loss-of-function mutant worms may increase the refolding of denatured proteins bound to the sHSPs hsp12.3 and hsp12.6.

RNAi knockdown of T19B10.2 consistently suppressed *age-1* hypertonic stress resistance to <10% of control animals (Table 1). The predicted T19B10.2 protein contains an NH$_2$-terminal signal peptide and a coiled-coil domain, suggesting that it may be a secreted protein that interacts with itself or other proteins. T19B10.2 exhibits 93% identity to a *C. briggsae* protein but has no apparent homologs outside of nematodes. Although the role of T19B10.2 in hypertonic stress resistance is not yet clear, we have observed that another putative secretory protein regulates the expression of osmotically induced genes (unpublished observations). Therefore, intercellular communication mediated in part by secretory proteins may play an important role in hypertonic stress signaling in *C. elegans*.

TPS genes also play an important functional role in hypertonic stress resistance of *age-1* loss-of-function mutants (Table

Fig. 6. Model illustrating DAF-16-mediated hypertonic stress resistance. Signaling from the DAF-2/insulin receptor to the AGE-1/phosphatidylinositol 3-kinase (PI3-kinase) is thought to induce phosphorylation of the DAF-16/FOXO3 transcription factor and inhibit its translocation into the nucleus. Loss-of-function mutations in either *daf-2* or *age-1* result in dephosphorylation and nuclear translocation of DAF-16. Nuclear localized DAF-16 activates the transcription of genes that protect against or repair hypertonic stress-induced damage. For simplicity, proteins involved in signaling between AGE-1 and DAF-16 have been omitted. TPS, trehalose 6-phosphate synthase.
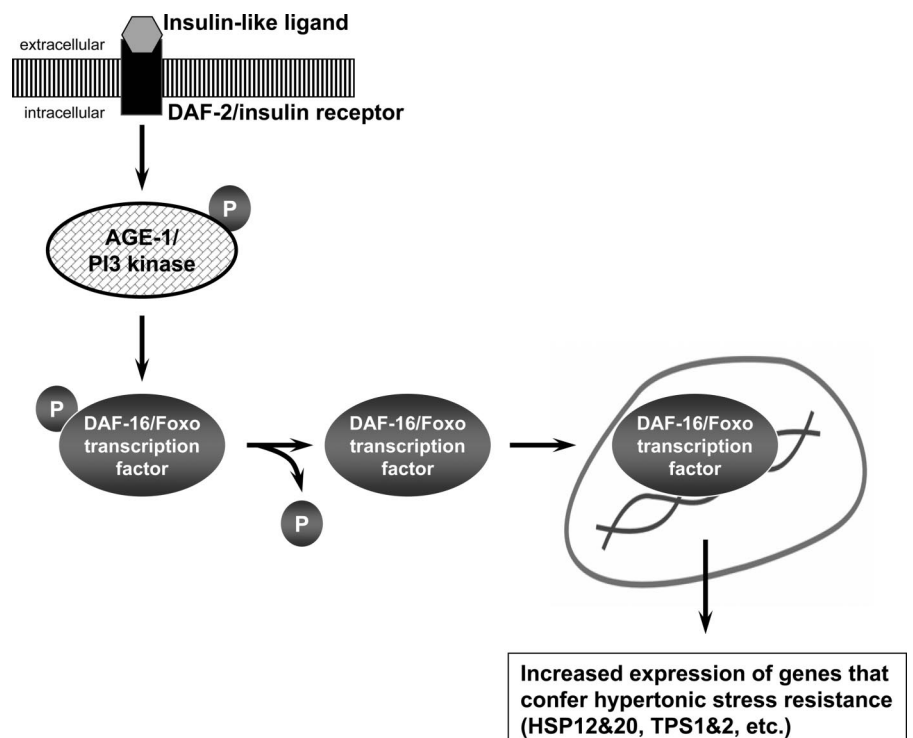
1). Trehalose functions as an organic osmolyte and cytoprotectant in diverse organisms (2, 8, 10). Certain species of yeast, nematodes, rotifers, tardigrades, plants, and insects are capable of surviving indefinitely in a completely desiccated state, a phenomenon termed anhydrobiosis (11). Survival under anhydrobiotic conditions is mediated in part by accumulation of large amounts of trehalose. Trehalose functions to protect macromolecular and cellular architecture during desiccation (9). Our studies demonstrate that trehalose content is elevated approximately twofold in *age-1* loss-of-function mutants under control conditions, with further increases observed on exposure to hypertonic stress (Fig. 5). Interestingly, basal glycerol levels were also elevated approximately twofold in *age-1* loss-of-function mutants (see RESULTS). As we showed previously (28), glycerol functions as an organic osmolyte during hypertonic stress in *C. elegans*.

Simultaneous RNAi knockdown of *tps-1* and *tps-2* reduces trehalose levels by >90% in *C. elegans* (40) and dramatically reduces hypertonic stress resistance of *age-1* mutant worms (Table 1). One mechanism by which trehalose could confer hypertonic stress resistance would develop if trehalose were accumulated to sufficiently high levels, thereby increasing total osmotic content and preventing water loss and shrinkage. However, we observed that wild-type and *age-1* loss-of-function mutant animals shrank to similar degrees during a 5-min exposure to 200 mM NaCl (see RESULTS). This suggests that increases in trehalose and possibly glycerol levels function to protect insulin signaling mutants against cellular and molecular damage caused by hypertonic stress. Consistent with this hypothesis, stress-resistant dauer larvae, whose development depends on DAF-16 activation, exhibit elevated trehalose concentrations and increased expression of glycerol synthesis enzymes (40, 51). Therefore, insulin signaling-regulated accumulation of trehalose and glycerol may play an important role in the ability of dauer larvae to protect themselves from environmental stressors such as water loss.

In conclusion, we have shown that activation of the DAF-16 transcription factor through loss-of-function mutations in the DAF-2 insulin receptor or the AGE-1 PI3-kinase can induce hypertonic stress resistance in *C. elegans*. Our findings are summarized in Fig. 6. Using reverse genetic strategies and the results of whole genome microarray studies, we have shown that transcriptionally upregulated targets of this signaling pathway confer protection against damage caused by hypertonic stress, and these targets suggest specific molecular mechanisms underlying hypertonic stress resistance. Heat shock genes, cytoskeletal genes, genes of unknown function(s), and trehalose biosynthesis genes all were shown to mediate the hypertonic stress resistance phenotype of *age-1* loss-of-function mutant worms. Stress resistance conferred by inhibition of insulin signaling appears to be evolutionarily conserved, because both *Drosophila* and mice carrying mutations in insulin signaling components are long-lived and stress resistant (16, 20, 21, 23). Therefore, the identification of insulin signaling targets that mediate hypertonic stress resistance in *C. elegans* should also provide a foundation for new investigations into mammalian cellular stress response mechanisms.

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