Physiol Genomics 14: 17–24, 2003.
First published April 9, 2003; 10.1152/physiolgenomics.00179.2002.

HIF-1 is required for heat acclimation in the nematode *Caenorhabditis elegans*

Millet Treinin,[1] Judith Shliar,[2] Huaqi Jiang,[3] Jo Anne Powell-Coffman,[3] Zohar Bromberg,[2] and Michal Horowitz[1,2]

[1]*Department of Physiology, Hadassah Medical School, and* [2]*Hadassah School of Dental Medicine, The Hebrew University, Jerusalem 91120, Israel; and*
[3]*Department of Zoology and Genetics, Iowa State University, Ames, Iowa 50011-3260*

Submitted 16 December 2002; accepted in final form 4 April 2003

Treinin, Millet, Judith Shliar, Huaqi Jiang, Jo Anne Powell-Coffman, Zohar Bromberg, and Michal Horowitz. HIF-1 is required for heat acclimation in the nematode *Caenorhabditis elegans*. *Physiol Genomics* 14: 17–24, 2003. First published April 9, 2003; 10.1152/physiolgenomics.00179.2002.—Chronic exposure to environmental heat improves tolerance via heat acclimation (AC). Our previous data on mammals indicate that reprogramming the expression of genes coding for stress proteins and energy-metabolism enzymes plays a major role. Knowledge of pathways leading to AC is limited. For their identification, we established a *Caenorhabditis elegans* AC model and tested mutants in which signaling pathways pertinent to acclimatory responses are mutated. AC attained by maintaining adult *C. elegans* at 25°C for 18 h enhanced heat endurance of wild-type worms subjected to heat stress (35°C) and conferred protection against hypoxia and cadmium. Survival curves demonstrated that both *daf-2* (insulin receptor pathway) showing enhanced heat tolerance and *daf-16* loss-of-function (a transcription factor mediating DAF-2 signaling) mutants benefit from AC, suggesting that the insulin receptor pathway does not mediate AC. In contrast, the *hif-1* (hypoxia inducible factor) loss-of-function strain did not show acclimation, and non-acclimated *vhl-1* and *egl-9* mutants (overexpressing HIF-1) had greater heat endurance than the wild type. Like mammals, HIF-1 and HSP72 levels increased in the wild-type AC nematodes. HSP72 upregulation in AC *hif-1* mutants was also observed; however, it was insufficient to improve heat/stress tolerance, suggesting that HIF-1 upregulation is essential for acclimation, whereas HSP72 upregulation in the absence of HIF-1 is inadequate. We conclude that HIF-1 upregulation is both an evolutionarily conserved and a necessary component of heat acclimation. The known targets of HIF-1 imply that metabolic adaptations are essential for AC-dependent tolerance to heat and heavy metals, in addition to their known role in hypoxic adaptation.

heat acclimation; cross-tolerance; HIF-1; HSP72

UPON TRANSFER FROM ONE TEMPERATURE to another for prolonged periods of time, most animals are able to adapt physiologically and biochemically to the new environmental conditions. This process is termed heat acclimation, and if successful, it enhances heat tolerance in terms of the upper extreme of tolerable temperature and the duration of heat endurance (15). This leads to delayed appearance of heat injuries (15, 16). In contrast to evolutionary adaptation, acclimation is mostly phenotypic adaptation, although genetic processes may be involved (3). In mammals, heat acclimation can be achieved via prolonged exposure to moderate ambient heat at the upper edge of the body's thermal comfort zone. The acclimated phenotype exhibits an extended operative thermoregulatory range compared with the preacclimation state and enhanced metabolic efficiency, thus improving cellular performance. Reprogramming of gene expression and posttranslational variations underlie the developing acclimatory responses (17). The signaling pathways and molecules targeted to acclimatory responses include G proteins, cytoprotection factors [e.g., via the heat shock protein HSP 72 kDa (HSP72)], antioxidants, several transcription factors, metabolic enzymes, and contractile proteins (2, 9, 10, 24, 25). Of significance are our findings of marked upregulation of HSF1 (heat shock transcription factor; unpublished observations, A. Maloyan and M. Horowitz), increased HSP72 cellular reserves, leading, upon stress, to the provision of cytoprotection without the need for de novo protein synthesis (24), and the stabilization of the hypoxia-inducible transcription factor (HIF-1α) at a high constitutive level (25). HIF-1α, a master regulator of oxygen sensitivity and controller of most glycolytic enzymes, has been shown to be targeted for proteosomal degradation in an oxygen-dependent manner (8, 30). Currently, its role in heat acclimation has not yet been directly assessed, and it is not clear whether an increased level of HIF-1α is functionally important or only an epiphenomenon.

An important inseparable consequence of heat acclimation is the "cross-tolerance phenomenon," in which adaptation to one stress also confers protection against an additional type of stress. In prior studies in mammalian systems we have substantiated the development of long-term cross-tolerance between heat acclimation and several other environmental stressors with one common denominator: oxygen deprivation and/or the consequences of reoxygenation (10, 15). This was shown, for example, in the rat when heat acclimation conferred cardioprotection upon ischemic reperfusion insults (10, 16) and delayed the threshold of oxygen

Article published online before print. See web site for date of publication (http://physiolgenomics.physiology.org).

Address for reprint requests and other correspondence: M. Horowitz and M. Treinin, Dept. of Physiology, Hadassah Medical School, The Hebrew Univ., POB12272, Jerusalem 91120, Israel (E-mail: horowitz@cc.huji.ac.il, millet_t@cc.huji.ac.il).

toxicity under subjection to hyperoxic conditions (1). Cross-tolerance has been previously described for several related phenomena, among which "preconditioning," providing short-acting tolerance, is generated when a short exposure to sublethal stress confers protection against a subsequent stress (29, 37). Extensive studies in respect to thermal and ischemic preconditioning have shown that upregulation of HSPs is involved in the cytoprotection achieved (37). By Selye's (29) definition, preconditioning can be counted as one facet of the general stress response. In contrast to this short-acting stress response, heat-acclimation-mediated cross-tolerance confers a long-lasting period of protection and seems to involve mechanisms other than merely cytoprotection (10). Our studies on mammalian models indicate that changes in the expression of metabolic enzymes and enhanced metabolic efficiency are involved (10). Our novel finding in the rat, having established that HIF-1α is also induced during heat acclimation (25), suggests that this transcription factor may also be a player in the development of cross-tolerance.

An elegant approach to examine the signaling pathways is to use genetic models. This approach has gained power in the last two decades when genome studies have revealed striking similarities between regulatory proteins in humans and those in animals more amenable to genetic manipulations, such as *Caenorhabditis elegans*, *Drosophila*, or zebrafish (23). The demonstration that the nematode *C. elegans* displays broad hormetic abilities (hormesis is the induction of beneficial effects by exposure to low doses of otherwise harmful chemical or physical agents) (6) challenged us to study signaling pathways leading to heat acclimation. Specifically genetics was used to test the hypothesis that HIF-1α indeed plays a role in heat acclimation. Although the responses of *C. elegans* to acute heat shock have been extensively studied (6, 20), no data were available in respect to its ability to acclimate to heat. The purpose of the present investigation was therefore twofold: *1*) to study whether *C. elegans* can acclimate to heat, and to develop an appropriate heat acclimation protocol; and *2*) given our findings in mammalian species, to test the hypothesis that the pathway to heat acclimation involves HIF-1 mediation. Our data provide the unequivocal finding that HIF-1, the *C. elegans* ortholog of the mammalian HIF-1α, is essential for heat acclimation, and they suggest that overexpression of HIF-1 may be sufficient for initial transient heat tolerance during subjection to heat stress.

MATERIALS AND METHODS

C. elegans mutants, experimental conditions, and in vivo response assessment. Experiments were conducted on *C. elegans* nematodes of either wild type (N2) or strains carrying the following mutations: *1*) *daf-2(e1370)*, *2*) *daf-16(mDf50)*, *3*) *daf-16(m26)*, *4*) *hif-1(ia4)*, *5*) *egl-9(n571)*, and *6*) *vhl-1(ok161)*. The strains *daf-16(mDf50)*, *hif-1(ia4)*, and *vhl-1(ok161)* are complete loss-of-function mutations. The mutations used can be categorized under one of two signaling pathways chosen on the basis of our findings in the mammalian species: *1*) the DAF-2 insulin receptor pathway, an effector of stress tolerance (*mutations 1–3*); and *2*) altered hypoxic tolerance (*mutations 4–6*).

The worms were bred under standard *C. elegans* growth condition (36) at 20°C. Response to environmental stress was assayed on age-synchronized groups, produced by placing reproductive adults onto fresh agar plates for 4–6 h and permitting the eggs laid to develop for 4 days into adults. Worms of each strain were divided into two groups while maintaining identical initial density: *1*) controls, maintained at 20°C; and *2*) heat acclimated, maintained at 20°C and then shifted to 25°C for 18 h. Assessment of heat acclimation or the response to other environmental stressors was done by the determination of percent survival of both the wild type and all mutants during exposure to one of the following stressors: *1*) heat stress at 35°C or 37°C for at least 5 h; *2*) $CdCl_2$ (0, 4, 8 or 24 mg% for 5 h at normothermic temperature); or *3*) chemical hypoxia (0.6 mg/ml sodium azide for 20 min at normothermic temperature). For the $CdCl_2$ exposure, worms were transferred into test tubes containing the $CdCl_2$ dissolved in a buffer solution containing 50 mM NaCl and 30 mM KCl. To assess mortality, we prodded animals with a platinum wire; nonresponding animals were scored dead; 100% survival signifies that all animals in the plate are alive. All experiments were repeated 3–4 times on independent days. During each repetition, 3–4 plates of both the wild type (N2) and the chosen mutant were studied. Each plate contained 30–50 animals.

Western immunoblot. HIF-1 and HSP72 levels were measured in transgenic worms containing all the *hif-1* coding sequence fused in-frame to green fluorescent protein (GFP) (pHJ06Ex6) and in *hif-1(ia4)* (18). For HSP72, polyclonal anti-HSP72 antibody (AB) 1:20,000 (Stressgen, Victoria, BC, Canada) with secondary AB goat anti-rabbit 1:1,000 were used. For GFP, anti-GFP 1:1,000 (Roche) was used with secondary AB goat anti-mouse 1:1,000. To further validate our data, CeHIF-1 level in the N2 worms was measured using a specific CeHIF-1 AB 1:1,000 (contributed by Peter Ratcliffe) with goat anti-rabbit 1:1,000 as a second AB. To ensure equal amounts of initial protein, each membrane was also probed with polyclonal anti-lamin-specific AB 1:1,000 [nuclear matrix protein (21) contributed by Y. Gruenbaum] with goat anti-rabbit 1:1,000 second AB.

Sample preparation. The worms were washed twice in M9 buffer and then once with standard double-distilled water. SDS+M9 (1:1) loading buffer was added immediately (2:1 volume-to-tissue ratio), samples were boiled for 5 min, ultrasonicated, and spun down, and the supernatant was quickly frozen and kept at −70°C until analysis. For SDS electrophoresis, 20 μl per lane was applied onto 7.5% polyacrylamide gels. After separation by electrophoresis (90 V, 2 h), the proteins were transferred onto nitrocellulose (190 mA, 4°C, 1 h), blocked for 2 h in phosphate-buffered saline (PBS) containing 0.5% dried skimmed milk powder, and probed overnight, at 4°C, with one of the primary antibodies. After repeated washings, the membranes were incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson) diluted 1:1,000. Specific antibody binding was detected using enhanced chemiluminescence (Amersham) and visualized by exposing X-ray film to the membrane. Following stripping (14), each membrane was probed with additional antibody as before. The density of the scanned antibody bands was calculated using Tina software (Raytest, Straubenhardt, Germany).

Statistical analysis. To assess significant changes, the commercial software package SigmaStat software (ver. 2.03, SPSS) with alpha level for significance set at 0.05 was used.

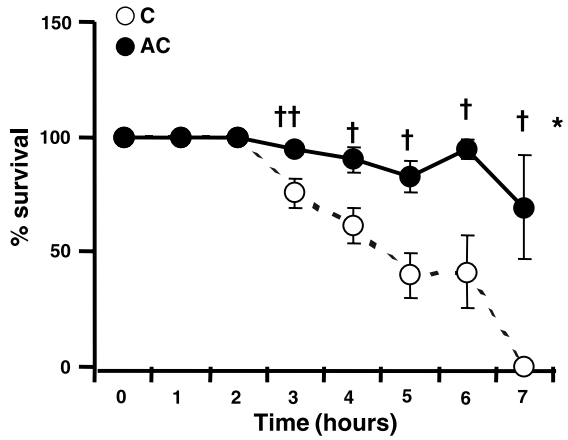

Fig. 1. The heat-acclimated *C. elegans* phenotype: survival curves of adult wild-type worms grown at 20°C (control, C) and following heat acclimation (18 h at 25°C, AC) during subjection to heat stress at 35°C. AC worms endured heat stress markedly better than control worms. Results are means ± SE (SE is standard error of the means). Asterisk denotes significant difference between control and AC worms (two-way ANOVA $P < 0.0001$); daggers denote significant difference between matched pairs. †$P < 0.003/0.001$. ††$P < 0.05$. Each time point is the average of at least 18 independent experiments. For further details, see MATERIALS AND METHODS.

For comparisons of survivorship curves of the matched control and AC worms, two-way ANOVA followed by multiple comparisons (Bonferroni) was conducted. Ambient treatments and heat stress sessions were taken as the fixed effects, and percent survival was used as the dependent variable. The individual worm plates were assumed to be random samples from the worm population. Student's paired *t*-test for specific time point was included as well.

RESULTS

Induction of heat-acclimated C. elegans phenotype. The *C. elegans* response to acute heat shock has been extensively studied. Nevertheless, no data were available with respect to the ability of *C. elegans* to acclimate nor with respect to a suitable acclimation protocol for this species. In analogy to homeotherms, in which heat acclimation is induced by exposure at the upper limit of the comfortable thermal environment, we decided to examine whether the upper limit of the temperature at which *C. elegans* is viable and fertile can induce heat acclimation. It is well established that *C. elegans* is viable and fertile in the temperature range 12–25°C (36). The effects of heat stress at 35°C on synchronized worms grown at 20°C (controls) and those grown at 20°C and then shifted to 25°C for 18 h (acclimation) is depicted in Fig. 1. Exposure of the control group to heat stress led to a quick decline in viability. More than 50% of the animals died after 5 h, and at 7 h no surviving animals were detected. In contrast, viability of the acclimated nematodes declined more slowly, and at 7 h survival was 70%. To rule out age-dependent differences in heat tolerance (in *C. elegans*, developmental rates are temperature dependent), an additional protocol in which heat endurance of synchronized control animals maintained at 20°C for 4 days was compared with that of worms maintained for 3 days at 25°C. Similar to our first protocol, this treatment also enhanced stress tolerance (data not shown).

The DAF-2 insulin-like receptor pathway influences heat tolerance but not heat acclimation. Mutations in the DAF-2 receptor reduce the activity of the insulin receptor pathway, leading to enhanced intrinsic thermotolerance, positively correlated with increased longevity, and increased tolerance to other stressors, e.g., hypoxia, as well (4, 20, 28). In agreement with the previous studies, we find that *daf-2(e1370)* mutants were not affected by heat stress at 35°C even prior to heat acclimation. No mortality was observed even after 6 h of heat exposure (Table 1). Acclimation at 25°C, however, enhanced the heat tolerance of this mutant. This was observed upon exposure to heat stress at 37°C. Following 6.5 h at this stress, the acclimated *daf-2* mutants maintained 100% survival, whereas in the non-acclimated worms about 5% of the worms survived (Fig. 2, *right*; Table 1). In comparison, the wild-type *C. elegans* showed 23% and 0% survival in the heat-acclimated and the non-acclimated worms, respectively (Fig. 2, *left*). It is noteworthy that during the initial hour of heat stress (at 37°C) non-acclimated *daf-2* mutants resembled heat-acclimated N2 worms under similar stress conditions (Fig. 2, *right* vs. *left*).

DAF-16 is a forkhead transcription factor that transduces the effects of the DAF-2 insulin receptor path-

Table 1. *Percent survival of the studied mutants during heat stress at 35°C or 37°C before and after acclimation to heat*

Genotype	Heat Stress	Survival at heat stress, %		*P*	AC/C	No. of Animals
		C	AC			
Wild type	35°C	41.3 ± 9.0	95.0 ± 6.6	<0.001	2.3	3,400; 3,400
Wild type	37°C	0	23 ± 23			351; 467
daf-2(e1370)	35°C	100	100		1	850; 550
daf-2(e1370)	37°C	5 ± 3.3	100	<0.001	20	850; 850
daf-16(mgDf50) (LF)	35°C	28.0 ± 13.8	80.0 ± 13.3	<0.001	2.8	1,000; 1,100
daf-16(m26)	35°C	51.5 ± 14.6	73.0 ± 0.005	<0.01	1.4	505; 558
hif(ia4) (LF)	35°C	58.3 ± 11.0	42.0 ± 4.5		0.7	550; 550
vhl-1(ok161) (LF)	35°C	37.0.5 ± 12.3.3	87.2 ± 11.2	<0.001	2.3	1,210; 960
egl-9(n571)	35°C	57.5 ± 16.9	97.3 ± 5.0	<0.004	1.7	960; 1,200

Data were taken at time interval 6–6.5 h. C, non-acclimated; AC, heat acclimated; LF, loss of function; the number of animals denotes the number of worms used to construct the survival curves. Acclimation conditions were 25°C.

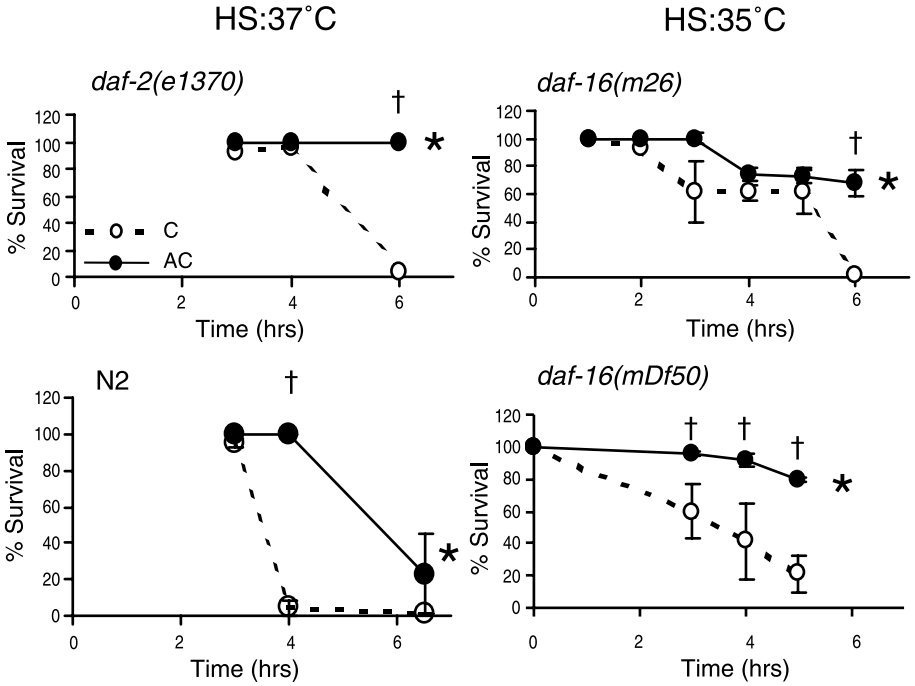

Fig. 2. Heat acclimation in mutants affecting the DAF-2 insulin receptor pathway. *Left*: survival curves of *daf-2(e1370)* subjected to heat stress at 37°C before and after heat acclimation (*top*) compared with the wild-type worms under similar conditions (*bottom*). The *daf-2(e1370)* mutants have high intrinsic thermotolerance, and in this investigation the effect of heat acclimation was only seen upon exposure to heat stress at 37°C. *Right*: survival curves of *daf-16(m26)* (*top*) and *daf-16(mDf50)* (*bottom*) subjected to heat stress at 35°C before and after heat acclimation. DAF-16 is a forkhead transcription factor that transduces DAF-2 effects; thus the results suggest that DAF-16 is not required for heat acclimation. Asterisks denote significant difference between control (C) and AC worms [two-way ANOVA *daf-2(e1370)*, N2, *daf-16(mDf50)* $P < 0.001$, *daf-16(m26)* $P < 0.002$]; daggers denote significant difference between matched pairs. †$P < 0.001$, 0.004. For acclimation conditions, see Fig. 1 and text.

way on stress resistance, and mutations in this gene suppress the enhanced longevity and hypoxic tolerance seen in *daf-2* mutants (26, 28). We examined *daf-16(m26)* and *daf-16(mgDf50)* mutations, which result in partial or complete loss of DAF-16 function, respectively (26). Both strains showed a marked acclimatory response to heat stress at 35°C (Fig. 2, *right*; Table 1). This suggests that DAF-16 is not required for heat acclimation.

Induction of HIF-1 expression is required for normal heat acclimation. To test the hypothesis that induction of HIF-1α is critical to heat acclimation, we assayed mutations in *hif-1*, the *C. elegans* ortholog of HIF-1α, and in its regulatory proteins. The HIF-1-associated mutations studied in this investigation included HIF-1 loss-of-function mutation *hif-1(ia4)* (18) and two mutations leading to HIF-1 overexpression: *egl-9(n571)* and *vhl-1(ok161)* (8). In an evolutionarily conserved pathway, HIF-1 is hydroxylated by the EGL-9 enzyme in an oxygen-dependent manner. This modification increases the affinity of HIF-1 for VHL-1, which targets HIF-1 for proteosomal degradation (8). Hence, loss of VHL function, as demonstrated by the *vhl-1(ok161)* mutant, and defective function of the prolyl hydroxylase, as shown in the *egl-9(n571)* mutant, bring about the constitutive overexpression of HIF-1 under normoxic conditions. As evident from Fig. 3*A*, the HIF-1 loss-of-function mutation eliminated the ability of *C. elegans* to acclimate to heat. Furthermore, heat endurance during exposure to heat stress was somewhat shorter than in the non-acclimated mutant (Fig. 3*A*). In contrast, both non-acclimated *egl-9(n571)* and *vhl-1(ok161)* mutants, which overexpress HIF-1, showed rightward shifts in their survival curves compared with the wild type. This was particularly pronounced in the *egl-9* mutants in which there was an initial plateau during which survival was close to 100%, followed by an abrupt fall in survival ratio. Although *egl-9(n571)* and *vhl-1(ok161)* mutants displayed a significant positive acclimatory response (Table 1), the pattern of their survival curves differed from that observed for the *daf-16* mutants subjected to 35°C (Fig. 3 vs. Fig. 2). The intrinsic thermotolerance of *egl-9(n571)* was as high as that exhibited by the *daf-2(e1370)* mutants. The *egl-9(n571)* mutants, unlike *vhl-1(ok161)*, are egg-laying defective. Therefore, EGL-9 is likely to have functions that are independent of HIF-1. Loss of these additional functions may contribute to the extremely high thermotolerance seen in the *egl-9* mutants. It is noteworthy that *egl-9(n571)* shows tolerance to cyanide poisoning as well (11). Collectively, the obtained data indicated to us that HIF-1, in addition to its well-known function during hypoxia, plays an important role in the induction of thermal tolerance and the heat acclimatory response.

To confirm the induction of HIF-1 upon heat acclimation, the level of HIF-1 was measured using Western blot analysis using GFP:HIF-1 transgenics. The data (Fig. 4) show upregulation of HIF-1 following 18 h of heat acclimation at 25°C. Experiments in which CeHIF antibody was used (data not shown) yielded similar results.

In all species studied, including *C. elegans*, increased level of protein members of the HSP70 superfamily is associated with enhanced cytoprotection (13). We have previously shown that heat acclimation in mammalian species increases HSP72 cellular reserves (24). We therefore measured the HSP72 level in the *hif-1(ia4)* mutant, vs. the wild-type worms. Data are presented in Fig. 4. Collectively, the data clearly show that acclimation in both wild type and *hif-1(ia4)* mutants leads to an increase in HSP72 levels. Hence HIF-1 is not re-

quired for the effects of acclimation on HSP70 expression.

Heat acclimation and cross-tolerance. In mammalian species, heat-acclimated animals display greater resistance to additional forms of environmental stressors (10, 16). This promoted us to extend this study and to test whether *C. elegans* demonstrates similar features.

A variety of heat treatments have been implicated to confer enhanced heavy metal stress tolerance (19). We therefore subjected *C. elegans* N2 nematodes to increasing concentrations of cadmium chloride. As shown in Fig. 5, greater percentages of heat-acclimated *C. elegans* survived increasing cadmium concentrations than the non-acclimated worms. This was particularly emphasized in the highest Cd concentration employed when the acclimated/control survival ratio (AC/C) was 3.8. A similar response was obtained for cross-tolerance with chemical hypoxia (% survival: 95 ± 5 vs. 41 ± 25 for AC and control nematodes, respectively). The *hif-1(ia4)* mutant, which lacks the ability to acclimate to heat, did not demonstrate cross-tolerance against heavy metals. Subjection to 8 mg% Cd demonstrated this lack of cross-tolerance, most significantly when AC_{hif}/C_{hif} survival ratio was 1:2.

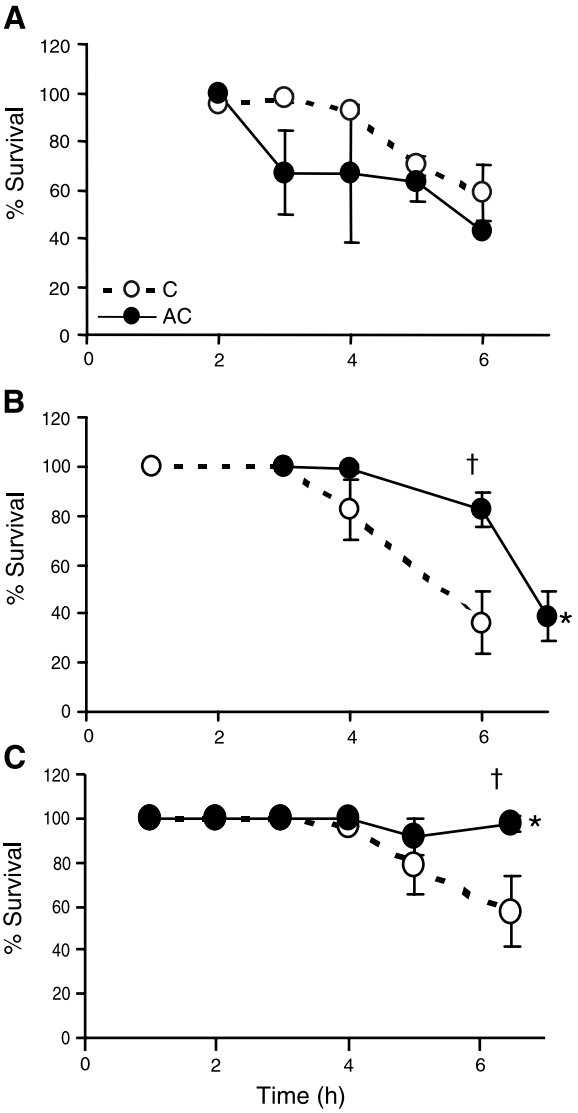

Fig. 3. HIF-1 loss of function eliminates the ability of *C. elegans* to acclimate to heat. Survival curves of the heat acclimated and non-acclimated mutants *hif-1(ia4)* (*A*), *vhl-1(ok161)* (*B*), and *egl-9(n571)* (*C*) subjected to heat stress at 35°C. The *hif-1(ia4)*, the HIF-1 loss-of-function mutant, was unable to acclimate to heat, whereas *vhl-1(ok161)* and (C) *egl-9(n571)*, both overexpressing HIF-1, show a rightward shift vs. N2 (see Fig. 1) in their survival curves. Data are means ± SE. Each time point is the average of 3–4 independent experiments. Asterisks denote significant difference between control and AC worms (two-way ANOVA $P < 0.01$); daggers denote significant difference between matched pairs. †$P < 0.001$. For acclimation conditions see Fig. 1 and text.

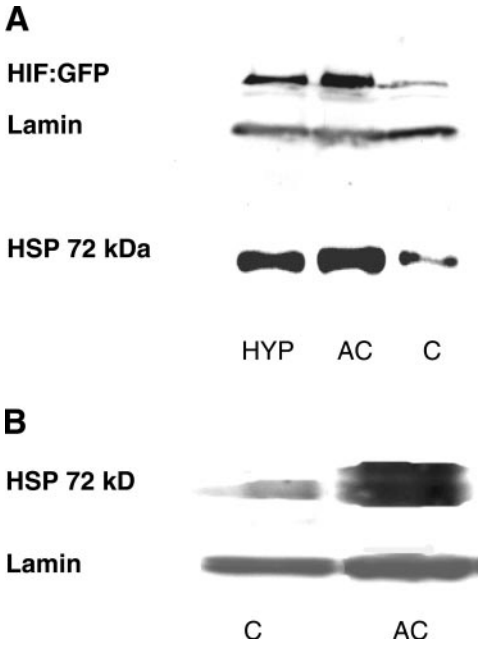

Fig. 4. Western blot analysis, representative blots of HIF-1 and HSP72 protein levels detected by Western immunoblotting. Three different independent experiments were conducted. *A*: HIF-1 and HSP72 in transgenic worms containing all the *hif-1* coding sequence fused in-frame to green fluorescent protein (GFP) (pHJ06Ex6). Three experimental groups were tested: non-acclimated (C), heat acclimated (AC), and those subjected to hypoxia (HYP) using an anaerobic jar (AnaeroGen). The last group was taken as an additional control to validate heat activation. *B*: HSP72 in *hif-1(ia4)* mutants before and after heat acclimation.

DISCUSSION

Many animals are adapted to their particular environmental temperature by genetic selection. In addition, most animals show compensatory responses when subjected to persistent changes in their thermal environment, to allow better coping with the new temperature. This is the acclimatory response defined in the introduction of this paper (15, 16). A comparison of our data on biochemical modalities of heat acclimation in mammals to those known for a wide spectrum of species from other taxa has led us to suggest that at the cellular or biochemical and molecular levels there is similarity in acclimatory responses among species regardless of their taxonomic assignment. The key signaling regulator(s) that mobilizes the acclimatory ma-

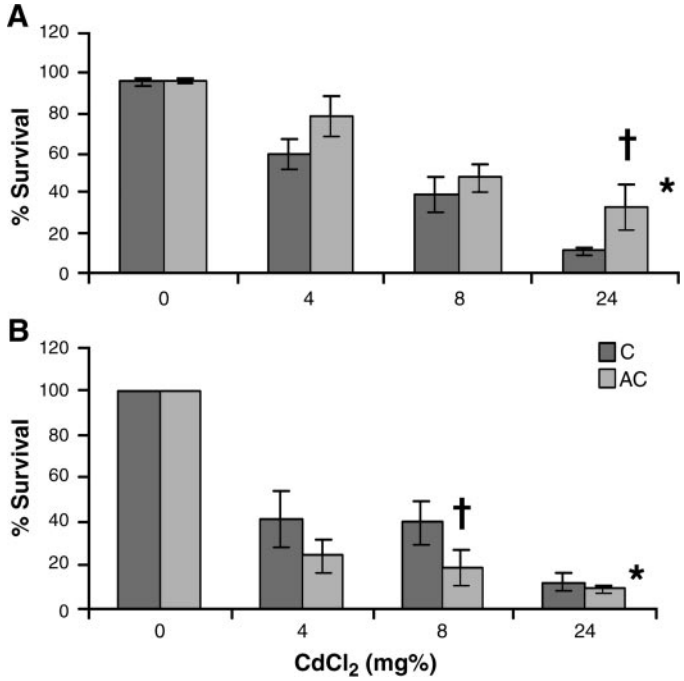

Fig. 5. The induction of cross-tolerance by heat acclimation. *A*: % survival of non-acclimated and heat acclimated wild-type *C. elegans* upon subjection to 4 different concentrations of $CdCl_2$ for 5 h. Survival of the heat-acclimated worms was enhanced (two-way ANOVA $P < 0.04$). Dagger denotes significant different from the matched control ($P < 0.05$). (Number of animals for each bar = 250–260; 3–4 independent experiments were conducted) *B*: % survival of *hif-1(ia4)* HIF-1 loss-of-function mutants to $CdCl_2$ before and after heat acclimation. The *hif-1(ia4)* mutants, which lack the ability to acclimate to heat, do not demonstrate cross-tolerance against $CdCl_2$. Survival of the heat-acclimated worms was significantly reduced compared with the non-acclimated worms (two-way ANOVA $P < 0.03$). Dagger denotes significant different from the matched control ($P < 0.05$); *n* for each bar = 190–200.

chinery is not adequately understood. Hence, our findings that *C. elegans* undergoes heat acclimation challenged us to use this well-known genetic model to identify pathways underlying this ecologically important trait, by testing mutants of potential interest for their acclimatory capacity. We showed in this investigation that loss of HIF-1 function eliminates the ability to acclimate to heat. This novel finding extends our knowledge of HIF-1, known as a master regulator of oxygen sensitivity (30, 31), and attributes to this transcription factor additional adaptive roles, as discussed below.

The criterion for successful acclimation was enhanced thermal tolerance of the nematodes when subjected to heat stress. Our data showed that 18 h at 25°C, the upper limit of the temperature at which *C. elegans* is viable and fertile (36), was sufficient to enhance thermal endurance (at 35°C) of the wild-type *C. elegans* by almost 100% (when calculated as the time at which survival is 40–50%). This protocol to enhance thermal tolerance by approximately twofold without pre-exposing the worms to thermal stress differs from that used by Lithgow et al. (20), who subjected the worms to heat stress at 30°C for at least 3 h. We could hypothesize that Lithgow's protocol yielded a preconditioning effect, rather than heat acclimation, which does not affect fecundity. Currently we do not know whether there are significant differences in the outcome, in terms of heat endurance/thermal injury, between the two protocols. The data obtained in this investigation provided us with the baseline for comparison of the ability to acclimate to heat among the various mutants used.

Mutations that reduce the activity of the insulin receptor homolog, DAF-2, an effect mediated by DAF-16, lead to enhanced stress tolerance, heat resistance, hypoxic-death tolerance, and longevity (30, 26, 28). Our data indicated that *daf-2(e1370)* mutants, in addition to their inherited profound enhanced heat tolerance, can also benefit from heat acclimation. The *daf-16(mDf50)* [and to some extent *daf-16(m26)*] mutants do not exhibit a priori enhanced thermotolerance and also maintain their ability to acclimate to heat. Taken together, these data indicate that the insulin receptor pathway is not required for heat acclimation, and the ability to acclimate depends on another pathway.

It is interesting to note that *daf-2* mutants showed both a priori greater intrinsic thermotolerance than that of the wild-type nematodes, as well as more profound enhanced heat tolerance after acclimation than the wild type. This is manifested by a higher AC/C survival ratio (at 37°C, see Table 1). The inherent difference in heat tolerance among the various mutants used is beyond the scope of this study and is discussed somewhat, e.g., in Walker et al. (35), who suggested that *C. elegans* mutants conferring thermotolerance and longevity show altered coordinated regulation of stress response genes such as reactive oxygen species, antioxidants, and upregulation of molecular chaperons, to protect/alter proteins conformation.

The only genetic background that showed inability to acclimate to heat was the *hif-1*-defective strain. In contrast, *egl-9(n571)* and *vhl-1(ok161)* worms, which constitutively overexpress HIF-1, did undergo heat acclimation. Their survival curves, when subjected to heat stress before acclimation, show an initial attenuated decline and then a rather abrupt fall (Fig. 4). This, together with inability of *hif-1(ia4)* to acclimate to heat, may provide a first clue that HIF-1 is an essential component in the pathway to heat acclimation, possibly via elevated levels of HIF-1, rendering short-term thermotolerance at the onset of acclimation. This conclusion is reinforced by our results showing elevation of HIF-1 in N2 worms after acclimation. We previously provided evidence that HIF-1α is constitutively upregulated in two heat-acclimated mammalian species: the rat and the mouse (Ref. 25; and Z. Bromberg, G. Semenza, and M. Horowitz, unpublished observations). This similar phenomenon, in two taxonomically distant groups, mammals and nematodes, supports our conclusion that HIF-1 is an evolutionarily conserved mediator in the pathway to heat acclimation.

In mammalian species HIF-1α controls the majority of the hypoxia-responsive genes, including glycolytic enzymes, glucose transporters, etc. (19). Heat acclima-

tion also involves changes in energy metabolism, plasticity of several of the glycolytic enzymes, upregulation of glucose transporters, glyconeogenesis, etc. (Ref. 27; and M. Horowitz and E. Levi, unpublished observations). Given the targets of HIF-1 regulation, its upregulation could account for the changes in metabolism seen following heat acclimation. Such metabolic adaptations may increase metabolic efficiency leading to increased stress tolerance.

The non-acclimated *egl-9(n571)* and *vhl-1(ok161)* mutants resemble the N2 heat-acclimated phenotype only at the initial period of heat stress. This may imply that the metabolic pathway to heat acclimation mediated by HIF-1 is essential for short-term endurance during heat stress but that (long-term) heat acclimation involves additional acclimatory pathways, delayed in their full expression after stress. The heat shock proteins, e.g., HSP72, might be the likely candidates. These proteins are known for their contribution to the second window of protection following, for example, thermal or ischemic preconditioning (37).

A large body of studies associates heat tolerance with the heat shock proteins, particularly from the HSP70 superfamily. Invertebrates, as well as ectotherm and homeotherm vertebrates living in hot environments, have higher HSP70 levels compared with the same species living in temperate environments (34). Heat acclimation increases cellular reserves of HSP72 [which is the inducible member of the HSP70 superfamily (24) and HSP90 (7)]. Hence, to find out whether the inability to acclimate to heat, as found in the *hif-1(ia4)* mutant, is associated with impairment of the upregulation of HSP70, we measured HSP72 levels in wild-type and *hif-1(ia4)* worms. Interestingly, both N2 worms, which undergo heat acclimation, and *hif-1(ia4)* mutants, which do not, showed HSP72 upregulation upon heat acclimation, suggesting that induction of HSP72 reserves is not sufficient for heat acclimation. Taken together, the data from the present study provide direct experimental evidence that in the absence of HIF-1, greater HSP72 expression is not sufficient to induce heat acclimation. Others have shown that thermotolerance can be generated in the absence of HSP accumulation (reviewed in Ref. 22), which is in agreement with our results.

An important inseparable consequence of heat acclimation is long-term cross-tolerance to several other environmental stressors with one common denominator: either oxygen deprivation and/or the consequences of reoxygenation/reperfusion. Other investigators have shown causal relationships between thermotolerance and protection against heavy metals, and the hormetic response displayed by heat-stressed *C. elegans* to reactive oxygen species or oxygen deprivation is well established (6). Bearing in mind that HIF-1 is essential for heat acclimation, we tested whether this pathway contributes to the cross-tolerance phenomenon as well. Our findings from this investigation confirmed that heat acclimation induces cross-tolerance to heavy metals (cadmium) and chemical hypoxia in the wild-type *C. elegans*. The novelty of our findings is that HIF-1 is required for the observed cross-tolerance. Thus we implicate HIF-1 in tolerance to both heat and cadmium in addition to its known previous specific role in hypoxic stress. Interestingly, Chun et al. (5) have demonstrated that cadmium increases HIF-1α degradation in Hep 3B cells. Whether the drop in HIF-1 expression plays an important role in cadmium toxicity, however, is not clear. Nevertheless, our findings of the absence of cross-tolerance to cadmium in the *hif(ia4)* mutants urge future studies in this respect. Scott et al. (28) assayed hypoxia tolerance at 28°C, and they demonstrated increased hypoxia tolerance in *daf-2(e1370)* mutants pre-incubated at 20°C compared with those held at 15°C. These findings are in agreement with our findings of cross talk between heat acclimation and hypoxic tolerance in both mammals and *C. elegans*.

To summarize, the hypoxia inducible factor, HIF-1α, initially identified in mammalian tissues, is known as a master regulator of oxygen homeostasis. It plays a central role in both cellular and systemic responses to hypoxia. In addition to mammalian cells, its activation in response to hypoxia was recently identified in some teleost (33) and avian species (12) and in the nematode *C. elegans* (its homolog HIF-1) (18). Its central role during development and neoplastic diseases has also been proved (32). In this investigation we demonstrate, for the first time, a new function of HIF-1: a key regulator in the pathway for heat acclimation. Future analysis may elucidate the molecular mechanisms that govern the pathway to both heat acclimation and heat acclimation induced cross-tolerance to other stressors.

DISCLOSURES

This study was supported by the Ring Fund for Environmental Sciences-The Hebrew University, and by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities grant no 511/01-1. J. A. Powell-Coffman and H. Jiang were supported by National Science Foundation Award 9874456.

REFERENCES

1. **Arieli Y, Eynan M, Gantz H, Arieli R, and Kashi Y.** Heat acclimation prolongs the time to CNS oxygen toxicity in the rat. Possible involvement of HSP 72. *Brain Res* 962: 15–20, 2003.
2. **Beit-Yannai E, Kohen R, Horowitz M, Trembovler V, and Shohami E.** Changes of biological reducing activity in rat brain following closed head injury: a cyclic voltammetry study in normal and heat acclimated rats. *J Cereb Blood Flow Metab* 17: 273–279, 1997.
3. **Bennett AF.** Adaptation and evolution of physiological characters In: *Handbook of Physiology, Comparative Physiology*. Bethesda, MD: Am. Physiol. Soc., 1997, sect. 13, vol. I, chapt. 1, p. 3–16.
4. **Braeckman BP, Houthoofd K, and Vanfleteren JR.** Insulin-like signaling, metabolism, stress resistance and aging in *Caenorhabditis elegans*. *Mech Ageing Dev* 122: 673–693, 2001.
5. **Chun YS, Kim MS, and Park JW.** Oxygen-dependent and independent regulation of HIF-1α. *J Korean Med Sci* 5: 581–588, 2002.
6. **Cypser JR and Johnson TE.** Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. *J Gerontol A Biol Sci Med Sci* 57: B109–B114, 2002.
7. **Dietz TJ and Somero GN.** The threshold induction temperature of the 90-kDa heat shock protein is subject to acclimatization in eurythermal goby fishes (genus *Gillichthys*). *Proc Natl Acad Sci USA* 89: 3389–3393, 1992.

8. **Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, and Ratcliffe PJ.** *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43–54, 2001.
9. **Eynan M, Gross H, Hasin Y, Palmon A, and Horowitz M.** Temporal changes in heart mechanics with heat acclimation: adrenergic signaling and SR-Ca regulatory proteins. *Am J Physiol Regul Integr Comp Physiol* 279: R77–R85, 2000.
10. **Eynan M, Knubuvetz T, Meiri U, Navon G, Gerstenblith G, Bromberg Z, Hasin Y, and Horowitz M.** Heat acclimation-induce elevated glycogen, glycolysis and low thyroxine improve heart ischemic tolerance. *J Appl Physiol* 93: 2095–2104, 2002. First published August 9, 2002; 10.1152/japplphysiol.00304.2002.
11. **Gallagher LA and Manoil C.** *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* 183: 6207–6214, 2001.
12. **Gross TS, Akeno N, Clemens TL, Komarova S, Srinivasan S, Weimer DA, and Mayorov S.** Selected Contribution: osteocytes upregulate HIF-1α in response to acute disuse and oxygen deprivation. *J Appl Physiol* 90: 2514–2519, 2001.
13. **Hashmi S, Hashmi G, Glazer I, and Gaugler R.** Thermal response of *Heterorhabditis bacteriophora* transformed with the *Caenorhabditis elegans* hsp70 encoding gene. *J Exp Zool* 281: 164–170, 1998.
14. **Hoffman l W, Jump AA, Kelly PJ, and Ruggles AO.** Binding of antibodies and other proteins to nitrocellulose in acidic, basic, and chaotropic buffers. *Anal Biochem* 198: 112–118, 1991.
15. **Horowitz M.** Do cellular heat acclimatory responses modulate central thermoregulatory activity? *News Physiol Sci* 13: 218–225, 1998.
16. **Horowitz M.** Heat acclimation: phenotypic plasticity and cues to underlying mechanisms. *J Therm Biol* 26: 357–363, 2001.
17. **Horowitz M.** From molecular and cellular to integrative heat defense during exposure to chronic heat. *Comp Biochem Physiol A Mol Integr Physiol* 131: 475–483, 2002.
18. **Jiang H, Guo R, and Powell-Coffman JA.** The *Caenorhabditis elegans* hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc Natl Acad Sci USA* 98: 7916–7921, 2001.
19. **Kapron-Bras CM and Hales BF.** Heat-shock induced tolerance to the embryotoxic effects of hyperthermia and cadmium in mouse embryos in vitro. *Teratology* 43: 83–94, 1991.
20. **Lithgow GJ, White TM, Melov S, and Johnson TE.** Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. *Proc Natl Acad Sci USA* 92: 7540–7544, 1995.
21. **Liu J, Ben-Shahar TR, Riemer D, Treinin M, Spann P, Weber K, Fire A, and Gruenbaum Y.** Essential roles for *Caenorhabditis elegans* lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. *Mol Biol Cell* 11: 3937–3947, 2000.
22. **Kregel K.** Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J Appl Physiol* 92: 2177–2186, 2002; 10.1152/japplphysiol.01267.2001.
23. **Ma E and Haddad GG.** Cell and molecular responses to hypoxic stress. In: *Environmental Stressors and Gene Responses*, edited Storey KB and Storey J. Elsevier Sci, 2000, chapt. 7, p. 87–97.
24. **Maloyan A, Palmon A, and Horowitz M.** Heat acclimation increases basal HSP72 level and alters its production dynamics during heat stress. *Am J Physiol Regul Integr Comp Physiol* 276: R1506–R1515, 1999.
25. **Maloyan A, Semenza G, Gerstenblith G, Stern M, and Horowitz M.** Heat-acclimation-ischemia cross-tolerance: does HIF 1 play a role? *J Mol Cell Cardiol* 33: A72, 2001.
26. **Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA, and Ruvkun G.** The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature* 389: 994–999, 1997.
27. **Sawka MN, Wenger CB, and Pandolf KB.** Thermoregulatory responses to acute exercise-heat stress and heat acclimation. In: *Handbook of Physiology. Environmental Physiology. Thermal Physiology. Adaptive Responses to Heat*. Bethesda, MD: Am. Physiol. Soc., 1996, sect. 4, vol. I, part 2, chapt. 9, p. 157–185.
28. **Scott BA, Avidan MS, and Crowder CM.** Regulation of hypoxic death in *C. elegans* by the insulin/IGF receptor homolog DAF-2. *Science* 296: 2388–2391, 2002.
29. **Selye H.** *The Physiology and Pathology of Exposure To Stress; Treatise Based on the Concept of the General Adaptation Syndrome and the Diseases of Adaptation*. Montreal: Acta Medical, 1951.
30. **Semenza GL.** Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. *Curr Opin Genet Dev* 8: 588–594, 1998.
31. **Semenza GL.** HIF-1, O_2, and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 107: 1–3, 2002.
32. **Semenza GL.** HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* 8: S62–S67, 2002.
33. **Soitamo AJ, Rabergh CM, Gassmann M, Sistonen L, and Nikinmaa M.** Characterization of a hypoxia-inducible factor (HIF-1α) from rainbow trout. Accumulation of protein occurs at normal venous oxygen tension. *J Biol Chem* 276: 19699–19705, 2001.
34. **Ulmasov KA, Shammakov S, Karaev K, and Evgenev MB.** Heat shock proteins and thermoresistance in lizards. *Proc Natl Acad Sci USA* 89: 1666–1670, 1992.
35. **Walker GA, Walker DW, and Lithgow GJ.** Genes that determine both thermotolerance and rate of aging in *Caenorhabditis elegans*. *Ann NY Acad Sci* 851: 444–449, 1998.
36. **Wood WB.** *The Nematode Caenorhabditis elegans*. Plainview, NY: Cold Spring Harbor Laboratory Press, 1998, p. 587–605.
37. **Xi L, Tekin D, Bhargava P, and Kukreja RC.** Whole body hyperthermia and preconditioning of the heart: basic concepts, complexity, and potential mechanisms. *Int J Hyperthermia* 17: 439–455, 2001.