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Anoxic stress and rapid cold hardening enhance cold tolerance of the migratory locust ☆

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ABSTRACT

Anoxia and rapid cold hardening (RCH) can increase the cold tolerance of many animals. However, mechanisms underlying these two kinds of stresses remain unclear. In this study, we aimed to explore the relationship of acclimation to cold stress with acclimation to anoxic stress in the migratory locust, *Locusta migratoria*. RCH at 0 °C for 3 h promoted the survival of cold stress-exposed locusts. Anoxic hypercapnia (CO_2 anoxic treatment) for 40 min exerted an effect similar to that of RCH. Anoxic hypercapnia within 1 h can all promote the cold hardiness of locusts. We investigated the transcript levels of six heat shock protein (Hsp) genes, namely, *Hsp20.5*, *Hsp20.6*, *Hsp20.7*, *Hsp40*, *Hsp70*, and *Hsp90*. Four genes, namely, *Hsp90*, *Hsp40*, *Hsp20.5*, and *Hsp20.7*, showed differential responses to RCH and anoxic hypercapnia treatments. Under cold stress, locusts exposed to the two regimens showed different responses for *Hsp90*, *Hsp20.5*, and *Hsp20.7*. However, the varied responses disappeared after recovery from cold stress. Compared with the control group, the transcript levels of six Hsp genes were generally downregulated in locusts subjected to anoxic hypercapnia or/and RCH. These results indicate that anoxic stress and RCH have different mechanisms of regulating the transcription of Hsp family members even if the two treatments exerted similar effects on cold tolerance of the migratory locust. However, Hsps may not play a major role in the promotion of cold hardiness by the two treatments.

Introduction

Temperature is among the main determinants of insect distribution and activity. Survival at low temperatures is a common challenge for insects and other invertebrates living in seasonally cold environments. Winter survival for many insect species in temperate regions largely depends on their ability to avoid freezing by supercooling of extracellular body fluids [6]. Freezing hampers oxygen delivery to tissues. Thus, well-developed anoxia resistance is a component of natural freeze tolerance [25]. Oxygen supply is a key component in freezing survival because oxygen diffuses very slowly through ice, and breathing is arrested when tissues freeze. Staying in low-oxygen conditions has several physiological and biochemical consequences, including severe metabolic depression, which significantly leads to reduced adenosine triphosphate (ATP) demands [38], decreased production of nicotinamide adenine dinucleotide phosphate enzyme and glutathione [12], and denaturation of enzymes caused by decreased pH [3]. Anoxia treatments can enhance the cold tolerance of insects [5]. Studies on goldenrod gall fly larva exposed to anoxia showed that increased HIF-1α expression can promote freezing survival [25]. However, information is lacking on the enhancement of insect cold tolerance by anoxia treatment.

Insects rely on various forms of cold hardening to cope with sub-zero temperatures [35]. Insects use environmental signals, such as decreasing temperature and photoperiod, to trigger seasonal cold hardening [7]. Insects are also capable of cold hardening on a short time scale, in a process known as rapid cold hardening (RCH) [20]. RCH is a physiological mechanism that enables survival of organisms under otherwise lethal low temperature conditions following pre-exposure to a less severe cold temperature [20]. RCH was initially regarded as physiologically interesting but ecologically irrelevant; currently, RCH is considered a common occurrence and is ecologically important in many insects [2]. Seasonal cold hardening controls the gradual and predictable transition to the overwintering phenotype, whereas RCH secures survival during sudden cold snaps, during which the insect is not in a cold

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hardy state. These two types of cold hardening result in similar endpoints and share many similar basic mechanisms, such as cryoprotectant synthesis [19,41], membrane restructuring [28,30], and adjustment to ion transport [1,18]. However, special mechanisms for RCH have been reported recently. RCH has little effect on gene transcription [41,43], and fails to upregulate stress-related genes, e.g., heat shock proteins (Hsps), unlike cold acclimation [36,41,43]. Hsp is an important group of molecular chaperones that repair misfolded proteins in response to numerous environmental stresses [8,34]. Instead, RCH activates cell signaling pathways, such as calcium signaling [40] and MAP kinase signaling [13], and inhibit apoptotic cell death [48].

Adaptations to cold and anoxic stresses are not physiologically independent. In *Drosophila melanogaster*, RCH can be induced by a period of anoxia [27]. In the housefly *Musca domestica*, anoxia induces cold tolerance in the same way as RCH, but cold tolerance develops faster with anoxia treatment than with RCH treatment [5]. Anoxia changes the expression of several Hsp genes in the crucian carp, *Carassius carassius*, which survives in ice-locked ponds in the winter [37]. This response suggests that low temperature induces expression of Hsps, thereby enhancing anoxia tolerance to long-term low oxygen exposure. However, the molecular mechanisms underlying adaption to cold and anoxic stresses remain unclear.

The migratory locust, *Locusta migratoria*, has a broad geographic distribution from tropical to temperate zones [16]. The first instar hoppers are often subjected to subzero temperatures in early April following their emergence from the soil [44]. Previous studies in our laboratory confirmed that the mean supercooling point of first instar hoppers is −13.0 ± 1.4 °C, and at this temperature, the hoppers suffer from freezing stress [44]. Overwintering eggs often showed high mortality during severely cold winter seasons under natural conditions. However, RCH can increase the cold tolerance of *L. migratoria* eggs, young hoppers, and adults [9,44]. RCH improves chill coma recovery of hoppers by inducing rapid recovery of hemolymph ion homeostasis [9].

In the present study, we used first instar hoppers of migratory locusts to explore the relationship between acclimation to cold stress and acclimation to anoxic stress. We determined whether the molecular stress responses of young hoppers subjected to anoxic hypercapnia are similar to or different from the stress responses of those subjected to RCH. We compared the effects of anoxic stress and RCH on the cold tolerance of locusts and explored the possible underlying mechanisms by monitoring the transcriptional levels of six Hsp genes.

Materials and methods

Locust rearing

The stock colony of the migratory locust (*L. migratoria* L.) used in this study originated from adults that were obtained in April 2003 from Huanghua County (38°25′N, 117°20′E), Hebei Province, China. The locusts were reared in large, well-ventilated wooden cages (height × length × width of 60 cm × 50 cm × 50 cm) at densities of approximately 1000 insects per container under a long-day photoperiod (14 h light/10 h dark cycle) and 30 ± 1 °C. First instar nymphs were used in all experiments. For each treatment, six replicates of 120 individuals were used.

RCH and anoxic hypercapnia treatments

For RCH treatment, 20 hoppers were maintained in a glass test tube (2.0 cm × 8.0 cm) covered with gauze at 0 °C for 3 h. The expression of *Hsp* genes after RCH treatment was investigated using quantitative real-time PCR (qPCR). To test the effect of RCH on cold hardiness, the hoppers were subjected to cold stress (−6 °C) for 2 h following RCH treatment, and the survival ratio was calculated after 2 h of recovery at the rearing temperature (30 °C) with signs of movement. The expression of *Hsp* genes was immediately measured after cold stress or after 1, 2, and 4 h of recovery at the rearing temperature. The temperature was controlled by a programmable refrigerated bath (Polyscience, U.S.A.). Each treatment was performed in six replicates.

For anoxic hypercapnia treatment, 20 hoppers were maintained in a vacuum bag full of CO_2 for different durations (5, 10, 20, 40, 60, 120, and 240 min). One hour after recovery from anoxic hypercapnia or without recovery, the hoppers were subjected to freezing temperature at −6 °C for 2 h, and subsequently, the survival rates were calculated after 2 h of recovery at 30 °C with signs of movement. The responses of *Hsp* genes were examined by qPCR after three treatments, as follows: (1) 40 min of anoxic hypercapnia; (2) 40 min of anoxic hypercapnia followed by 1 h of recovery from anoxic hypercapnia, and subsequent 2 h of exposure to −6 °C; and (3) 40 min of anoxic hypercapnia followed by 1 h of recovery from anoxic hypercapnia, and 2 h of exposure to −6 °C followed by 1, 2, or 4 h of recovery from cold stress. Each treatment was performed in six replicates.

qPCR

Total RNA was isolated from the hoppers using an RNeasy Mini kit and treated with DNase I (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was reverse transcribed from 1 μg of total RNA using MLV reverse transcriptase (Promega, Madison, WI, USA). The transcriptional levels of six *Hsp* genes, including *Hsp90*, *Hsp70*, *Hsp40*, *Hsp20.7*, *Hsp20.6*, and *Hsp20.5*, and the β-actin reference gene, were quantified in six biological replicates of various treated and control groups (without anoxic hypercapnia or RCH treatment) with specific primers and methods, as described in previous studies [45,46]. The transcript levels of six Hsps were normalized by the β-actin transcripts.

Statistical analysis

Differences in survival ratios (after the arcsine and square root transformations) and *Hsp* transcriptional levels were compared by *t*-test for comparison of two means or by one-way ANOVA followed by a Tukey's test for multiple comparisons using SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Treatment differences were considered significant at $P < 0.05$. Values are reported as mean ± SE.

Results

Anoxic stress and RCH have similar effects on locust cold hardiness

The first instar hoppers were subjected to anoxic hypercapnia for 10, 20, or 40 min, and then exposed to −6 °C for 2 h immediately or 1 h after recovery from anoxic hypercapnia. The survival rate of the locusts with 1 h of anoxic hypercapnia recovery was significantly higher than that of the locusts that did not undergo anoxic hypercapnia recovery at each time point (10 min: $T = 9.61$, $P < 0.001$; 20 min: $T = 15.33$, $P < 0.001$; 40 min: $T = 21.24$, $P < 0.001$) (Fig. 1A). Anoxic hypercapnia recovery benefits the cold hardiness of locusts. Therefore, 1 h of anoxic hypercapnia recovery was used in later anoxic hypercapnia treatments. RCH (0 °C for 3 h) increased the survival rate of locusts under cold stress (−6 °C for 2 h) from 36% in the control to 74% ($P < 0.001$) (Fig. 1B). Anoxic hypercapnia treatment for 40 min resulted in a locust survival rate of 79% under cold stress, indicating that the effect of this treatment was comparable to that of

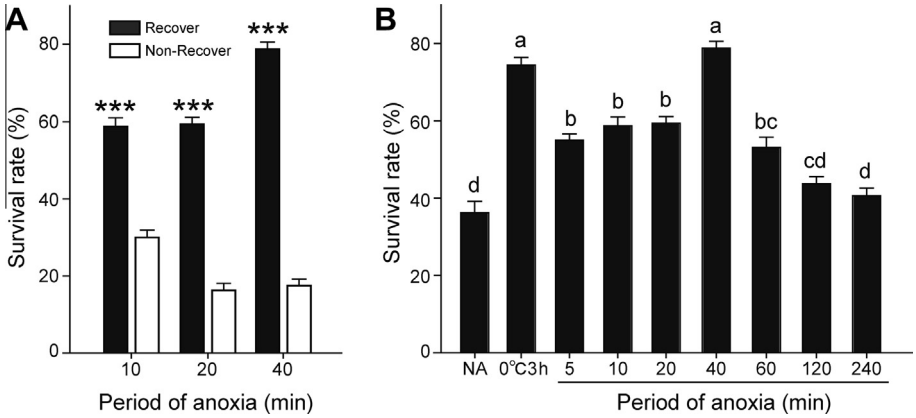

Fig. 1. Survival ratios of first instar hoppers under cold stress at −6 °C for 2 h with anoxic hypercapnia or RCH pretreatments. (A) Survival ratios were measured under cold stress 1 h after recovery from different periods of anoxia pretreatments (recovery) or directly (non-recovery). ***$P < 0.001$. (B) Survival ratios were measured under cold stress 1 h after recovery from different periods of anoxic hypercapnia pretreatments or RCH pretreatment (0 °C for 3 h). NA, control group without anoxia or RCH pretreatment. Letters indicate multiple comparisons among groups. Values are presented as the mean ± SE. Statistical analysis was performed on the transformed survival ratios treated with the arcsine and square root.

RCH ($P = 0.78$) (Fig. 1B). Anoxic hypercapnia treatments for 5, 10, 20, 40, and 60 min can promote the cold hardiness of locusts, whereas anoxic hypercapnia treatment for 2 h or more failed to promote the cold hardiness of locusts (Fig. 1B). A recovery period is required to obtain the beneficial effect of anoxic hypercapnia.

Expression of six Hsp genes in anoxic stress or RCH treatments

The transcription of Hsp20.5, Hsp20.6, Hsp20.7, Hsp40, Hsp70, and Hsp90 was quantitatively measured after RCH and 40 min of anoxic hypercapnia treatments (Fig. 2A). Four genes, *Hsp90*, *Hsp40*, *Hsp20.5*, and *Hsp20.7*, showed differential responses to the two treatments. Among these genes, only *Hsp90* was significantly downregulated after RCH treatment. After anoxic hypercapnia treatment, *Hsp90*, *Hsp40*, and *Hsp20.6* were significantly downregulated, whereas *Hsp20.5* was significantly upregulated. The Hsp transcript levels were generally not upregulated after the two treatments, indicating that these inducible Hsps failed to play a major role in the promotion of cold hardiness caused by RCH and anoxic hypercapnia.

Expression of six Hsp genes under cold stress with anoxic stress or RCH pretreatments

The extent of responses of three Hsp genes differed between RCH- and anoxic hypercapnia-pretreated (40 min) locusts under cold stress. When the locusts were subjected to −6 °C cold stress, the responses of *Hsp70*, *Hsp40* and *Hsp20.6* in locusts pretreated with RCH and anoxic hypercapnia were the same, and the transcript levels of *Hsp70* and *Hsp40* were downregulated compared with that of the control group (no pretreatment) (Fig. 2B). Differential responses were observed in the *Hsp90*, *Hsp20.5*, and *Hsp20.7*, where anoxic hypercapnia induced higher transcriptions of *Hsp20.5* and *Hsp20.7*, whereas RCH induced higher transcription of *Hsp90* (Fig. 2B). Furthermore, compared with the control group, the transcript levels of *Hsp90* and *Hsp20.5* were downregulated in the anoxic hypercapnia- and RCH-pretreated locusts, respectively, whereas *Hsp20.7* was upregulated in the anoxic hypercapnia-pretreated locusts (Fig. 2B).

Expression of six Hsp genes after recovery from cold stress with anoxic stress or RCH pretreatment

The transcription of the six *Hsps* was checked at three recovery time points (1, 2, and 4 h) after −6 °C cold stress treatment (Fig. 2C). The responses of six *Hsp* genes in locusts subjected to the two treatments were nearly not significantly different after cold stress recovery. Within the first 2 h of recovery, the transcript levels of the six *Hsps* were significantly downregulated in RCH- and anoxic hypercapnia-pretreated locusts compared with those in the control group. The downregulation was no longer observed at 4 h after recovery from cold stress.

Discussion

Successful adaptation to anoxic condition and cold stress is a contributing factor to the broad ecological and geographical distribution of insects. Adverse factors sometimes affect one another and exert convergent effects on insect physiology. In the present study, less than 1 h of anoxic hypercapnia by CO_2 and RCH treatments exerted similar effects in promoting the cold hardiness of locusts. RCH for 3 h at 0 °C produced a level of larval survival equivalent to that obtained after 40 min of anoxic hypercapnia under cold stress. Even 5 min of anoxic hypercapnia significantly promoted the survival rate. CO_2 induced physiological and metabolic stress partly due to the decrease in body fluid acidity [4]. Nitrogen is another frequently used gas that induces anoxia [5,49]. A similar phenomenon was observed in *M. domestica*, where 1.5 h of RCH at 0 °C elicited effects equivalent to those obtained after 40 min of anoxia by nitrogen [5]. Therefore, anoxic stress more rapidly induces insect cold tolerance than RCH.

As important and integral components of the minimal stress proteome of cells, Hsps have the necessary regulatory capacity to acclimate the insects under different environmental conditions [34]. Hsps fall into several major families, including Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsps (less than 30 kDa) families [8]. Previous studies show that Hsp71/72 proteins were induced by anoxia in *Spodoptera frugiperda* cell lines [15]. *Hsp26* and *Hsp70* were remarkably upregulated (up to a thousand-fold; especially *Hsp70*) in response to anoxic stress in the central nervous system of *Drosophila* sp. [22]. In our study, among the six previously cloned Hsps [46], only *Hsp20.5* was significantly upregulated after anoxic hypercapnia, whereas *Hsp90*, *Hsp40*, and *Hsp20.6* were downregulated. Each member of the Hsp families served a different function in the response to anoxic stress in different insect species. However, the differences in reactions to anoxia might result from Hsp tissue-specificity or cellular-specificity in expression.

RCH induces changes in compatible osmolytes [23,29] and in membrane composition or fluidity [28] to improve the organism's

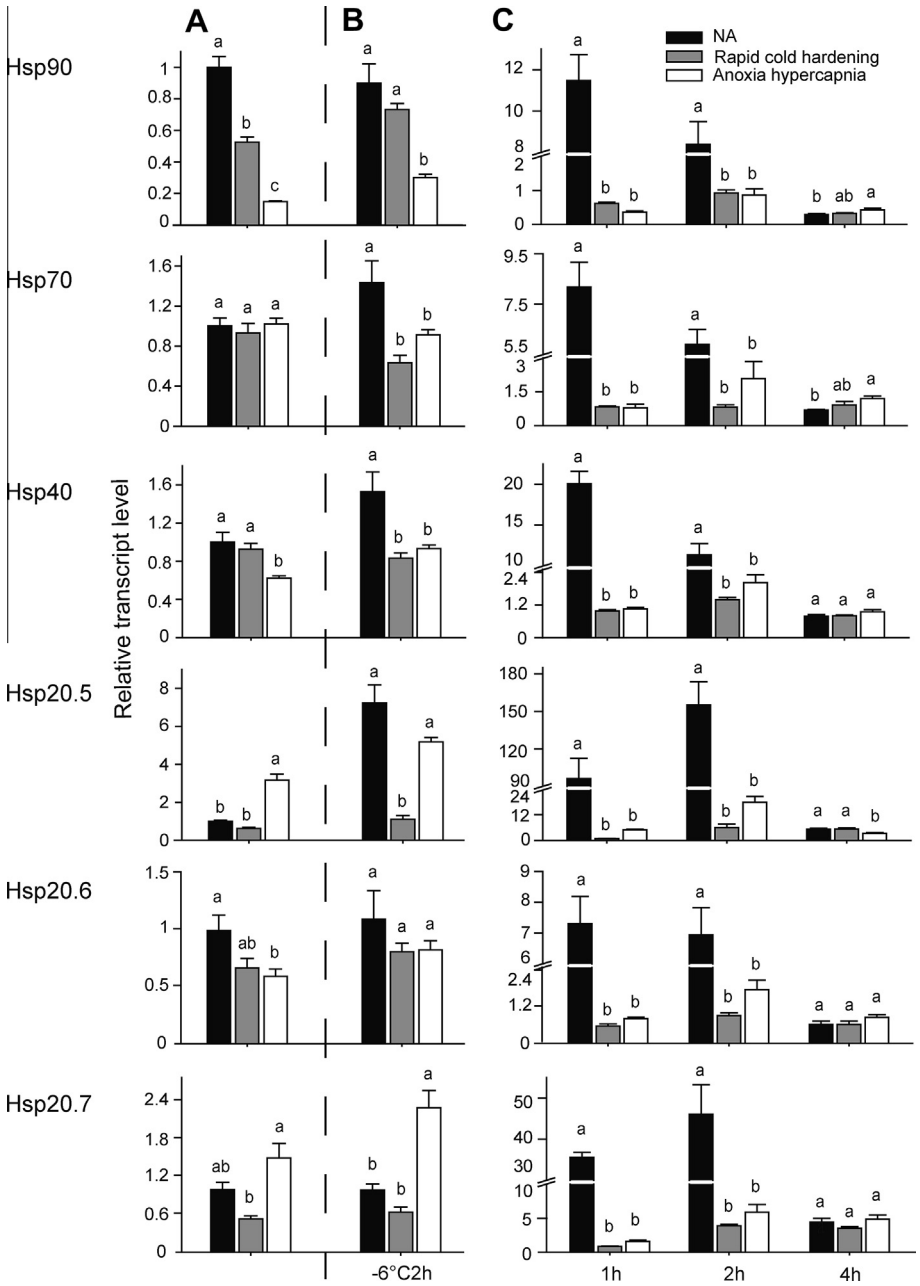

Fig. 2. Relative transcript levels of six *Hsp* genes in locusts. (A) With anoxic hypercapnia or RCH treatment. (B) Under cold stress at −6 °C for 2 h with RCH or anoxic hypercapnia pretreatment. (C) After different periods of recovery from cold stress with anoxic hypercapnia or RCH pretreatments. The transcript levels of *Hsp* genes are normalized with that of β-actin transcript and presented as mean ± SE. NA, the control group without anoxic hypercapnia or RCH treatment. Letters indicate multiple comparisons among groups.

ability to maintain ionic and metabolic homeostasis [1,9,29,41,39], and to prevent cold-induced apoptosis [48]. Usually, Hsp expression remains constant in RCH, e.g., Hsp70 in *D. melanogaster* [17,26]. However, RCH upregulates Hsps, e.g., increased Hsp23 abundance in the brain of flesh fly *Sarcophaga crassipalpis* [21]. In our study, we found that the transcript levels of the studied *Hsps*, except that of *Hsp90*, were not affected by RCH. *Hsp90* transcript level was downregulated by RCH. We could not exclude the possibility of the change in protein levels for Hsps because RCH may exert a complex regulation across different levels of the biological organization. A new and powerful case that supports this possibility is the complex regulation of glycogen phosphorylase by RCH in *D. melanogaster*; fly cold tolerance is associated with protein abundance and metabolite concentrations, but not with the level of transcript and enzyme activity [31].

The Hsp90 response differed from the responses of the other two large Hsps after anoxic stress and RCH. This phenomenon was also observed in flesh fly, *S. crassipalpis*; most Hsps of the flesh fly were upregulated except Hsp90, which was downregulated during pupal diapause [33]. Evolutionary analysis of eukaryotic Hsps in our previous study demonstrated that large Hsps have evolved according to cellular localizations and diverged at an early stage of eukaryotic cell formation [14]. The unusual response of Hsp90 may be due to its cellular localization, which is different from those of the other two large Hsps.

The response patterns of Hsp70 and Hsp40 in our present study were similar. Hsp70 required an adenosine triphosphatase (ATPase) cycle of ATP binding, hydrolysis, and nucleotide exchange to properly deliver its chaperone function [32]. However, Hsp70 required Hsp40 as a co-chaperone to stimulate its ATPase activity.

The N-terminal region (referred to as the J-domain) of Hsp40 cloned from *L. migratoria* is postulated to interact with the Hsp70 protein to stimulate ATPase activity [46]. This finding can explain the convergent response of these two Hsps under adverse conditions. The nearly identical expression profiles of Hsp70 and Hsp40 in *S. crassipalpis* during exposure to anoxia support the role of Hsp40 as an essential modulator of Hsp70 function [24].

In vitro, most small Hsps show chaperone-like activity, i.e., the capacity to interact with unfolding proteins to keep them in a folding and competent state [11]. In vivo, small Hsps have been implicated in various processes, such as enhancing cellular stress resistance [8], regulating actin and intermediate filament dynamics [47], inhibiting apoptosis, modulating membrane fluidity [42], and regulating vasodilation [10]. The three small Hsps, namely, *Hsp20.5*, *Hsp20.6*, and *Hsp20.7*, have been cloned from *L. migratoria* in our previous studies [46]. In the present study, the reactions of *Hsp20.5* and *Hsp20.7* to RCH and anoxic hypercapnia treatments with or without cold stress were different, whereas *Hsp20.6* showed similar reactions to both treatments. Thus, these three small Hsps served different functions after the RCH and anoxic stress.

In conclusion, anoxic stress and RCH regulated the expression of genes encoding different Hsp family members via different mechanisms. Hsps may not play a major role in the promotion of locust cold hardiness caused by anoxic stress and RCH.

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