Apoptosis (2007) 12:1183–1193
DOI 10.1007/s10495-006-0048-2

Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis

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Published online: 24 January 2007

Abstract The rapid cold-hardening (RCH) response increases the cold tolerance of insects by protecting against non-freezing, cold-shock injury. Apoptosis, or programmed cell death, plays important roles in development and the elimination of sub-lethally damaged cells. Our objectives were to determine whether apoptosis plays a role in cold-shock injury and, if so, whether the RCH response protects against cold-induced apoptosis in *Drosophila melanogaster*. The present study confirmed that RCH increased the cold tolerance of the adults at the organismal level. No flies in the cold-shocked group survived direct exposure to –7°C for 2 h, whereas significantly more flies in the RCH group survived exposure to –7°C for 2 h after a 2-h exposure to 5°C. We used a TUNEL assay to detect and quantify apoptotic cell death in five groups of flies including control, cold-shocked, RCH, heat-shocked (37.5°C, 30 min), and frozen (–20°C, 24 h) and found that apoptosis was induced by cold shock, heat shock, and freezing. The RCH treatment significantly improved cell viability by 38% compared to the cold-shocked group. Cold shock-induced DNA fragmentation shown by electrophoresis provided further evidence for apoptosis. SDS-PAGE analysis revealed an RCH-specific protein band with molecular mass of ∼150 kDa. Western-blotting revealed three proteins that play key roles in the apoptotic pathway: caspase-9-like (apoptotic initiator), caspase-3-like (apoptotic executioner) and Bcl-2 (anti-apoptotic protein). Consequently, the results of this study support the hypothesis that the RCH response protects against cold-shock-induced apoptosis.

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Keywords *Drosophila melanogaster* · Apoptosis · TUNEL assay · Rapid cold-hardening · Cold shock

Abbreviations

Apaf-1	apoptosis protease-activating factor-1
Bcl-2	B cell leukemia/lymphoma-2
Caspases	cysteinyl-directed aspartate-specific proteases
DAPI	4′,6-diamidino-2-phenylindole · 2HCl
Hsf-1	heat-shock factor 1
Hsps	heat shock proteins
RBP	rat brain proteins
RCH	rapid cold-hardening
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated uridine 5′-triphosphate-biotin (dUTP) nick end labeling

Introduction

The survival of an organism depends on its capacity to cope with various types of environmental stress including exposure to high and low temperatures. Insects survive extreme winter cold using various behavioral and physiological adaptations that have been relatively well characterized [1, 2]. In addition, many insects have another cold-hardening mechanism, termed the rapid cold-hardening (RCH) response, that functions even during the summer to enhance cold tolerance within minutes to hours after exposure to modest chilling [3, 4]. The RCH response not only protects against non-freezing, cold shock injury but also preserves and fine-tunes organismal performance, including courtship behaviors, by tracking even slight changes in environmental temperature [5].

Although the RCH response was discovered nearly two decades ago and is well documented in diverse taxa of insects, little is known about how this response prevents lethal cold shock injury. At the cellular level, injury due to chilling or cold shock is characterized by loss of membrane integrity, leakage of cytoplasmic contents and damage to intracellular organelles [6]. Injury is believed to result from thermotropic phase transitions in membrane lipids that cause a transition from the liquid-crystalline phase to a lethal gel phase [6].

Using fluorescent vital dyes, we demonstrated recently that organismal cold-hardening is matched closely by increases in the cold tolerance of their tissues [7, 8]. Furthermore, isolated cells and tissues are capable of cold-sensing and exhibit the RCH response *in vitro* [8]. Consequently, chilling can directly induce RCH in isolated insect cells and neuroendocrine mediation is not required.

Apoptosis, or programmed cell death, is a precisely regulated complex cascade of molecular events for eliminating unwanted and damaged cells [9], and plays important roles in the development of insects, nematodes, and vertebrates [10–13]. In addition, apoptosis removes cells damaged by stroke, cancer, neurogenerative disorders, and various chemical and mechanical stresses [10, 14]. Relatively long exposure to low temperature leads to apoptosis in some mammalian cells [15–17]. Furthermore, one study using Chinese hamster fibroblast cells found that a brief cold shock of 0°C for 1 h induced apoptosis [18].

The mechanism by which cold shock leads to cell death in insects is poorly known. Likewise, the process by which the RCH response protects against cold shock is also unclear. Consequently, the aim of our study was to determine (1) whether apoptosis is associated with cold-shock injury and, if so, (2) whether RCH protects against this cold-induced apoptosis; and (3) what molecular mechanisms underly this cryoprotective response in *Drosophila melanogaster*. To expand our understanding of mechanisms of temperature-induced injury, we also examined apoptosis in heat-shocked and frozen flies.

Materials and methods

Organismal cold tolerance

Fruit flies, *D. melanogaster* Oregon-R strain, were reared on a standard cornmeal-agar-yeast medium in 500-ml bottles (approximately 200 adults per bottle) at 23°C and a photoperiod of 15L:9D [19]. Two-to-four-day-old adult flies were used in all experiments. To determine the effect of rapid cold-hardening (RCH) on the cold tolerance of the whole animals, adults were divided into three groups: control (untreated at 23°C), cold-shocked (directly exposed to –5°C or –7°C for 2 h), and RCH (exposed to 5°C for 2 h prior to a 2-h exposure to –5°C or –7°C). Five replicates of 20–30 adults each were placed in 1.5-ml Eppendorf tubes, suspended in a programmed cold-bath. Survival was assessed at 2 h and 24 h post-treatment. We judged flies to have survived if they were able to stand.

Cryosections and TUNEL assay

Apoptotic cell death was detected by the terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) assay [12, 20] on cryosections of adult flies. Cryosections of thoracic flight muscles were prepared from 5 groups of flies: the untreated control, the cold-shocked, the RCH, the heat-shocked (37.5°C, 30 min), and the frozen (–20°C, 24 h) followed by recovery for 2 h at room temperature. After briefly rinsing in PBS (137 mM NaCl, 4.0 mM KCl, 0.6 mM Na_2HPO_4, 0.15 mM KH_2PO_4, pH 7.2), flies were fixed in 4.0% paraformaldehyde for 2 h on a shaker at room temperature. The fixed flies were cryoprotected in 30% sucrose-PBS solution (w:v) overnight after 1-h washing in PBS. The next day, the flies were embedded in Histo Prep, a frozen tissue embedding medium (Fisher, Fair Lawn, NJ) in plastic cubic base-molds (7 × 7 × 5 mm) (Fisher, Florence, KY). After sitting at room temperature for 15 min, the embedded molds were frozen at –80°C in a flat bottomed metal tray for at least 4 h. Ten μm thick cryosections were processed on a HM 505 N Microm at –22°C and placed on Superfrost/plus microscope slides (Fisher), which provide good adhesion for the cells. The slides were stored at –80°C until analyzed.

TUNEL assay was carried out using the *in situ* cell death detection kit, TMR Red (Roche, Indianapolis, IN) as per the manufacturer's instructions. Slides were brought to room temperature for 15 min and washed 3 times, 5 min each, in PBS. Permeabilization was done by incubating the slides in a solution of 0.1% sodium citrate and 0.1% Triton X-100 at 4°C for 15 min followed by washing three times in PBS. Fifty microliters of TUNEL reaction mix (enzyme:label solution = 1:9) were added to each slide. The slides were then placed in a moist box and incubated for 1 h in the dark at 37°C. After incubation, the slides were washed twice in a blocking buffer [0.05% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS] and counterstained with 200 μl of 4′,6-diamidino-2-phenylindole · 2HCl (DAPI) (Pierce Biotechnology, Rockford, IL) solution for 5 min followed by 3 washes in PBS. Then the slides were covered with cover slips and a drop of Vectashield, a mounting medium for fluorescence (Vector Laboratories, Burlingame, CA). Individual apoptotic cells were stained red by the detection kit, while all nuclei were stained blue by DAPI when observed with a fluorescence microscope. Cell apoptosis was quantified by comparing the ratio of red to blue cells using ANOVA Bonferroni-Dunn test (Statview, SAS Institute) with

statistical significance set at $P < 0.01$. Each mean value is based on the counts of ten fields of 50–300 cells for each of three replicates.

DNA isolation and gel electrophoresis

DNA was isolated as described by Birren et al. [21] from three groups of flies: the untreated control, the cold-shocked and the RCH. Flies were rinsed 3 times with 0.16 M NaCl and homogenized in a 2-ml Pyrex glass homogenizer containing buffer (100 mM Tris, 100 mM EDTA, 1% SDS, pH 8.8) in a ratio of 10 μl buffer per mg of fresh body mass. The homogenates were incubated in 1.5-ml Eppendorf tubes at 65°C for 30 min, and then 56 μl of 8 M potassium acetate were added and mixed by vortex. After sitting on ice for 30 min, the homogenates were centrifuged at 12,000 *g* in an Eppendorf centrifuge at 4°C for 15 min. Supernatants were collected and 200 μl of isopropanol were added and mixed by inverting the tube. The mixture was centrifuged for 5 min before the pellet was collected and washed with 70% ethanol. After another 5-min centrifugation, the pellet was collected and dissolved in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4). DNA was extracted with an equal volume of phenol/chloroform/isopentyl alcohol (25:24:1) and precipitated with 1/3 volumes of 7.5 M ammonium acetate (pH 7.4) and 2.5 volumes of absolute ethanol on dry ice for 10 min. The DNA was washed with 70% ethanol, air dried, and stored at –80°C until electrophoretic analysis.

Before electrophoresis, the DNA was dissolved in TE buffer and its concentration was measured on a Nanodrop automatic system. DNA damage was evaluated by electrophoresis on 2% agarose gel containing ethidium bromide in TAE buffer (40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.1) and visualized under ultraviolet light [22]. Fifteen micrograms of DNA samples were loaded onto each lane of the gel using a 100 Base-pair ladder (Fisher, Fair Lawn, NJ) as standard markers.

Protein extraction and SDS-PAGE

Soluble proteins were extracted using *Drosophila* extract buffer (25 mM Hepes, pH 6.8, 50 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 250 mM sucrose) [23] containing the following protease inhibitors: 5 μg/ml aprotinin, 5 μg/ml antipain, 5 μg/ml leupetin, and 1 mM PMSF. Briefly, flies were washed with 0.16 M NaCl and homogenized in the *Drosophila* extract buffer containing protease inhibitors in a ratio of 4 μl of buffer per mg of fresh body mass. After 15-min centrifugation at 10,000 *g* at 4°C, the supernatant, which contained soluble proteins, was collected.

Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) as per the manufacturer's instruction and using BSA as a standard. The proteins were analyzed by SDS-PAGE on a 4–20% gradient gel using 80 μg of proteins in each lane. A broad range molecular weight standard kit (Bio-Rad, Hercules, CA) was used as references. Rat brain proteins (RBP), kindly provided by Phyllis A. Callahan (Department of Zoology, Miami University, Oxford, OH), were used as positive control proteins. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250.

Immuno-blotting

Following electrophoresis, proteins were transferred to a cellulose membrane (Bio-Rad) for western blotting. Non-specific protein antigens were blocked at 4°C overnight by a 10% dehydrated non-fat milk in western wash buffer (10 mM Tris, 100 mM NaCl, pH 7.5, containing 0.1% Tween 20). The membrane was cut into strips according to the expectant antigens' molecular sizes and incubated with corresponding primary antibodies in a 5% non-fat milk-western wash buffer solution at room temperature for 2 h. Mouse anti-Hsp90 (1:1,000), rabbit anti-Hsp110 (1:1,000), anti-Hsf-1 (heat-shock factor 1) (1:1,000), and anti-Hsp27 (1:2,000) were purchased from Sigma (Saint Louis, MS); whereas mouse anti-procaspase-9 (1:1,000), anti-Bcl-2 (1:1,000), rabbit anti-Hsp70 (inducible) (1:3,000), anti-caspase-3 active form (1:500), and anti-survivin (1:1,000) were purchased from Stressgen (Victoria, BC, Canada). After washing, the membranes were incubated for 2 h with either goat anti-mouse or anti-rabbit IgG-HRP conjugates (Sigma, Saint Louis, MS) diluted 1:1,000 with 5% dehydrated non-fat milk in western wash buffer followed by three 10-min washes in the western wash buffer. Immuno-reactive bands were detected by 2-min incubation of the membranes in a chemiluminescent substrate mixture, the ECL detection reagents (Amersham Biosciences, Piscataway, NJ), exposed to an autography film and developed.

Results

Organismal cold tolerance

RCH increased the cold tolerance of adults at the organismal level (Fig. 1). No flies in the cold-shocked group survived direct exposure to –7°C for 2 h, whereas significantly more (18.8%) flies in the RCH group (2 h at 5°C) survived a 2-h exposure at –7°C. Similarly, under a less severe cold-shock treatment (–5°C), 41.3% more flies survived in the RCH group (92.8%) than in the cold-shocked group (ANOVA, Bonfferoni-Dunn test, $P < 0.01$), indicating cryoprotection by the RCH treatment.

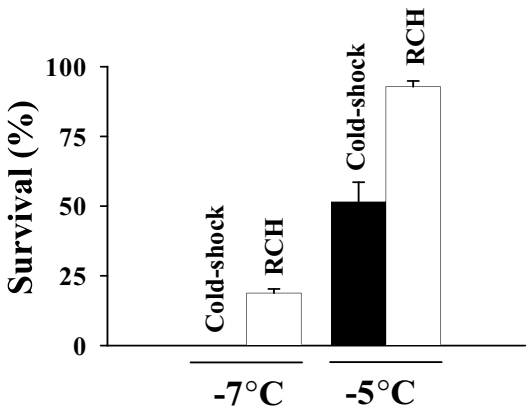

Fig. 1 Effect of cold shock and rapid cold-hardening (RCH) on adult survival at –5°C or –7°C for 2 h. There was a significant difference in the survival between cold-shocked and RCH groups (ANOVA Bonferroni-Dunn test, $P < 0.01$) at both temperatures

Temperature stress and apoptotic cell death

Cold shock treatment markedly increased the incidence of apoptotic cell death in flight muscles of adult flies (Figs. 2 and 3). We used the TUNEL assay to determine whether apoptosis occurred in response to temperature stress, especially injury caused by cold shock. Whereas 15.7 ± 3.5% (mean ± SEM) of cells in the untreated control group (Fig. 2(A)–(C)) stained positively for TUNEL, a significantly higher level (78.3 ± 4.3%) of apoptotic cell death was induced by the cold-shock treatment (Fig. 2(D)–(F)), which indicates that apoptosis is involved in cold shock injury.

Rapid cold-hardening significantly decreased apoptotic cell death in flight muscles of flies exposed to –7°C. Only 40.3 ± 4.8% of apoptotic cells were present in the RCH-treated flies (Fig. 2(G)–(I)), corresponding to a significant improvement of cell viability by 38% compared to the cold-shocked group. The heat-shock treatment caused apoptosis in 82.3 ± 5.6% (Fig. 2(J)–(L)) of cells, a level similar to that induced by the cold-shock treatment. Cells in the freezing-treatment group (Fig. 2(M)–(O)) were intensely labeled by the TUNEL staining, indicating an even more severe level of apoptosis (94.4 ± 8.9%). These results suggest that apoptosis plays a role in heat-shock and freezing injury, in addition to cold shock injury.

DNA fragmentation

Gel electrophoresis of fly DNA revealed different banding patterns among treatment groups (Fig. 4). A band of 400–600 base pairs (bp) was present in both control and RCH treatment groups, but it was absent from the cold-shock treatment group. Instead, the major band was at the ∼150 bp DNA segment position. This pattern of DNA fragmentation into short segments (approximately 180 bp) is characteristic of apoptosis [22, 24], and provides additional evidence that RCH response protects against cellular apoptosis. These observations are consistent with the results obtained from the TUNEL assay (Figs. 2 and 3), in which we found a significant difference in the frequency of apoptotic cells between the RCH and the cold-shocked treatments.

RCH-specific protein band

To determine whether certain proteins were specifically expressed in association with the RCH response, we extracted soluble proteins from control, cold-shocked, and RCH flies and analyzed them by SDS-PAGE on 4–20% gradient gel (Fig. 5). Our electrophoretic data indicated changes in two protein bands. A novel protein band with a molecular size of ∼150 kDa (Band 1) appeared only in the RCH group, whereas Band 2 (∼130 kDa) became less dense in the cold-shocked flies compared to either the control or the RCH group. In separate experiments, we did not find any significant changes in banding patterns in the lower molecular mass regions.

Immunological identification of heat-shock proteins (Hsps)

We used western-blotting to identify whether and which Hsps were involved in the RCH response. After electrophoresis, the proteins were blotted onto a cellulose membrane and reacted with specific monoclonal or polyclonal antibodies against Hsps (Hsp110, 90, 70 and 27), or against heat-shock factor 1 (Hsf-1) (Fig. 6). These proteins were selected for identification based on their molecular sizes and the fact that they are commonly up-regulated in flies exposed to various environmental stresses [25–28]. Neither cold shock nor RCH treatment induced a significant increase in the expression of the Hsps and Hsf-1. Immuno-blotting showed the presence of Hsp110 (Fig. 6(A)), and Hsp70 (Fig. 6(D)) but not Hsp90 (Fig. 6(B)) in all treatment groups, although the amount and pattern for each Hsp protein varied among the groups. Interestingly, Hsf-1 (Fig. 6(C)) and Hsp27 (Fig. 6(E)) shared a very similar immuno-reactive pattern: they were abundant in the first three groups (control, cold-shocked and RCH), but absent from both heat-shocked and frozen flies. These immunological patterns suggest potential roles for Hsp70 and Hsp27, for enhancing organismal cold tolerance and protection against cellular apoptosis.

Immunological identification of caspases and apoptosis inhibitors

The protein extracts also were immuno-blotted to test for the expression of proteins that play key roles in the apoptotic pathway. Two caspases (procaspase-9-like and active caspase-3-like), and two inhibitors of apoptosis proteins

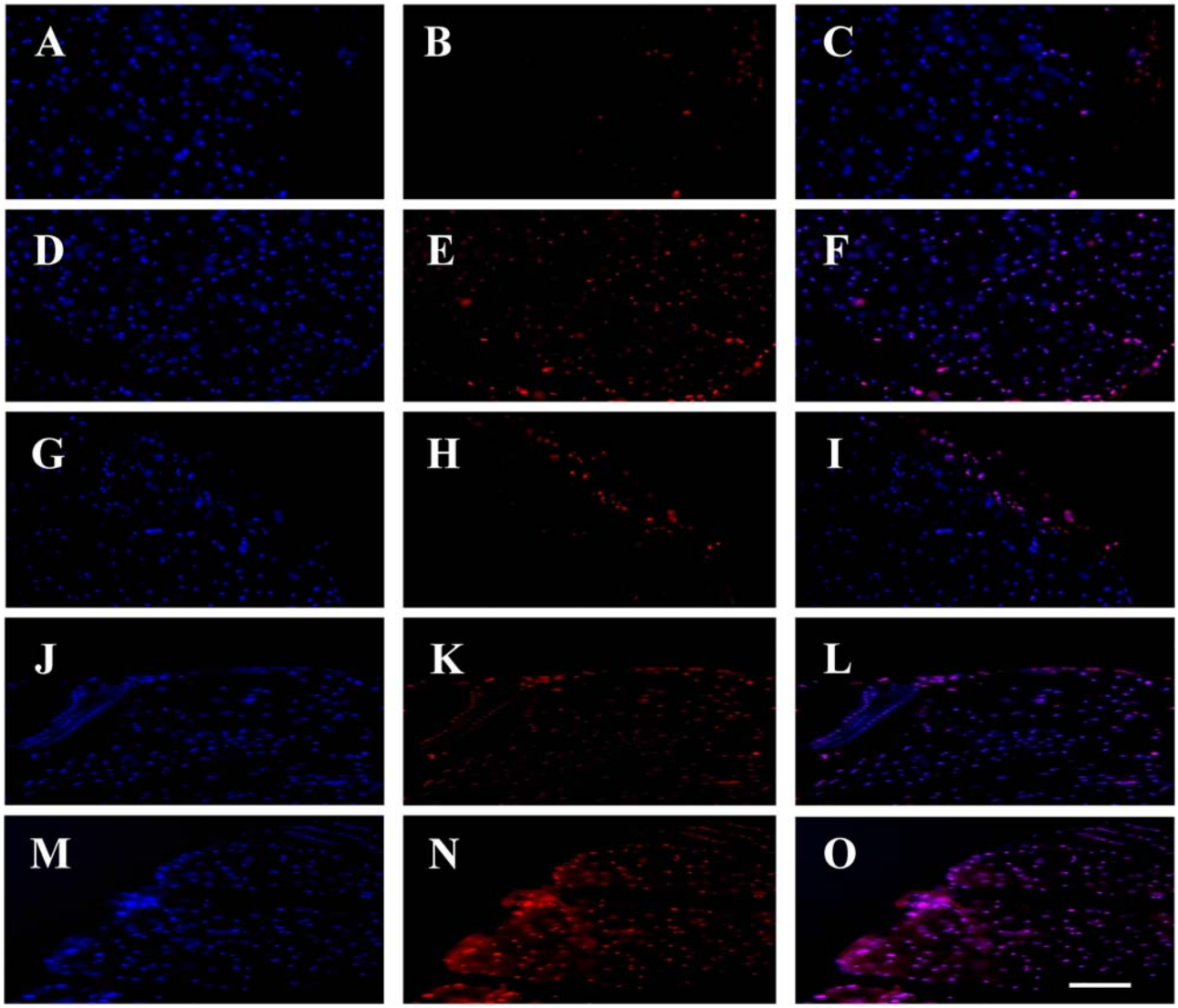

Fig. 2 Comparison of apoptosis in cryosections of flight muscles from adult flies in control (A, B, C), cold-shocked (D, E, F), rapidly cold-hardened (G, H, I), heat-shocked (J, K, L), and frozen (M, N, O) treatment groups detected by TUNEL assay with *in situ* cell death detection kit, TMR red. Blue color in A, D, G, J, and M shows nuclear staining with DAPI. Red color in B, E, H, K, and N shows cell apoptosis stained by the TUNEL reagents. Both blue and red are shown in the merged images in C, F, I, L, and O. Scale bar = 50 μm

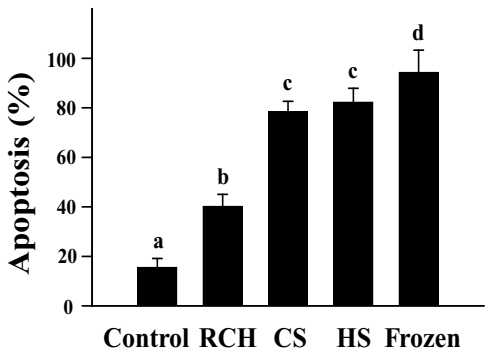

Fig. 3 Comparison of apoptotic cell death among control, rapidly cold-hardened (RCH), cold-shocked (CS), heat-shocked (HS), and frozen treatments. Different letters indicate significant differences between treatment groups (ANOVA Bonferroni-Dunn test, $P < 0.001$)

(IAP) (Bcl-2 and survivin), were detected using specific antibodies (Fig. 7). Both caspases belong to the family of cysteine proteases with specificity for aspartate residues [29]. According to their mode of activation, the former is an initiator whereas the latter is an executioner in the apoptotic cascade. Although both caspase-like proteins were present in each treatment group, they were least abundant in the RCH group (Fig. 7(A) and (C)). As expected, the active form of caspase-3-like was most abundant in the cold-shocked group (Fig. 7(C)). Not surprisingly, the apoptotic inhibitor Bcl-2 was down-regulated in both cold-shocked and heat-shocked groups compared to the control and RCH groups (Fig. 7(B)), thus showing a pattern of immunological reactivity opposite to the caspase-3-like protein. Finally, survivin (Fig. 7(D)),

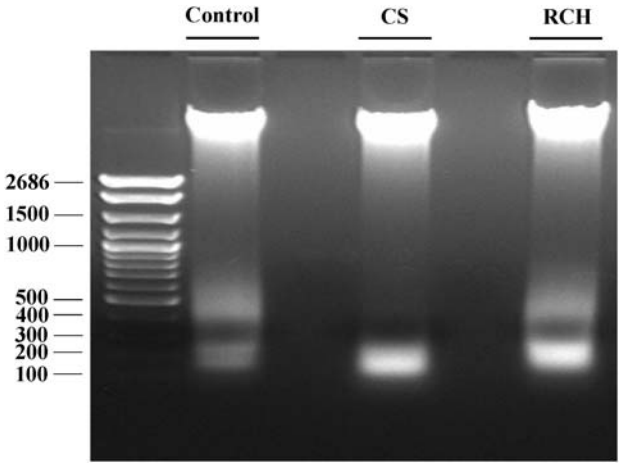

Fig. 4 DNA electrophoresis of control, cold-shocked (CS), and rapidly cold-hardened (RCH) adults. A band ranging in size from 400–600 base pairs (bp) presented in both control and RCH treatment groups was absent from cold-shocked flies

the other apoptotic inhibitor we examined, appeared at a low level in all treatment groups.

Discussion

Apoptosis is associated with cold injury

In addition to its role in the development and removal of cells damaged by disease, recent studies related to cryosurgery and cryopreservation have reported cold-induced apoptosis in mammalian cells [22, 30–33]. In nearly all of these reports, tissues were exposed to 0–4°C and/or freezing over extended periods of many hours or days. In contrast, in Chinese hamster V79 fibroblast cells cold shock-induced apoptosis can occur without freezing after only 1 h at relatively mild temperatures of 0–10°C [18, 34].

A hallmark of apoptosis is damage to DNA in the form of cleavage by endonucleases into oligonucleosoma-sized fragments. Consequently, we tested for DNA damage using the TUNEL assay and gel electrophoresis [20]. Cold-shocked flies exhibited significantly more apoptotic cells than controls (Figs. 2 and 3). Furthermore, heat-shock and freezing also induced apoptosis, indicating that both high and low temperature stress can serve as triggers. Freezing at –20°C for 24 h induced the highest level of apoptotic cell death compared to the cold- or heat-shock treatments. Cold-shock resulted in the disappearance of the 400–600 DNA base-pair band, leaving only a 100–200 base-pair band (i.e., the DNA was cut into short fragments; Fig. 4). Consequently, the TUNEL and electrophoretic results are consistent with cold shock-induced apoptosis. To our knowledge these molecular data are the first such reported for cold-induced apoptosis in insects. Cold shock-induced apoptosis was reported in two species of reduviid bugs as evidenced by changes in nuclear phenotypes (nuclear fusion and heterochromatin unraveling) in the Malpighian tubules [35, 36]. However, no molecular evidence for apoptosis was provided in these studies.

RCH response protects cells against cold-induced apoptosis

Our previous studies on adult flesh flies, *Sarcophaga crassipalpis* and *S. bullata,* demonstrated that RCH protected

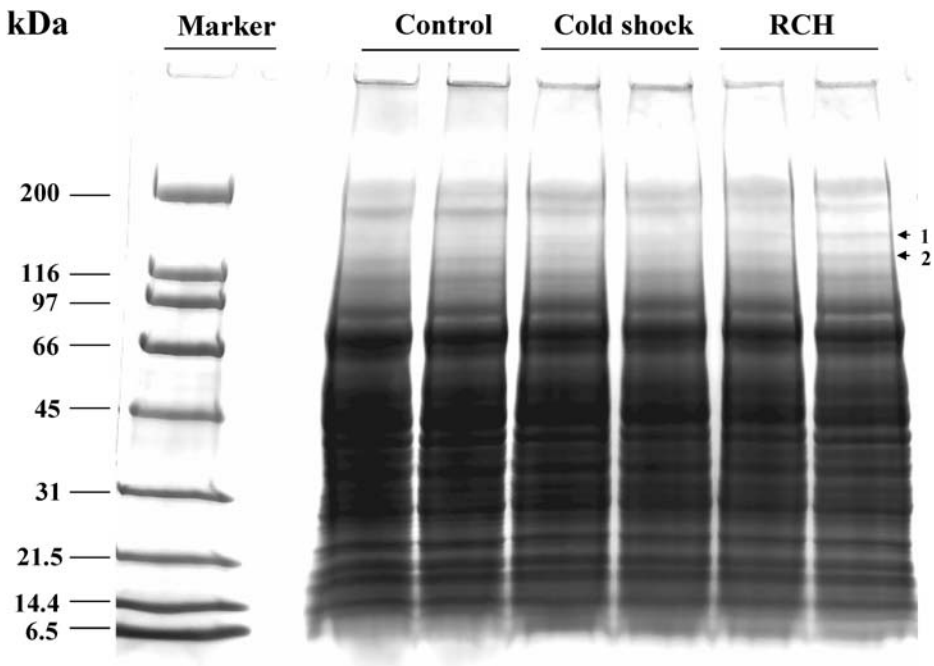

Fig. 5 A representative of SDS-PAGE on 4–20% gradient gel of proteins from control, cold-shocked, and rapidly cold-hardened (RCH) treatment groups. Eighty micrograms of protein samples were loaded onto each lane. Numbers on the right indicate changes in protein bands between groups. Their estimated molecular masses are: Band 1 = ~150 kDa; and Band 2 = ~130 kDa, respectively. Protein Band 1 (~150 kDa) appears to be unique to the RCH flies

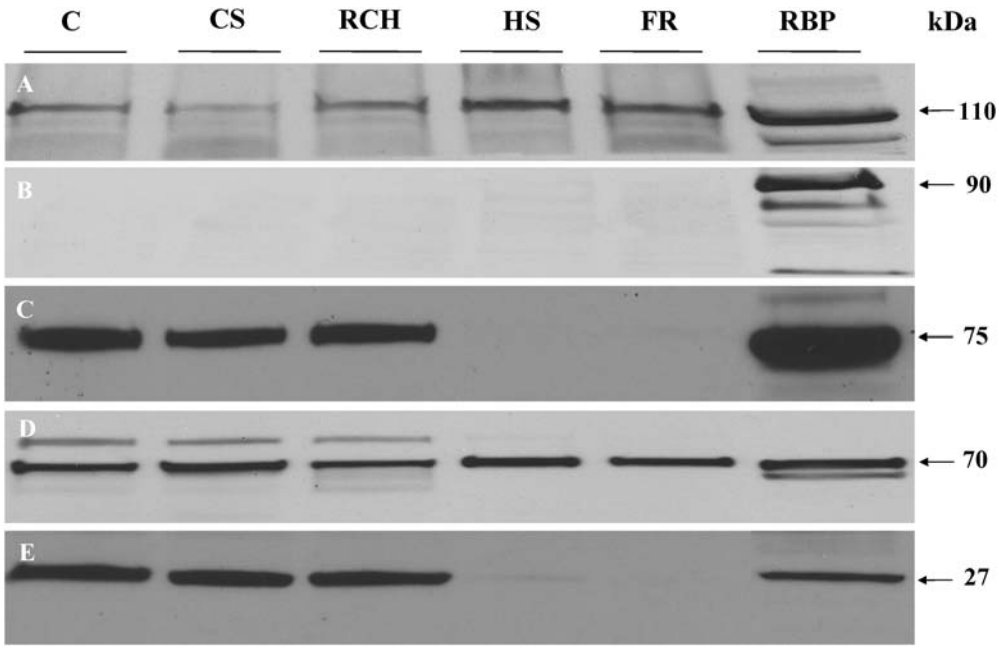

Fig. 6 Immuno-blotting of fly proteins with anti-Hsp110 (A), Hsp90 (B), Hsf-1 (C), Hsp70 inducible (D), and Hsp27 (E) antibodies. Molecular masses are indicated on the right. Proteins were extracted from: C = control, CS = cold-shocked, RCH = rapidly cold-hardened, HS = heat-shocked, and FR = frozen treated flies. RBP = rat brain proteins. Both RBP and purified Hsp27 were used as positive controls

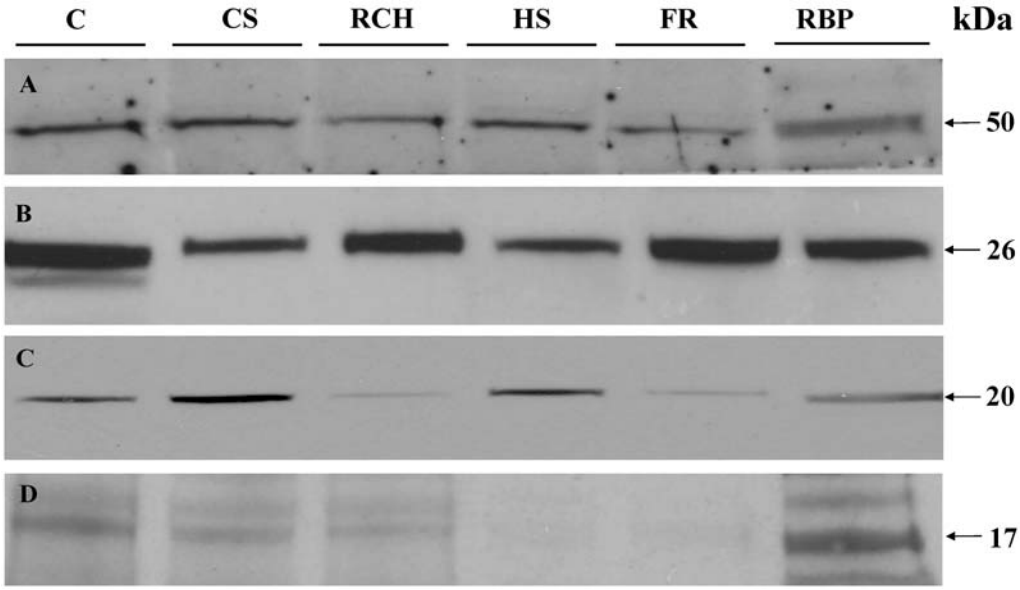

Fig. 7 Immuno-blotting of fly proteins with anti-procaspase-9 (A), Bcl-2 (B), active caspase-3 (C), and survivin (D) antibodies. Molecular masses are indicated on the right. Proteins were extracted from the same sources as in Fig. 6. Rat brain proteins (RBP) were used as positive controls

adults and their tissues from cold shock injury in the fat body, gut, salivary gland and Malpighian tubules both *in vivo* and *in vitro* [8, 37]. In addition, RCH also significantly increases organismal and cellular cold tolerance in freeze-tolerant larvae of the Antarctic midge, *Belgica antarctica* [38]. In the present study, RCH markedly increased adult survival of exposure to –5°C and –7°C (Fig. 1), as we have demonstrated previously in *D. melanogaster* [4, 5]. By means of TUNEL staining, we showed that protection provided by the RCH response at the organismal level was closely paralleled at the cellular level, as the incidence of apoptosis dramatically decreased by 38% in *D. melanogaster* adults compared to flies in the cold-shocked group (Figs. 2 and 3).

Studies with mammalian tissues have also demonstrated that brief pre-exposure to moderately severe temperatures blocked heat shock- or cold shock-induced apoptosis. Brief pre-exposure of Chinese hamster ovary and human adenocarcinoma cervical cells to 40°C for 3 h increased thermotolerance to the otherwise lethal exposure to 43°C by blocking heat shock-induced apoptosis [39]. Xu et al. [31] reported that moderate postinjury hypothermia (32°C) inhibits apoptosis following cold-induced brain injury in rats.

Do Hsps play a role in blocking apoptosis during RCH?

A number of heat shock proteins are involved in either developmentally or stress-induced apoptosis [40]. For instance, a small Hsp-like B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation [41]. In L6 myoblast cells, Suzuki et al. [42] reported that a pretreatment (heat shock at 42°C for an hour) prior to the hypoxia-reoxygeneration insult significantly reduces the percentage of apoptotic cells [42]. Furthermore, Hsp70 and Hsp27 inhibit apoptosis and thereby increase the survival of cells exposed to a wide range of lethal stimuli [43]. In another study, Hsp70 content was negatively correlated to caspase-3 activity and positively correlated to Bcl-2 protein and its mRNA [44].

In our study, immuno-blotting did not reveal the presence of any RCH-specific Hsp band, although a protein band with molecular mass of $\sim$150 kDa appeared to be unique to the RCH treated flies (Fig. 5). Furthermore, neither cold-shock nor RCH treatment induced a significant increase in the expression of the heat shock proteins Hsp110, Hsp70 and Hsp27 or the heat shock factor, Hsf-1 (Fig. 6). These data are consistent with previous studies of cold shock in this species. Kelty and Lee [45] reported no change in the level of the most responsive heat-inducible protein, Hsp70. Similarly, Overgaard et al. [46] studied the RCH's role in Hsp expression after cold shock and found no induction of Hsp70 following cold-shock treatment irrespective of the pre-treatment. In *Drosophila auraria*, Yiangou et al. [47] found no induction of small Hsp genes during recovery after transient cold shock. Taken together, these results suggest that the RCH response does not require a significant increase in the Hsps.

Nevertheless, one cannot exclude the possibility of the Hsps' involvement in RCH protection because Hsps are expressed following transient cold shock and a return to higher temperatures [48]. Indeed, other studies have demonstrated that the major heat-shock proteins, Hsp83, Hsp70, Hsps 27 (or 26), Hsp23 and Hsp22 in *D. melanogaster* tissues or cells were induced following cold shock or cold acclimation [25, 27, 28, 49, 50]. Both Hsps and their mRNA accumulate during the recovery period after cold treatment [49, 51, 52]. Thus, Hsps likely play a role in blocking apoptosis and in other ways protecting insects that are exposed to repeated cycles of chilling and warming.

Evidence for caspase-9 and caspase-3

A central step in the apoptotic pathway is the activation of a class of highly conserved cysteine proteases, the caspases [53]. The activation and regulation of this suicide program involves numerous intra- and extra-cellular signals and factors [54–56]. Induction of apoptosis frequently requires the activation of the "initiator" caspases-2, -8 and -9 [57]. These initiator caspases lead to downstream activation of "executioner" caspases, such as caspase-3, which incite the proteolytic cascade that kills cells [55].

Since caspase-9 is associated with a variety of apoptosis-inducing agents [57], we selectively tested for the expression of caspase-9-like as well as caspase-3-like proteins using immuno-blotting analysis (Fig. 7). We found procaspase-9-like protein in all treatment groups, and the up-regulation of active caspase-3-like protein in the cold-shocked and heat-shocked flies. In contrast, flies in the RCH group had significantly lower levels of caspase-3-like protein suggesting that the RCH response confers cytoprotection by blocking apoptotic progress.

Bcl-2 is involved in RCH protection against apoptosis

During the course of caspase activation in the apoptotic pathway, there are multiple barriers to prevent inappropriate cell death. Endogenous inhibitors block the initiation of the caspase cascade and/or interrupt its forward progression [55]. Proteins in the Bcl-2 (B cell leukemia/lymphoma-2) family are key regulators of apoptosis and include both pro-apoptotic and anti-apoptotic members in mammalian cells [58, 59], but few such proteins have been described in insects. Bcl-2 protein and its anti-apoptotic relative Bcl-x_L, located in the mitochondrial outer membrane and the endoplasmic reticulum/perinuclear membrane, protect cells from apoptosis in response to stress stimuli by acting upstream of caspases to prevent their activation [55, 56]. These inhibitory proteins may function by preventing cytochrome *c* release from the mitochondria in some situations or by inhibiting cytochrome *c* from inducing caspase activation in others [55].

In our study, protein validation by western blotting demonstrated an up-regulation of the anti-apoptotic protein Bcl-2 in concert with down-regulation of both procaspase-9-like and caspase-3-like proteins by the RCH treatment in *D. melanogaster*, suggesting an anti-apoptotic function of Bcl-2 (Fig. 7). Our data provide the first evidence that Bcl-2 plays a role in the RCH protection against cold-induced apoptosis.

We found that the anti-apoptosis protein survivin was down-regulated in both cold-shock and RCH treatment

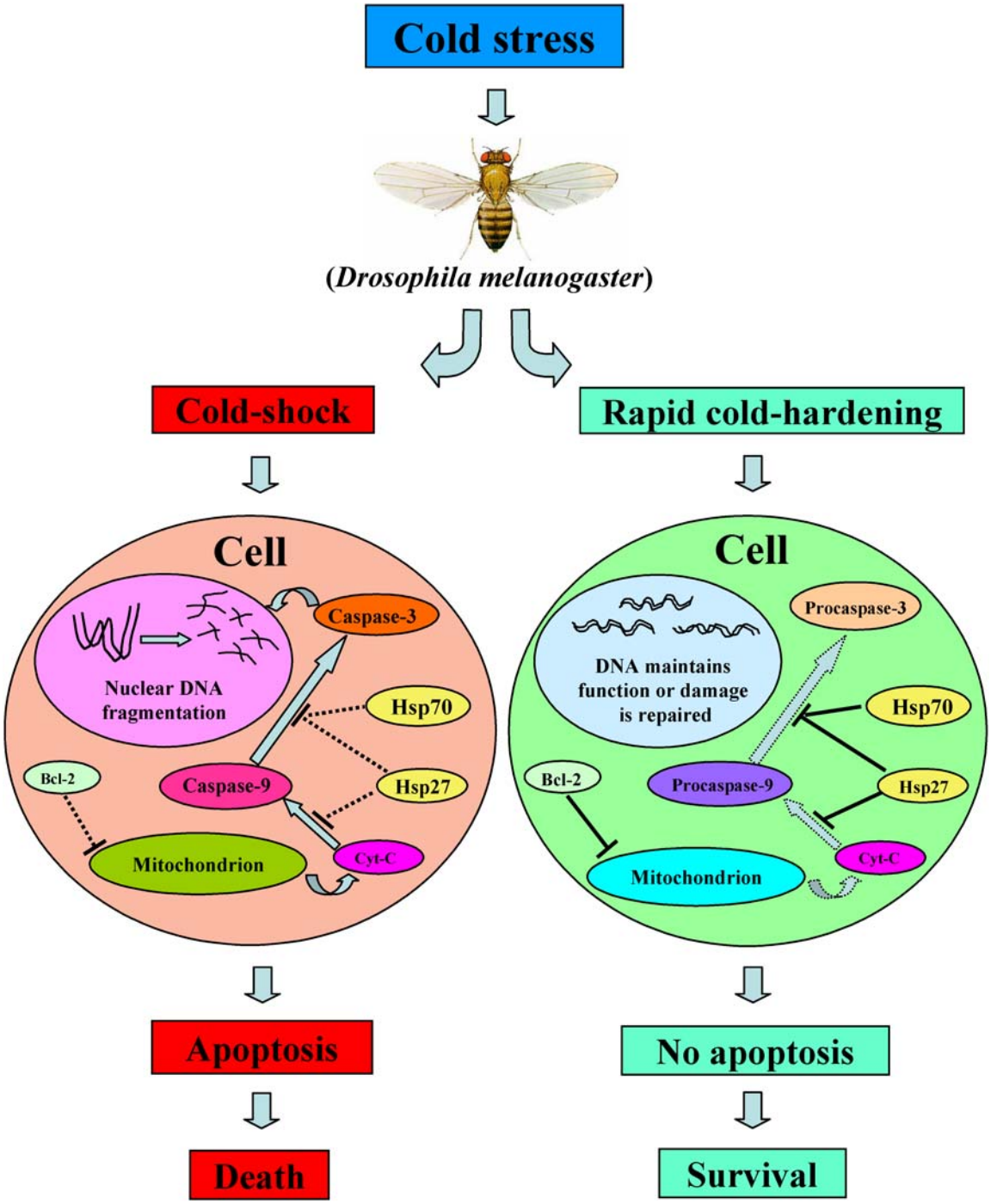

Fig. 8 Proposed mechanism by which RCH protects against cold-induced apoptosis in *D. melanogaster*. Apoptotic cell death is triggered by many stimuli including cold shock. One characteristic of apoptosis is DNA fragmentation of the nuclear genome. Two key members in the signaling pathways are caspase-9 (an initiator) and caspase-3 (an executioner). In the RCH flies, heat-shock proteins (Hsp70 and Hsp27) and apoptosis inhibitors (Bcl-2) may play a role as anti-apoptosis proteins that inhibit apoptosis activation process and thereby protect cells from apoptotic death

groups compared with the control group (Fig. 7). This result suggests that survivin is not directly involved in the RCH protection against apoptosis in the flies, although it is reported to have a role in resistance to apoptosis in human malignancies [60].

Conclusion

In conclusion, based on our data in combination with the available literature, we propose a working model (Fig. 8) for cold-induced apoptosis and RCH protection in insects

using *D. melanogaster* as a representative. The first step in the apoptotic pathway is initiated by a cold-shock that triggers mitochondrial release of cytochrome *c* into the cytoplasm [61–63]. Studies by Liu et al. [61], Yang et al. [62] and Morita-Fujimura et al. [63] found that cytochrome *c* is rapidly released from the mitochondrial intermembrane space into the cytoplasm during the induction of apoptosis by diverse stimuli. Then, cytochrome *c* interacts with other factors such as Apaf-1 and caspase-9 to form a caspase-3 activating complex [29, 64, 65]. Caspase-3 is synthesized as a precursor and converted into an active enzyme of 17–20 kDa and a small 12 kDa subunit by apoptotic signals [66]. Finally, the activated caspase-3 causes DNA fragmentation and cell death [62, 66].

In contrast, the RCH response blocks apoptosis by up-regulation of the anti-apoptotic protein Bcl-2 (and possibly other factors such as Hsps [29, 67]) that inhibits the release of cytochrome *c* [68] and the activation of procaspase-9 [55, 56]. These actions would prevent the formation of the caspase-3 activating complex and thereby, blocks apoptosis [63].

Acknowledgments This research was supported by NSF grant #IOB-0416720. We thank Katia Del Rio-Tsonis, Natalia Vergara and Juanita Constible for a critical reading of the manuscript.

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