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ORIGINAL PAPER

Rapid cold-hardening blocks cold-induced apoptosis by inhibiting the activation of pro-caspases in the flesh fly *Sarcophaga crassipalpis*

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Abstract Apoptosis plays important roles in the selective elimination of sub-lethally damaged cells due to various environmental stresses. The rapid cold-hardening (RCH) response protects insects from the otherwise lethal consequences of injury due to cold-shock. We recently demonstrated that cold shock induces apoptotic cell death in insects and that RCH functions to specifically block cold-shock-induced apoptosis. In the present study we used isolated fat body, midgut, muscle, and Malpighian tubules from adult flesh flies *Sarcophaga crassipalpis* to test the following hypotheses: (1) cold-induced apoptosis varies among different tissues and (2) RCH blocks the apoptotic pathway by preventing the activation of pro-caspases. Cold-shock induced substantial amounts of apoptotic cell death that matched with tissue damage as determined using vital dyes. RCH treatment significantly reduced apoptotic cell death in all tested tissues. Caspase-3 (executioner) activity was 2–3 times higher in the cold- and heat-shocked groups than in control and RCH groups. Likewise, the activity of caspase-9 (initiator) showed a similar trend as for caspase-3 in all tissues but midgut. In addition, cold-shock and heat-shock treatments also increased caspase-2 activity 2–3 folds in both soluble and membrane fractions of fat body and muscle extracts compared to controls.

Keywords Apoptosis · Caspases · Cold shock · RCH · *Sarcophaga crassipalpis*

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Introduction

The rapid cold-hardening (RCH) response that protects insects from non-freezing cold-shock injury was reported more than two decades ago [1]. RCH occurs very rapidly requiring only minutes to hours for its induction, and is elicited in diverse taxa of freezing intolerant insects and related arthropods [2, 3]. This acclimatory response functions to enhance organismal performance during environmental cooling [4, 5].

At the cellular level, RCH results in changes in the composition of membrane lipids [6–8] and increases in membrane fluidity [9]. This response also requires calcium for cold-sensing and the induction of cold-hardening [10]. Despite the generality of this response, the nature of the underlying physiological mechanisms of RCH is not clear.

The RCH response also blocks cold-induced apoptosis [11]. Apoptosis, also called programmed cell death, plays a critical role in the development of multicellular organisms [12]. Apoptosis is a highly conserved, precisely regulated cascade of biochemical and molecular events for eliminating aged and sub-lethally damaged cells [13, 14], and can be induced by various stresses, including environmental, chemical and mechanical damage [11, 15]. Morphological changes in the apoptotic cells include blebbing, loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation [16].

A vital component in the apoptotic process is a cascade of caspases (cysteinyl aspartate-specific protease) that cleave many cellular substrates [17]. Thus far, 15 caspases have been identified in mammals, 4 in *Caenorhabditis elegans*, and 7 in *Drosophila melanogaster* [18]. All caspases are synthesized prior to the induction of apoptosis and maintained as inactive precursors

(pro-caspases). Induction of apoptosis triggers proteolysis of pro-caspases to produce two peptides, 17–25 and 10–12 kDa, which dimerize to form the active caspases. The initiators (caspase-2, -8, -9, -10) begin the cascade, whereas the executioners (caspase-3, -6, -7, -14) are critical for final execution of the cell death program [19, 20]. Activation of pro-caspase-3 is a central step in the execution phase and serves as the convergence point of different apoptotic signaling pathways [21]. Therefore, caspase activation is an indicator of apoptosis and can be used to determine whether, and when, apoptosis occurs in tissues/cells [22].

Although the effects of RCH protection against cold-shock injury are well known at the organismal level for many species, the underlying molecular mechanisms conferring increased cold tolerance remain poorly understood. Previously, we demonstrated that both high and low temperature stimuli including heat shock, cold shock, and freezing induced apoptotic cell death in *D. melanogaster* [11]. We also discovered that cold-shock-induced apoptosis was prevented by RCH treatment by suppressing levels of caspase-like proteins and elevating anti-apoptotic protein, Bcl-2. In this study, we used isolated tissues (fat body, midgut, muscle, and Malpighian tubule) from adults of the flesh fly *Sarcophaga crassipalpis* to determine whether: (1) cold-induced apoptosis varies among different tissues and (2) RCH blocks the apoptotic pathway by preventing the activation of pro-caspases.

Materials and methods

Insect culture and assessment of cell viability

The flesh flies, *S. crassipalpis* (Diptera, Sarcophagidae), were reared as described by Lee and Denlinger [23]. All experiments used 5- to 8-day-old adult flies. To determine the effect of RCH on cold tolerance of tissues and cells, adults were divided into three groups: control (untreated at 23°C), cold-shocked (directly exposed to −8°C for 2 h), and RCH (exposed to 0°C for 2 h prior to a 2 h exposure to −8°C). 2 h after each treatment, fat body, midgut, and Malpighian tubules were dissected from adults in Coast's solution. Following dissection, cell viability of each tissue was assessed with the LIVE/DEAD sperm viability kit (Molecular Probes, Eugene, OR) as described by Yi and Lee [24, 25]. Green or yellow green fluorescence indicates live cells with intact cell membranes, while dead cells fluoresced red or orange-red. Percent cell survival of each tissue from individual flies was based on the mean value of three counts of 100 cells. Three to five individuals ($n = 3$–5) were used in each treatment group. Means were compared using ANOVA Bonferroni–Dunn test (Statview from SAS Institute) with statistical significance set at $P < 0.05$.

Detecting apoptosis by TUNEL assay

Terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick end labeling (TUNEL) assay [26, 27] was used to detect the apoptotic cell death on cryosections of adult tissues, including fat body, muscle, midgut, and Malpighian tubules. Preparation of cryosections and TUNEL assay were performed as previously described by Yi et al. [11] from three groups of flies: the untreated control, the cold-shocked, and the RCH, using the in situ cell death detection kit, TMR Red (Roche, Indianapolis, IN). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole·2HCl (DAPI) (Pierce Biotechnology, Rockford, IL) solution. Then the slides were covered with cover slips and Vectashield (Vector Laboratories, Burlingame, CA). Apoptotic cells were stained red by TUNEL reagent, while all nuclei were stained blue by DAPI when observed with a fluorescence microscope [11]. 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 $P < 0.05$. Each mean value is based on the counts of eight fields of 50–200 cells for each of three replicates.

DNA isolation and gel electrophoresis

DNA was isolated as described by Yi et al. [11] from fat body and midgut tissues of three groups of flies: control, cold-shocked and RCH. The isolated DNA was washed with 70% ethanol, air dried, and stored at −80°C. Before electrophoresis, the DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and its concentration was measured on a Nanodrop automatic system. DNA fragmentation 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 [28]. Fifteen micrograms of DNA samples were loaded onto each lane of the gel using 100 bp Ladder (Fisher, Fair Lawn, NJ) as standard markers.

Caspase activity assays

Enzymatic activities of three caspases: caspase-2, caspase-3, and caspase-9, were determined, respectively, using the ICH-1/Caspase-2 colorimetric protease assay kit, Caspase-3 colorimetric activity assay kit (DEVD), and Caspase-9 colorimetric activity assay kit (Chemicon International,

Temecula, CA) as per the manufacturer's instructions. In these assays, caspase-2 proteases in both soluble and membrane-bound protein samples recognized the sequence of VDVAD (Val-Asp-Val-Ala-Asp) and specifically cleaved at the C-terminal side of the aspartate residue. The assay is based on spectrophotometric detection (405 nm) of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate VDVAD-*p*NA. Comparing the absorbance of *p*NA from an apoptotic sample with an untreated control allows determination of the increase in caspase-2 activity. Similarly, caspase-3 protease in the samples recognized the sequence DEVD (Asp-Glu-Val-Asp) and cleaved *p*NA from the labeled substrate DEVD-*p*NA; while caspase-9 protease recognized the sequence LEHD (Leu-Glu-His-Asp) and cleaved the labeled substrate LEHD-*p*NA to release free *p*NA. In each case, the protein samples were diluted to a protein concentration of 100–200 µg in a volume of 90 µl with the provided assay buffer. Then 10 µl of caspase-2 substrate (VDVAD-*p*NA), caspase-3 substrate (DEVD-*p*NA) or caspase-9 substrate (LEHD-*p*NA) was added to the sample solutions. After incubation for 1.5 h at 37°C, the mixtures were diluted to 500 µl with dilution buffer in a 1-ml black wall cell (Starna Cells, Inc, Atascadero, CA). Background reading from sample solutions and buffers was subtracted from the readings of both control and treated groups before calculation of caspase activity, which were expressed in A405 per mg of protein.

The standard curve was generated by preparing a series of *p*NA solution in the concentration range of 10 µM–1 mM by diluting the provided *p*NA stock solution in the assay buffer. Then, the A405 values were recorded using the assay buffer as a blank. A linear relationship ($r^2 = 0.999$) between *p*NA concentration and A405 value was obtained.

We used ANOVA post hoc tests (Statview from SAS Institution) and *t*-test from SigmaPlot to compare difference between groups with significance set at $P < 0.05$. All values are reported as mean ± SEM.

Results

Cell viability of tissues

As demonstrated previously [25], RCH treatment significantly ($P < 0.05$) improved cell viability in fat body, midgut, and Malpighian tubules compared to the cold-shocked groups (Fig. 1). The mean values for cell death decreased by 31.0% in the fat body, 21.1% in the midgut, and 24.5% in the Malpighian tubules, respectively, resulting in an average decrease of 25.5% for all tissues.

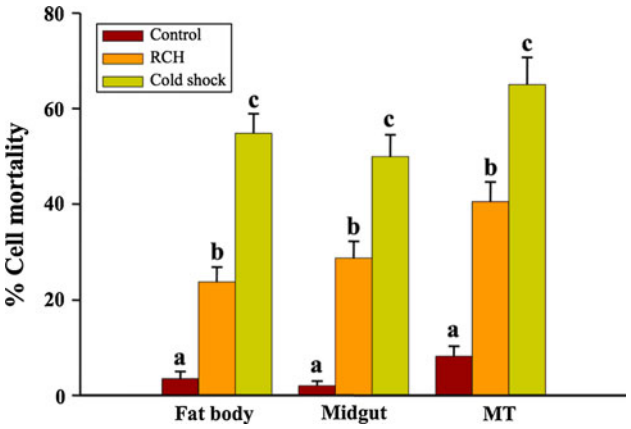

Fig. 1 Cell death detected in the fat body, midgut, and Malpighian tubules (*MT*) from control, RCH, and cold-shock treated adults by Live/Dead viability kit. A significant difference (ANOVA Bonferroni–Dunn test, $P < 0.05$) between treatment groups in each tissue is indicated by *different letters*

Apoptosis is induced by cold shock but suppressed by RCH

Cold shock significantly increased the incidence of apoptotic cell death, however, the RCH treatment significantly blocked this effect in all tested tissues including fat body, midgut, muscle, and Malpighian tubules ($P < 0.05$) as indicated by TUNEL staining (Fig. 2). These data are consistent with RCH protection observed at the tissue level as assessed using vital dyes (Fig. 1).

In fat body, the RCH treatment significantly reduced the number of apoptotic cells by 26% compared to the cold-shocked group (Fig. 2). Whereas 7.9 ± 1.6% (mean ± SEM) of fat body cells in the untreated control group stained positively for TUNEL, a significantly higher level

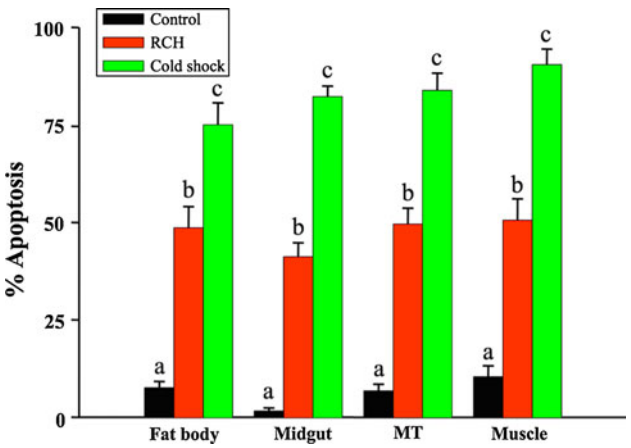

Fig. 2 Apoptotic cell death detected in the fat body, midgut, Malpighian tubules (*MT*), and muscle from control, RCH, and cold-shock treated adults by the TUNEL assay. A significant difference (ANOVA Bonferroni–Dunn test, $P < 0.05$) between treatment groups in each tissue is indicated by *different letters*

(75.4 ± 5.6%) of apoptotic cell death was induced by the cold-shock treatment. In contrast, RCH-treated flies had significantly lower levels of apoptotic cells (49.0 ± 5.4%), indicating that RCH protects against cold-shock-induced apoptosis.

Cells in the midgut were also protected from apoptosis by the RCH response (Fig. 2). Only very low levels of apoptosis (1.9 ± 0.8%) were observed in control flies. In contrast, cold-shock treatment resulted in 82.6 ± 2.7% apoptotic cells. RCH treatment significantly improved cell survival by 41%. A similar response was observed in Malpighian tubule cells (Fig. 2). Significantly different levels of apoptosis were shown among tested groups: 7.1 ± 1.7% in the control, 84.2 ± 4.4% in the cold-shocked, and 49.9 ± 4.1% in the RCH treatment group. In muscle cells (Fig. 2), the apoptotic rate was 10.7 ± 2.8% for the control, 90.8 ± 4.0% for cold-shock, and 50.9 ± 5.5% for the RCH group.

Overall, no statistical difference was found among tissues within the same treatment group except for the control, in which the apoptotic rate (1.9%) in fat body was significantly lower ($P < 0.05$) than in other tissues. The pooled mean rate of cellular apoptosis in the four tissues was 47.8% in the RCH and 83.3% in the cold-shocked group; these values were significantly different ($P < 0.05$) from each other.

DNA fragmentation

Gel electrophoresis of DNA showed different banding patterns among tissues from different treatment groups (Fig. 3). In fat body, the RCH treatment prevented DNA fragmentation as DNA in the RCH and control groups showed an identical banding pattern that was distinctly different from the cold-shock group. There were four major DNA bands in this tissue: Bands 1 (>>2.7 Kbp), 2 (>2.7 Kbp), 3 (~2.7 Kbp), and 5 (~700 bp). In the cold-shocked group, all these bands became less dense because of DNA degradation, particularly for Band 5, which almost completely degraded into small bands, such as Band 6 (100–200 bp). These observations are consistent with the results obtained from the TUNEL assay (Fig. 2). In midgut tissue three bands were observed including two major DNA bands: Bands 4 (~1.2 Kbp) and 5, as well as a small band (Band 6). The difference in banding patterns was not as obvious between RCH and cold-shock, suggesting a tissue-specific effect for the RCH response.

Caspase activities

The activity of three caspases was determined in different tissues using specific peptide substrates. Initially, the synthetic sequence of VDVAD-*p*NA (Val-Asp-Val-Ala-Asp-*p*NA)

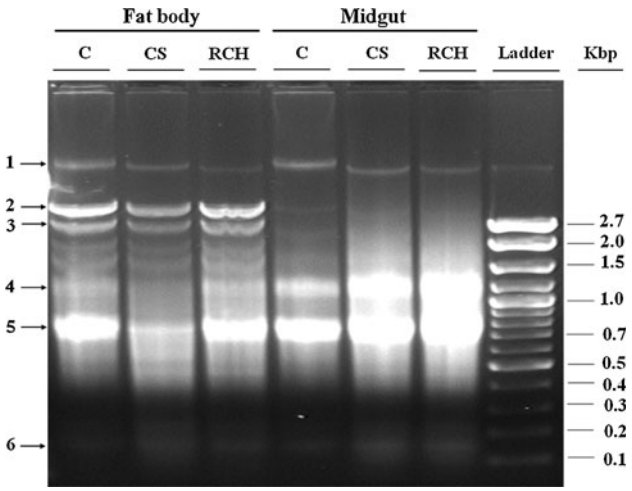

Fig. 3 Electrophoresis of DNA in the fat body and midgut tissues from control (*C*), cold-shocked (*CS*), and rapidly cold-hardened (*RCH*) adults. Fifteen micrograms of DNA samples were applied per lane using a 100 bp ladder as molecular markers. Band 1, larger DNA; Band 2, >2.7 Kbp; Band 3, ~2.7 Kbp; Band 4, ~1.2 Kbp; Band 5, ~700 bp; and Band 6, ~100–200 bp. This is a representative gel from three replicates of electrophoresis

was used to determine caspase-2 protease activity in both soluble (Fig. 4a) and membrane-bound protein samples (Fig. 4b). In both fractions, cold-shock and heat-shock treatments increased caspase-2 activity 2–3-fold in fat body and muscle compared to the control group. In contrast, the enzyme activity in the RCH group remained at the same level as control. In the midgut there were no significant differences between treatment and control groups. In all tissues, caspase-3 activity was 2–3 times higher in the cold-shocked and heat-shocked groups than in either the control or RCH groups (Fig. 5a). Compared to cold shock, RCH treatment greatly suppressed the activation of caspase-3 and remained at a level similar to those in the control group. There was no difference in the enzyme activity between cold shock and heat shock treatments. Likewise, the activity of caspase-9 (Fig. 5b) showed a similar trend as for caspase-3 (Fig. 5a) in all tissues but midgut, where no significant differences were found among tested groups.

Discussion

RCH improves cellular cold tolerance and protects cells from apoptosis

RCH significantly enhanced cellular cold tolerance by an average of 25.5% for fat body, midgut, and Malpighian tubules (Fig. 1). These results are consistent with our previous findings using fluorescent vital dyes in *S. crassipalpis* [25] and *S. bullata* [9], which demonstrated that

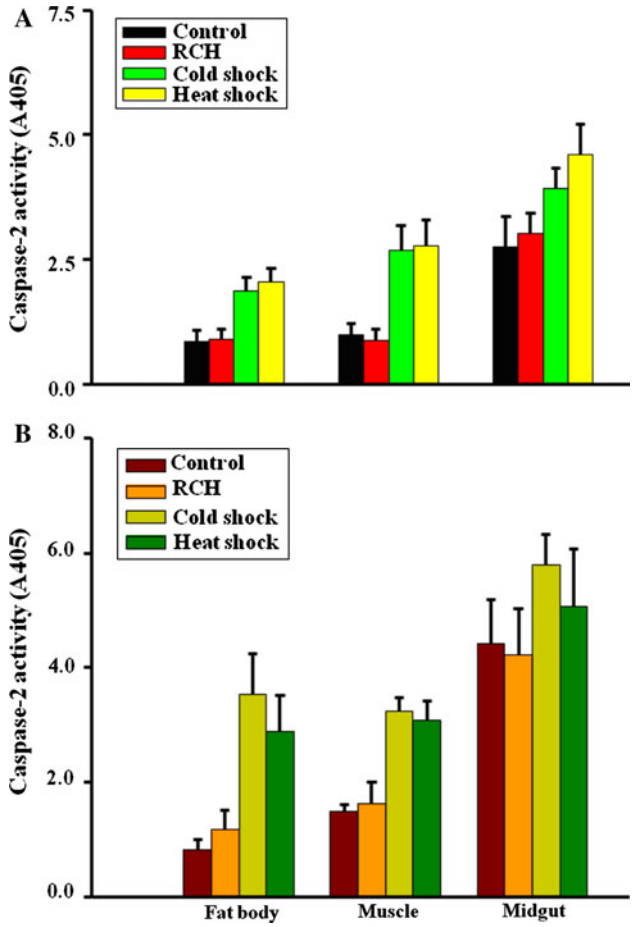

Fig. 4 Caspase-2 activities in the soluble (**a**) and membrane proteins (**b**) of fat body, muscle, and midgut from control, RCH, cold-shocked, and heat-shocked adults

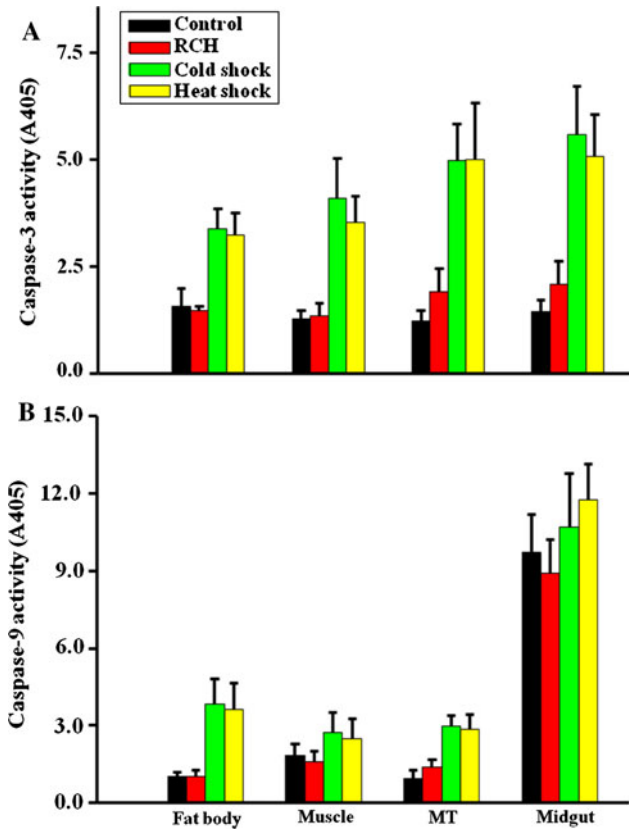

Fig. 5 Caspase-3 (**a**) and caspase-9 activities (**b**) in the soluble proteins of fat body, muscle, Malpighian tubules (*MT*), and midgut from control, RCH, cold-shocked, and heat-shocked adults

RCH improved cold tolerance at −8°C at both organismal and cellular levels and that the RCH response was independent of the central nervous system and neuroendocrine regulation [25]. RCH also increases levels of unsaturated phospholipid fatty acids that would be expected to maintain membrane fluidity at low temperature [6, 7]. Using solid-state NMR spectroscopy, Lee and colleagues [9] confirmed this expectation by directly measuring an increase in membrane fluidity after RCH.

A major finding in our study was that the RCH response protected cells (fat body, midgut, Malpighian tubules and muscles) from cold-shock induced apoptosis characterized by nuclear DNA fragmentation and degradation, as shown by both TUNEL staining (Fig. 2) and DNA electrophoresis (Fig. 3). To our knowledge, this is the second such report in an insect confirming the findings of our previous study with the fruit fly, *D. melanogaster* [11]. Apoptosis causes DNA cleavage by endonucleases producing oligonucleosoma-sized fragments. In *D. melanogaster*, cold-shock induced apoptosis results in the disappearance of the 400–600 bp DNA band, leaving only a 100–200 band [11]. Similarly, in the fat body of *S. crassipalpis*, DNA bands (larger than 700 bp) were degraded by cold shock into 100–200 bp fragments (Fig. 3). The damaging fragmentation in fat body was prevented by RCH treatment (Fig. 3) as we showed previously in *D. melanogaster* [11]. However, although cold-shock damaged DNA in midgut tissue of *S. crassipalpis*, RCH's protection was not as obvious (Fig. 3), suggesting a tissue-specific manner in the RCH response.

RCH blocks apoptosis by inhibiting the activation of pro-caspases

Activation and regulation of apoptosis involve a sophisticated orchestra of intra- and extra-cellular signals and factors that either promote or block forward progression of the cascade [29]. Critical steps in the apoptotic pathway require activation of specific caspases. We selected two initiator caspases (-2 and -9) that begin the cell death pathway and converge to proteolytically activate caspase-3 for final execution of the program [30, 31]. Changes in levels of these caspases are useful indicators of apoptotic induction or inhibition and progress through the cascade.

In the present study, we found evidence that caspases-2, -3, and -9 were activated by cold shock and heat shock, and that activation was associated with apoptotic cell death in fat body, muscle, midgut, and Malpighian tubules from *S. crassipalpis* (Figs. 4, 5). In all tested tissues, except midgut, hydrolytic activity of the three caspases increased 2–3 times in response to either cold-shock or heat-shock treatment, compared to the untreated controls. Caspase activities in the RCH groups, however, remained at a level similar to the controls, indicating that RCH response greatly suppressed the cold shock-induced activation of caspases-2, -3 and -9 in most of the tissues we tested. Similarly, in *D. melanogaster* both cold shock and heat shock increase the level of active caspase-3 protein, while the RCH treatment significantly lowered levels of this caspase suggesting that the RCH response confers cyto-protection by blocking apoptotic progress [11]. From the available evidence we conclude that caspases-2, -3 and -9 play key roles in both heat shock and cold shock-induced apoptosis, and that RCH blocks the initiation and interrupts the progression of the cell death cascade by inhibiting the activation of these key caspases.

Conclusions

A schematic diagram of cellular responses to cold (Fig. 6) summarizes our previous and present results and also provides a conceptual map for future research. The signaling pathway responsible for cellular cold-sensing/transduction and the RCH response has not been identified in insects, but calcium/calmodulin [10], p38MAPK [32], and caspases and apoptosis-inhibiting proteins (Bcl-2 and Hsps) [11] are likely major components in the process.

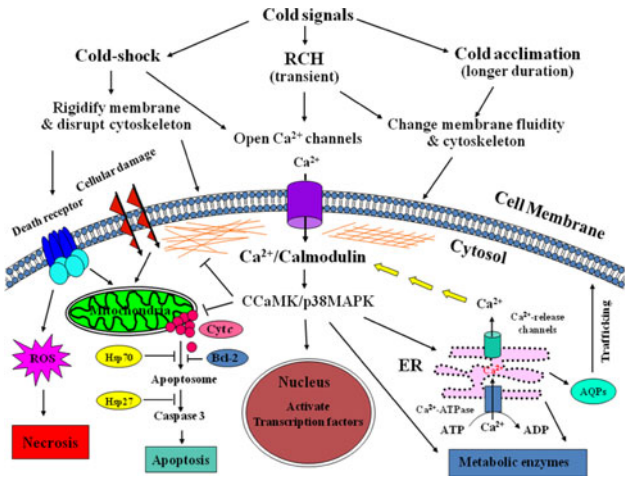

Fig. 6 Proposed pathway for cellular cold perception and transduction: cold shock injury causes apoptotic and necrotic cell death whereas RCH and cold acclimation protect and rescue cells for normal function

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