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INCREASED DIETARY CHOLESTEROL ENHANCES COLD TOLERANCE IN *Drosophila melanogaster*

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Abstract

For many years, non-freezing cold shock injury has been associated with damage to the cell membrane. In this study, we enhanced membrane cholesterol levels of *Drosophila melanogaster* by raising larvae on a cholesterol-augmented diet. Diet augmentation significantly increased the amount of cholesterol in the cell membranes of the adult flies (1.57 ± 0.17 nmol mg^{-1} vs. 0.93 ± 0.11 nmol mg^{-1}). Flies on the cholesterol-augmented diet exhibited a greater intrinsic cold tolerance: this group had a higher survival rate after a 2-h cold shock of -5°C than did the control group (71.0 ± 6.6% vs. 36.0 ± 8.1%). Cholesterol-augmented flies also had a significantly greater capacity to rapidly cold-harden to -7°C (36.7 ± 4.4%) compared to flies on a control diet (20.0 ± 2.9%). These results suggest a mechanistic link between protection from chilling or cold shock injury and modifications to the cellular membrane.

Keywords: cholesterol, membrane, insect cold tolerance, cold shock

INTRODUCTION

Only recently has the complex nature and functional significance of non-randomly distributed protein and lipid components of cell membranes begun to be appreciated (2,24). In both plants and animals, the cell membrane has long been known as the site of chilling or cold shock injury due to thermotropic changes in constituent lipids (8,13,16). Cold shock injury, characterized by ion leakage across cell membranes and damage to intracellular organelles, has been intensively studied in mammalian sperm in connection with the development of cryopreservation protocols. However, few studies have examined mechanisms of chilling injury and cold hardening at the cell and organ level in insects or other ectotherms that are naturally exposed to low temperature.

Membrane lipid re-ordering has been investigated as the mechanism for long-term seasonal adjustments to temperature. For example, larvae of the goldenrod gall fly, *Eurosta solidaginis,* acquire freeze tolerance over a two-week period in early autumn (11). During that time, there is an increase in monoenic fatty acids and a proportionate decrease in saturated species (1). It is becoming clear that during temperature adaptation in ectothermic animals, cholesterol influences membrane function in multiple ways, including regulation of membrane fluidity and maintenance of sphingolipid rafts (5,20,21).

Although numerous investigators have documented membrane restructuring during temperature acclimation, few studies have directly tested the consequences of experimentally varying membrane composition on cold tolerance in ectotherms. Insects are unable to synthesize cholesterol *de novo*, and so must obtain all necessary cholesterol through their diet.

We enhanced membrane cholesterol levels by adding cholesterol to the larval rearing medium, which allowed us to determine its effect on cold shock tolerance and rapid cold-hardening (RCH) in *Drosophila melanogaster*. We hypothesized that increased membrane cholesterol levels would increase cold shock tolerance and the capacity to rapidly cold-harden as reported in mammalian sperm (8,27).

MATERIALS AND METHODS

Drosophila melanogaster Oregon-R strain were reared on a control diet of cornmeal-yeast-agar medium in pint-sized bottles at 23°C and 15:9 L:D (9). The cholesterol-augmented diet was made by adding cholesterol to the control diet at 1.0 mg per ml. All flies in the cholesterol treatment group were reared on the cholesterol-augmented diet for three generations before experiments were conducted to allow flies to adapt to the cholesterol-enriched medium (14).

Cold tolerance and rapid cold-hardening (RCH) capacity were tested using the protocol developed by Czajka and Lee (7). Each replicate consisted of 20 adult flies (2 to 4 days post-eclosion) placed in a test tube that was immersed directly in a refrigerated bath (NesLab RTE-8, Portsmouth, NH, USA) set to -5° or -7°C for 2 h. Cold-shocked flies were directly exposed to subzero temperatures while a second group of flies was rapidly cold-hardened by a pre-treatment of 5°C for 2 h prior to sub-zero treatment. The relative level of intrinsic cold tolerance was determined by comparing survival rates after cold-shock. Flies were judged to have survived if they were able to right themselves after 24 h at 23°C.

To determine cholesterol levels, membrane preparations were conducted according to the method modified by Yi and Lee (26) from Sørensen (22,23). Adult flies were anesthetized, weighed to the nearest 0.1 mg, and washed with a 0.16 M NaCl solution to remove any media adhering to their bodies. Flies were then homogenized in 0.01 M Tris-HCl buffer (pH 7.2) containing 0.15 M NaCl and 3 mM $MgCl_2$. After sonication, the homogenate was centrifuged at 500 *g*, 4°C for 5 min. The supernatant was collected and centrifuged at 700 *g*, 4°C for 5 min. The resulting supernatant was centrifuged at 25,000 *g* in a Beckman L5-50 B Ultracentrifuge for 15 min at 4°C. The pellet, containing the plasma membrane fraction, was suspended in 0.01 M Tris-HCl buffer (pH 7.2) containing 0.1 M choline chloride, and the ultracentrifugation step was repeated. After discarding the supernatant, the pellet was re-suspended in 80 µl of the same buffer (0.01 M Tris-HCl, 0.1 M choline chloride).

The cholesterol assay was carried out as described by Yi and Lee (26). Briefly, 1.0 ml of reagent (Cholesterol Assay Kit, Diagnostic Chemicals, Ltd, Oxford, CT, USA) and 10 µl of membrane preparation sample were added to a 1.5 ml Eppendorf tube. Standards and reagent blanks were prepared by replacing the sample with 10 µl of calibrator (standard) (Sigma Chemical Co.) or 10 µl of 0.01 M Tris-HCl buffer (blank), respectively. Cholesterol concentration was determined colorimetrically at 505 nm using a spectrophotometer. Each replicate for the cholesterol assay consisted of the isolated membrane fractions of 90 to 140 adult flies in a 1:1 male to female ratio. There were 10 replicates of the control group and 14 replicates in the cholesterol-augmented group.

Survival data was analyzed using the arcsin-square root transformation of the original proportions. All pairwise comparisons of control and cholesterol-augmented flies were performed using a Student's *t*-test, with significant set at $P < 0.05$. Adult masses were compared using ANOVA and a Bonferroni multiple comparison post-test, with overall $P < 0.05$. All statistical analyses were performed using StatView 5.0.

RESULTS

Females reared on a cholesterol-augmented diet were smaller (0.92 ± 0.04 mg) than those reared on the control diet (1.08 ± 0.04 mg). However, cholesterol-augmentation did not have a significant effect on male mass (0.46 ± 0.01 mg versus 0.50 ± 0.03 mg, respectively).

We measured membrane cholesterol levels to determine whether augmentation of the flies' diet resulted in cholesterol being incorporated into cell membranes. Flies from the cholesterol-augmented group had about 50% more membrane cholesterol per mg body mass than control flies (1.57 ± 0.17 nmol mg^{-1} vs. 0.93 ± 0.11 nmol mg^{-1}, $P < 0.05$).

To test for differences in their intrinsic cold tolerance due to cholesterol-augmentation, we compared the survival of flies reared on control versus cholesterol-augmented diets following a 2-h exposure to -5°C (Fig. 1). A significantly greater portion of cholesterol-augmented flies (71.0 ± 6.6%) survived direct transfer to the sub-zero temperature than control flies (36.0 ± 8.1%). Both RCH groups were able to fully cold-harden and survive exposure to -5°C (Fig. 1).

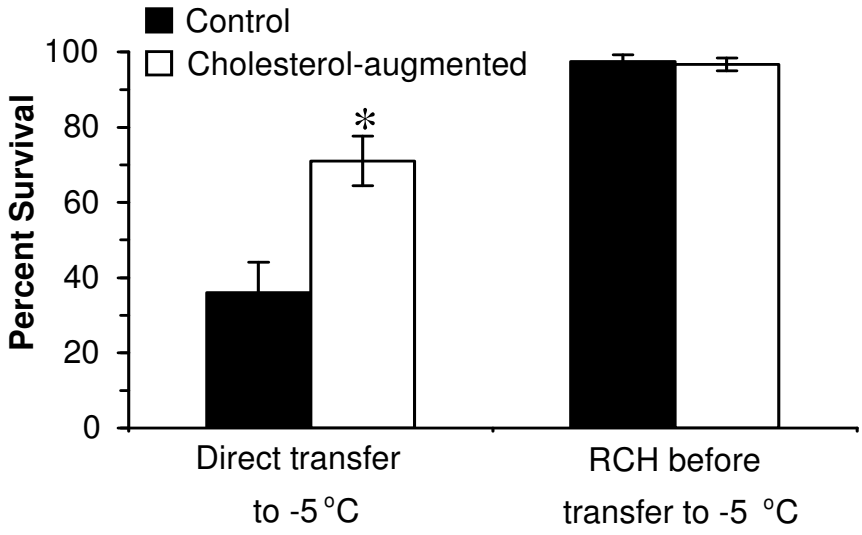

Figure 1. Percent survival of control versus cholesterol-augmented flies either directly transferred to -5°C for 2 h or rapidly cold-hardened, by a 2-h pre-treatment at 5°C, prior to transfer to -5°C for 2 h. Bars represent mean Each bar represents the mean ± SEM percentage of five replicates of 20 flies. Asterisk denotes significance at $P < 0.05$, Student's *t*-test.

To discern differences in RCH capacity between control and cholesterol-augmented flies, we compared survival rates between the two groups after a 2-h exposure to -7°C. All flies were killed by a step transfer from 23°C to -7°C (Fig. 2). In contrast, RCH flies from both groups survived -7°C; a significantly higher percentage of adults in the cholesterol-augmented group survived compared to the control group (36.7 ± 4.4% vs. 20.0 ± 2.9%, $P < 0.05$).

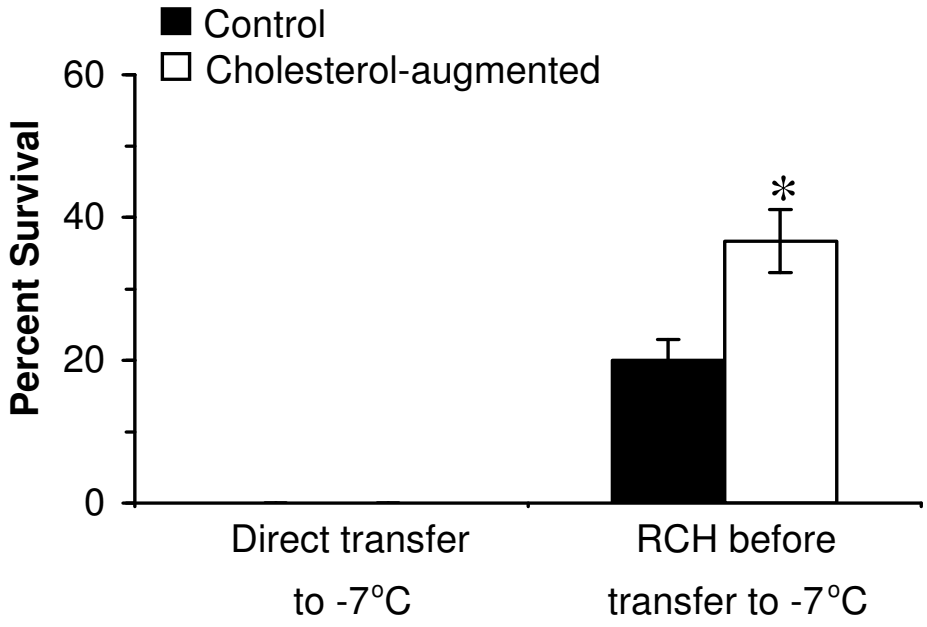

Figure 2. Percent survival of control versus cholesterol-augmented flies either directly transferred to -7°C for 2 h or rapidly cold-hardened, by a 2-h pre-treatment at 5°C, prior to transfer to -7°C for 2 h. Each bar represents the mean ± SEM percentage of three replicates of 20 flies. No flies from either group survived -7°C. Asterisk denotes significance at $P < 0.05$, Student's *t*-test.

DISCUSSION

The cholesterol-augmented diet enhanced the intrinsic cold tolerance of flies to direct exposure to -5°C and also made it possible for them to rapidly cold-harden and better survive chilling to a lower temperature than flies reared on the control diet (Figs. 1 and 2). Since chilling and cold shock damage the cell membranes (13,16), the increase in membrane cholesterol for flies fed the cholesterol-augmented diet presumably accounted for the observed increase in cold tolerance.

Despite advances that clearly demonstrate the ecological importance of RCH (4,9,10,19), the physiological mechanism underpinning this process remains poorly understood. Although Chen et al. (3) documented modest levels of glycerol production during RCH in *Sarcophaga crassipalpis*, Kelty and Lee (9) found no changes in glycerol levels, or in any other sugar or polyol cryoprotectant in *D. melanogaster* during RCH.

Recent studies with adult flies found a significant increase in unsaturated phospholipid fatty acids or membrane fluidity during RCH (12,15,17). In the present study, we demonstrated that increased membrane cholesterol levels were associated with an increased capacity to tolerate low temperature and to rapidly cold-harden (Figs. 1 and 2).

Our data support several lines of evidence indicating that cholesterol plays a role in cellular and organismal cold tolerance. Elevation of cholesterol in the membranes of mammalian sperm enhances chilling tolerance and their resistance to cold shock injury (8,27). In plasma membranes of trout, acclimation to low temperature increases cholesterol levels in one tissue while decreasing them in others (6,18). In larvae of the gall fly *E. solidaginis*, seasonal cold-hardening is closely correlated with increases in hemolymph cholesterol, but not the Malpighian tubule membranes (26). However, given the multiplicity of potential effects of cholesterol on the function of membrane-bound enzymes and other membrane proteins (5,25) and the fact that different tissues within a single organism respond

differently to a single acclimation regime, it is unlikely that a simple or a universal response will be sufficient to explain its role in cold hardening. Further research is needed to discern the role of cholesterol and cold tolerance at the cellular level.

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