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SHORT COMMUNICATION

Evidence for a rapid cold hardening response in cultured *Drosophila* S2 cells

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ABSTRACT

The ability to quickly respond to changes in environmental temperature is critical for organisms living in thermally variable environments. To cope with sudden drops in temperature, insects and other ectotherms are capable of rapid cold hardening (RCH), in which mild chilling significantly enhances cold tolerance within minutes. While the ecological significance of RCH is well established, the mechanisms underlying RCH are still poorly understood. Previous work has demonstrated that RCH is regulated at the cellular level by post-translational signaling mechanisms, and here we tested the hypothesis that cultured cells are capable of RCH. A 2 h cold shock at −8°C significantly reduced the metabolic viability of *Drosophila* S2 cells, but pre-treatment with RCH at 4°C for 2 h prevented this decrease in viability. Thus, S2 cells are capable of RCH in a similar manner to whole insects and provide a new system for investigating the cell biology of RCH.

KEY WORDS: Environmental stress, Cold tolerance, Cell culture, Phenotypic plasticity

INTRODUCTION

Environmental temperature varies on multiple time scales, and as small-bodied ectotherms, insects are particularly susceptible to fluctuating temperatures (Teets and Denlinger, 2013; Colinet et al., 2015; Overgaard and MacMillan, 2017). Long-term, seasonal cold acclimation occurs over a period of days to weeks in response to gradually decreasing temperature and/or changes in photoperiod (Denlinger, 1991), but insects must also cope with rapid changes in temperature that occur on diurnal time scales (Teets and Denlinger, 2013). Rapid cold hardening (RCH) is a plastic response in which brief chilling enhances cold tolerance within minutes to hours (Lee et al., 1987; Lee and Denlinger, 2010), and in addition to RCH in insects, RCH-like responses have been observed in fish (Hazel and Landry, 1988), amphibians (Layne and Claussen, 1987; McCann et al., 2014) and turtles (Muir et al., 2010). RCH is one of the fastest known physiological responses to temperature, rivaling that of the well-studied heat shock response (Morimoto, 1998), making it a robust response for studying rapid phenotypic plasticity at low temperatures.

While the ecological significance of RCH is well established in insects (e.g. Kelty and Lee, 1999; Kelty, 2007; Overgaard and Sorensen, 2008), the regulatory mechanisms are largely uncharacterized. Unlike many stress responses, RCH does not appear to be regulated by transcription and translation or require nervous or hormonal input (Sinclair et al., 2007; Teets and Denlinger, 2013; Teets et al., 2012; Vesala et al., 2012; Yi and Lee, 2004). At the level of cell signaling, the onset of RCH in the flesh fly *Sarcophaga crassipalpis* is accompanied by rapid activation of p38 mitogen-activated protein (MAP) kinase (Fujiwara and Denlinger, 2007). Temperatures that elicit RCH also cause an influx of calcium into the cytoplasm, and blocking this calcium entry prevents RCH in the Antarctic midge, *Belgica antarctica*, the goldenrod gall fly, *Eurosta solidaginis*, and the flesh fly *Sarcophaga bullata* (Teets et al., 2008, 2013). Apoptotic signaling is modulated by RCH in both *Drosophila melanogaster* and *S. crassipalpis*, although the exact molecular regulation of this pathway in response to cold is unknown (Yi and Lee, 2011; Yi et al., 2007). Using phosphoproteomics, we previously quantified changes in protein phosphorylation in response to chilling that elicits RCH in *S. bullata* and observed phosphorylation changes in cytoskeletal proteins, heat shock proteins and proteins involved in degrading damaged cellular components through the proteasome and autophagosome (Teets and Denlinger, 2016). However, the functional significance of these phosphorylation changes is unclear and requires further validation.

The existing literature points to a prominent role for post-translational cell signaling in the regulation of RCH, but these processes can be difficult to study *in vivo*. Thus, there is a need for a simplified cell culture system to complement existing methods for investigating the mechanisms of RCH. In the current study, we present evidence that cultured cells are capable of RCH and can be used in subsequent work to investigate underlying mechanisms. *Drosophila* S2 cells experience a significant decline in metabolic viability in response to cold shock at subzero temperatures, but a brief period of RCH prior to cold shock improves viability. The measurable RCH response in S2 cells, coupled with their ability to be routinely cultured, make this system an excellent complement to whole organisms and tissues for studying the mechanisms underlying RCH.

MATERIALS AND METHODS

Drosophila S2 cells, originally derived from 20–24 h old *D. melanogaster* embryos (Schneider, 1972), were purchased from the Drosophila Genomics Resource Center at Indiana University. Cells were maintained in custom M3+BPYE medium (containing a combination of powdered Shields and Sang medium, $KHCO_3$, yeast extract and bactopeptone; all from Sigma-Aldrich, St Louis, MO, USA) with 10% fetal bovine serum (GE Healthcare, Chicago, IL, USA) at 25°C in T25 flasks with bi-weekly passages (Luhur et al., 2019). The composition of Shields and Sang medium was as follows (g l^{-1}): 0.76 $CaCl_2$, 2.15 $MgSO_4$, 0.88 NaH_2PO_4, 0.25 β-alanine, 1.5 L-alanine, 0.5 L-arginine, 0.3 L-asparagine, 0.3 L-aspartic acid, 0.02 L-cysteine hydrochloride, 7.88 L-glutamic acid potassium salt, 6.53 L-glutamic acid sodium salt, 0.6 L-glutamine, 0.5 glycine, 0.55 L-histidine, 0.25 L-isoleucine, 0.4 L-leucine, 0.85

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L-lysine hydrochloride, 0.25 L-methionine, 0.25 L-phenylalanine, 0.4 L-proline, 0.25 L-serine, 0.5 L-threonine, 0.1 L-tryptophan, 0.26 L-tyrosine disodium salt, 0.4 L-valine, 0.05 choline chloride, 0.25 oxalacetic acid, 10.0 D(+)-glucose, 1.05 Bis-Tris and 1.0 yeast extract.

In a preliminary experiment, we established that cells suspended in 25 µl media (the minimum volume required to resuspend the cell numbers needed for our experiments) could remain supercooled down to −16°C for 2 h. We then tested the baseline cold tolerance of S2 cells by exposing them to 0, −4, −8, −12 and −16°C for 2 h in a programmable bath. Control cells were maintained at 25°C for the duration of the experiment. For this experiment, 1.05×10^3 confluent cells were suspended in 25 µl M3+BPYE, and after cold exposure 1.375 ml M3+BPYE was added to each tube, and cells were returned to 25°C for 20 h of recovery. After 20 h, metabolic viability was assessed using alamarBlue (Thermo Fisher Scientific, Waltham, MA, USA), a resazurin-based dye that is reduced to fluorescent resorufin by the mitochondria of healthy cells. Thus, metabolic viability can be measured by the ability of mitochondria to metabolize resazurin. This method is used extensively to assay cell health in a variety of contexts (Riss et al., 2013) and has been used previously in *Drosophila* S2 cells (Fujii-Taira et al., 2009; Sharma and Fitzgerald, 2010). Further, in a pilot experiment we assessed viability with Trypan Blue, a dye exclusion assay, and the viability patterns detected were similar to those obtained with alamarBlue (Fig. S1). For alamarBlue measurements, 200 µl aliquots of each sample were transferred to a black, 96-well plate and incubated with 20 µl of alamarBlue reagent at 25°C for 4 h in the dark. Each sample was measured in triplicate. Fluorescence was measured on a BMG Labtech CLARIOstar microplate reader with an excitation wavelength of 540±15 nm and an emission wavelength of 580±20 nm.

In our initial cold tolerance experiment, metabolic viability was relatively high (73.2% of controls) even at the lowest temperature (−16°C). This result was contrary to our expectations from working on whole flies, which succumb to cold around −6°C (Teets and Hahn, 2018). Thus, in a subsequent experiment, we included a freezing treatment to verify that S2 cells are freeze intolerant and that our assay can detect high levels of cell mortality 24 h after cold exposure. In this experiment, we quantified both metabolic health using alamarBlue and cell density using CyQUANT GR (Thermo Fisher Scientific). Cells were either supercooled at −8°C or frozen at −20°C for 2 h, with controls maintained at 25°C. Cells were treated the same as above, with the exception that they were kept at a 10-fold higher density during recovery to promote cell growth. Following recovery, each sample was split and measured with alamarBlue and CyQUANT GR. For CyQUANT GR, plates were centrifuged for 5 min at 1000 ***g***, then gently inverted onto a paper towel to remove the medium. The plate was frozen at −80°C for 15 min to lyse cells, then 200 µl of CyQUANT GR reagent (prepared as per the manufacturer's instructions) was added to each well. The plate was wrapped in foil to protect it from direct light and incubated at room temperature for 5 min. After the final incubation, fluorescence was measured using an excitation wavelength of 485 ±10 nm and an emission wavelength of 530±12.5 nm.

To test the extent to which S2 cells are capable of RCH, we used the following conditions: control (25°C), cold shock (−8°C for 2 h) and RCH (4°C for 2 h followed by −8°C for 2 h). As in previous experiments, cells were given 24 h recovery at 25°C before metabolic viability and cell number were measured. For each treatment, 16 biological replicates spread over two blocks were measured on consecutive days; a few replicates were lost because of technical issues that resulted in incomplete aspiration of the medium during the CyQUANT GR measurements. Cells were treated at the same density as above, and alamarBlue and CyQUANT GR measurements were conducted as described previously with the following exception: alamarBlue reagent was added to each sample prior to dispensing samples in triplicate onto the plate.

All statistical analyses were conducted in JMP Pro 14 (SAS Institute, Cary, NC, USA). Cold shock survival data were analyzed with ANOVA followed by Tukey's pairwise comparisons. Data for the RCH experiment were analyzed with a linear model with treatment as a fixed effect and experimental block as a random effect. Tukey's pairwise comparisons were used to compare groups. All data are included in Dataset 1.

RESULTS AND DISCUSSION

In an initial experiment, we measured the baseline cold tolerance of S2 cells. Metabolic viability, as determined by alamarBlue fluorescence, reached a minimum value at −8°C (Fig. 1A), so in subsequent experiments we used this condition as our discriminating temperature to test for RCH capacity. The relatively high rate of metabolic viability (∼73%) in S2 cells at −16°C was surprising, considering flies cultured at 25°C die from cold shock at −6°C (Gerken et al., 2015; Teets and Hahn, 2018). Thus, in a subsequent experiment, we included a freezing condition (−20°C) to verify that the S2 cells are freeze intolerant and that our assay can detect high levels of injury. Once again, metabolic viability after supercooling at −8°C was relatively high (∼80%), but when cells were frozen, viability decreased to 35% of controls (Fig. 1B). In this experiment we also measured cell density after cold exposure, based on DNA content through CyQUANT GR fluorescence, and the patterns for cell density were similar to those observed for metabolic health (Fig. 1C). Further, in a pilot experiment we assessed viability after cold shock with Trypan Blue, and the viability patterns were quite similar to those obtained with alamarBlue (Fig. S1A).

To test for RCH capacity in S2 cells, groups of cells were exposed to control (25°C), cold shock (−8°C for 2 h) or RCH (4°C for 2 h followed by −8°C for 2 h) conditions. After cold treatment, we measured metabolic viability through alamarBlue fluorescence and cell number through CyQUANT fluorescence. In this experiment, cold shock caused an 11% decrease in cell metabolic viability, and this difference was statistically significant (Fig. 2A; linear model, Tukey's HSD, P=0.0006). However, when cells were treated with RCH, metabolic viability was significantly higher than in cold shocked cells (linear model, Tukey's HSD, P=0.0048) and indistinguishable from controls (P=0.72). Thus, RCH preserves metabolic activity of cells following cold shock. As with metabolic activity, cold shock reduced the growth rate of cells, with 27% fewer cells than controls 24 h after treatment (Fig. 2B; linear model, Tukey's HSD, P=0.0002). However, unlike metabolic function, RCH had no effect on cell growth, and cell densities were statistically indistinguishable between cold shock and RCH cells 24 h after treatment (Fig. 2B; linear model, Tukey's HSD, P=0.99). We speculate that the increased metabolic activity of RCH cells, despite a slowed growth rate, reflects energetic investment in repair processes. Previous work on whole insects has identified energetic costs associated with repairing cold injury, which may explain the increased metabolic activity of RCH cells at the expense of growth (e.g. Marshall and Sinclair, 2010; MacMillan et al., 2012; Teets et al., 2011). In recent work in a freeze-tolerant Antarctic insect, we showed that larvae treated with RCH have higher metabolic rates during recovery than those directly cold shocked (Teets et al., 2019), indicating that preservation of metabolic activity may be a general

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feature of RCH. Another possible explanation for the inconsistency between the metabolic viability and cell quantification is that CyQUANT GR quantifies cell density via DNA content but is unable to distinguish between the DNA of healthy dividing cells and that of dead or dying cells. Thus, the alamarBlue results may better reflect the physiological status of the cells, and indeed in a pilot experiment, RCH also improved viability as measured by Trypan Blue, a membrane integrity assay (Fig. S1B). However, additional experiments are needed to test the extent to which RCH restores normal cell growth over time.

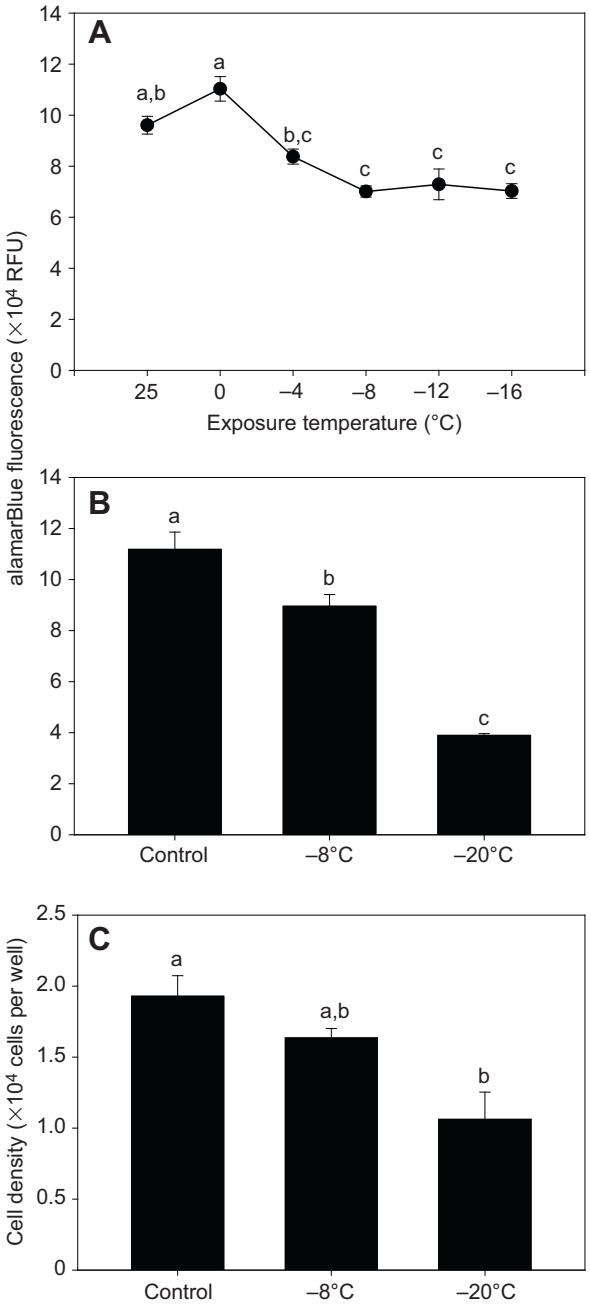

Fig. 1. Baseline cold tolerance of *Drosophila* S2 cells. Cells were exposed to the indicated temperatures for 2 h, and metabolic viability (A,B) and cell density (C) were assessed 24 h later with alamarBlue and CyQUANT GR fluorescence, respectively. In B and C, the –20°C treatment was used to include a temperature at which the cells froze. Bars represent means±s.e.m., *n*=3 biological replicates per group. Groups that do not share a letter are significantly different (ANOVA, Tukey's HSD, *P*<0.05). RFU, relative fluorescence units.

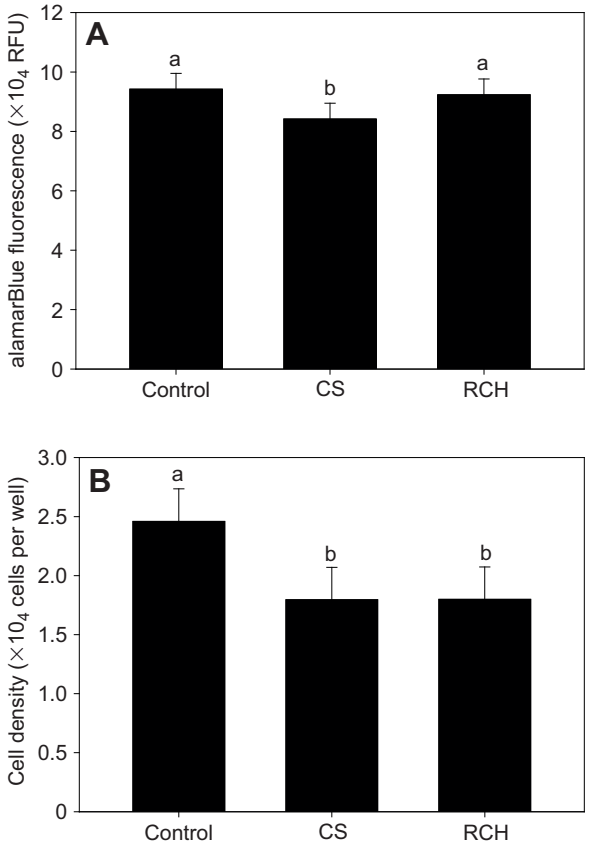

Fig. 2. Rapid cold hardening improves viability of cells after cold shock. (A) Metabolic viability and (B) cell density were measured following control, cold shock (CS) and rapid cold hardening (RCH) treatments. Bars represent means±s.e.m., *n*=14 biological replicates for control and *n*=15 biological replicates for CS and RCH. Different letters indicate significant differences between treatments (linear model, Tukey's HSD, *P*<0.05).

Our results indicate that cultured cells can detect and quickly respond to low temperatures in a manner analogous to whole insects. Previous work has demonstrated that insect tissues retain the capacity for RCH *ex vivo* (Teets et al., 2008; 2013; Yi and Lee, 2004), and our results demonstrate this capacity is also present in a clonal cell culture. Remarkably, S2 cells were first isolated nearly 50 years ago (Schneider, 1972), yet have retained the capacity for RCH outside of the context of the organism. Whether cultured cells use the same cold-sensing and protective mechanisms remains to be seen, but our work indicates that S2 cells are a potentially useful tool for investigating the mechanisms of low temperature stress. RCH is likely regulated by cell signaling pathways, but the exact mechanisms have been difficult to pinpoint. Cells detect chilling via calcium influx (Teets et al., 2008; 2013), and RCH leads to phosphorylation of p38 MAP kinase (Fujiwara and Denlinger, 2007), but the extent to which these pathways interact, and the downstream processes they activate, is unknown. These signaling processes are more readily manipulated in cell culture, and thus cell culture provides a powerful system for investigating signal transduction at low temperatures and screening for potential mechanisms. An added advantage of working with *Drosophila* cell lines is that candidate pathways can be readily validated *in vivo* using the plethora of available genetic tools in flies.

While we observed a consistent, detectable RCH response in cells, we acknowledge that the effect size was relatively small (~10% increase in metabolic viability compared with cold shock; Fig. 2A). The small effect size is due to the high cold tolerance of S2 cells. Normally, RCH is assessed at a discriminating temperature that causes high mortality (Sinclair et al., 2015), but even at the lowest temperature at which we could keep cells supercooled (−16°C), cell metabolic viability was ~73% of controls (Fig. 1A), and this high baseline tolerance constrains the potential phenotypic effects of RCH. Thus, additional work is needed to determine whether the RCH response we are measuring in cells is the same process as that measured *in vivo*, in which a near-complete rescue of lethality is observed (e.g. Lee et al., 1987; Czajka and Lee, 1990; Teets and Denlinger, 2016). Different tissues have different cold sensitivity (Yi and Lee, 2004; Teets et al., 2013), and it appears S2 cells are a particularly cold-resistant cell type. S2 cells were derived from embryos and have macrophage-like properties (Schneider, 1972), and cell lines derived from other tissues may show a more dramatic RCH response. Another possibility is that the cell culture medium is providing cryoprotection. However, proline is the only potent cryoprotectant in the media, and its levels (~3.5 mmol l^{-1}) are much lower than those observed in cold-acclimated flies (i.e. >15 mmol l^{-1}; see Koštál et al., 2011).

Cultured cells lack an organismal context, so the surprisingly high cold tolerance of S2 cells may reflect the importance of cell–cell interactions in tissues and organs for determining cold tolerance. For example, cold stress leads to epithelial barrier failure in the gut that causes paracellular ion leak, and the resulting hyperkalemia leads to cold injury (MacMillan et al., 2017). Furthermore, plastic acclimation responses that increase cold tolerance lead to structural changes in cytoskeletal and epithelial morphology that improve ion balance in the cold (MacMillan et al., 2017; des Marteaux et al., 2018). Together, these results suggest that cold tolerance may be limited more by a failure of specific organs and disruption in physiological systems than by the survival of individual cells. A cell culture system provides a potential means to tease apart the direct effects of cold stress on cell physiology versus effects on cell–cell interactions and organ function.

While cell culture has limitations, we have demonstrated that isolated cells retain the capacity to physiologically respond to low temperature in a manner similar to intact organisms. Intracellular signaling processes and the extracellular environment can be readily manipulated in cell culture, allowing for potential mechanisms to be screened *in vitro* before organismal experiments are attempted. The cell physiology of insect cold tolerance is a ripe area of research (Teets and Denlinger, 2013; Overgaard and MacMillan, 2017), and cell culture provides a powerful tool to complement *in vivo* studies for dissecting the cell biology and biochemistry of these processes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.A.W.N., N.M.T.; Methodology: E.A.W.N., N.M.T.; Validation: E.A.W.N.; Formal analysis: N.M.T.; Investigation: E.A.W.N.; Writing - original draft: E.A.W.N.; Writing - review & editing: N.M.T.; Visualization: N.M.T.; Supervision: N.M.T.; Project administration: N.M.T.; Funding acquisition: N.M.T.

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