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Sub-lethal heat stress causes apoptosis in an Antarctic fish that lacks an inducible heat shock response

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ABSTRACT

The endemic fish fauna of the Southern Ocean are cold-adapted stenotherms and are acutely sensitive to elevated temperature. Many of these species lack a heat shock response and cannot increase the production of heat shock proteins in their tissues. However, some species retain the ability to induce other stress-responsive genes, some of which are involved in cell cycle arrest and apoptosis. Here, the effect of heat on cell cycle stage and its ability to induce apoptosis were tested in thermally stressed hepatocytes from a common Antarctic fish species from McMurdo Sound in the Ross Sea. Levels of proliferating cell nuclear antigen were also measured as a marker of progression through the cell cycle. The results of these studies demonstrate that even sub-lethal heat stress can have deleterious impacts at the cellular level on these environmentally sensitive species.

1. Introduction

The notothenioid fishes endemic to the Southern Ocean are a species flock comprising relatively few, closely related species. As one approaches continental Antarctica the number of species drops dramatically as few fishes have successfully adapted to the icy waters of the southern polar ecosystem. These fishes are profoundly cold-adapted and possess some of the lowest upper thermal limits of any species (Somero and DeVries, 1967; Bilyk and DeVries, 2011). Physiological parameters perhaps responsible for this thermal sensitivity include the deleterious impact of heat on the affinity of acetylcholinesterase for acetylcholine (Baldwin, 1971; MacDonald et al., 1998) and the inability to provide sufficient oxygen to meet increased metabolic demands at higher temperatures (Pörtner, 2001, 2010).

At the cellular level, some Antarctic fishes are distinguished by the lack of a typical heat shock response (HSR), which is characterized by the heat-induced up-regulation of the family of molecular chaperones called heat shock proteins (Hsps) (Hofmann et al., 2000; Buckley et al., 2004; Clark et al., 2008). The Hsps are responsible for preventing aggregation of thermally denatured proteins and have been related to organismal thermal tolerance in various taxa (Feder and Hofmann, 1999; Fangue et al., 2011). Although Hsps are not induced in these fishes, the genes encoding them are intact and the proteins are being produced constitutively (Place et al., 2004). This may be due to problems folding proteins in the extreme cold and the requirement for constant chaperoning of the cellular protein pool by Hsps (Todgham et al., 2007).

Despite not displaying a heat shock response, at least one common Antarctic fish, the emerald rock cod *Trematomus bernacchii*, has retained the ability to up-regulate hundreds of genes in response to heat (Buckley and Somero, 2009), many of which are involved in the coordinated, conserved cellular stress response (CSR). The CSR concept, supported by genomic and proteomic analyses, posits that there are four basic aspects to the cellular response to environmental stress. These include macromolecular protection via molecular chaperoning (e.g. the HSR), increases in metabolic activity, the temporary and reversible arrest of the cell cycle and, potentially during severe stress, programmed cell death (apoptosis). We proposed the existence of a modified cellular stress response in *T. bernacchii* (Buckley and Somero, 2009). This model proposes that in the absence of an inducible HSR, elevated but sub-lethal heat stress may activate pathways that determine cell fate, including arrest of the cell cycle and/or apoptosis. In a cDNA microarray study on gene expression patterns in *T. bernacchii* exposed to 4 °C, numerous genes encoding cell cycle regulators and proapoptotic proteins were induced (Buckley and Somero, 2009). Furthermore heat induces the production of a key regulator of both cell cycle arrest and apoptosis, CCAAT/Enhancer Binding Protein – delta (C/EBP-∂) in this same species (Sleadd and Buckley, 2013).

In other systems, it has been shown that cell cycle arrest can be a response to heat stress (e.g. Smith and Fornace, 1996; Aldsworth et al., 1999). It may be beneficial during periods of environmental

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perturbation to halt cell division and repair damage to macromolecules. Furthermore, this would be a mechanism by which energetic resources can be prioritized on cell survival processes. To be adaptive, this response would be reversible so that normal growth can be resumed upon return to non-stressful conditions.

It is well established that heat stress can create reactive oxygen species (ROS) in cells and that oxidative stress can lead to apoptosis. There are two primary pro-apoptotic pathways. The intrinsic pathway involves mitochondrial outer membrane permeabilization and activation of pro-caspase 9. The extrinsic pathway begins at the cell membrane with the binding of ligands such as FasL and TRAIL and the subsequent cascade ends in active caspase 8. There is a balance in the stressed cell between survival via cytoprotective mechanisms-including the HSR-and cell death. In addition to their role in protecting cellular proteins, Hsps can directly antagonize both the intrinsic and extrinsic pathways at a variety of steps, blocking apoptosis (Beere, 2005). This begs the question: what does this mean for organisms, like the target species of this study, that lack an inducible HSR? It is possible that in the absence of an HSR, apoptosis may be favored in Antarctic fishes during heat stress. Alternatively, because these fishes produce Hsps constitutively, it is possible that their presence may provide anti-apoptotic effects.

Among marine ectotherms, the issue of heat-induced oxidative stress is particularly critical for species inhabiting cold, oxygen-rich polar waters (Viarengo et al., 1995; Ansaldo et al., 2000; Abele and Puntarulo, 2004; Lesser, 2006). Studies on Antarctic marine invertebrates demonstrate that heat stress elicits oxidative stress in the bivalve *Laternula elliptica* (Abele et al., 1998; Heise et al., 2003) and antioxidant genes are induced by heat in the limpet *Nacella concinna* (Truebano et al., 2010). The ability of the cell to tolerate oxidative stress depends on the activity of antioxidant enzymes and is often characterized as the total oxyradical scavenging capacity (TOSC). The TOSC has been measured in Antarctic invertebrates (Regoli et al., 2002) and fishes, including *T. bernacchii* (Regoli et al., 2005; Benedetti et al., 2010). While this demonstrates that Antarctic ectotherms appear to have the traditional cellular defenses against oxidative stress, the links between heat stress and cell cycle arrest and apoptosis in Antarctic fishes are not understood. This is especially true for the fishes that lack an HSR.

To directly test whether sub-lethal heat stress causes cell cycle arrest and/or apoptosis in cold-adapted Antarctic fishes, isolated hepatocytes from *T. bernacchii* were subjected to a range of temperatures for a range of durations and fluorescent assisted flow cytometry was used to stage cell cycle progression and to quantify apoptotic cells. Levels of proliferating cell nuclear antigen (PCNA) – a marker of progression through the cell cycle – were quantified in the liver tissue from *T. bernacchii* exposed to elevated, sub-lethal temperatures for a range of durations. This protein was chosen as a regularly employed marker of progression through the cell cycle as its production peaks during S-phase. Therefore, if cell cycle arrest is occurring, we would predict PCNA levels to decrease. Our findings support the concept of a modified cellular stress response in some cold-adapted poikilotherms.

2. Materials and methods

2.1. Animal collection and maintenance

Specimens of *T. bernacchii* were collected through holes drilled in the sea ice via hook-and-line in the shallow, near-shore waters at Inaccessible Island, McMurdo Sound, Antarctica (77° 53′S, 166° 40′W). Fish were transported to the United States Antarctic Program's McMurdo Station at Ross Island in aerated coolers and were maintained in flow-through seawater tanks at the ambient temperature of -1.0 ± 0.5 °C. Fish were allowed to acclimate to laboratory conditions for at least 4 days before use in experiments.

2.2. Heat exposures of hepatocytes and whole animals

One set of heat exposures was conducted on isolated, suspended hepatocytes (for flow cytometry), and a second set of exposures was conducted on pieces of whole liver tissue (for western blotting). For hepatocyte preparations, specimens were anesthetized with MS-222 (1 g/L seawater at -1.5 °C) and their livers perfused with perfusion buffer (290 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, 0.5 mmol l^{-1} EGTA and 25 mmol l^{-1} Tricine [pH 7.8]). Following perfusion, livers were excised and incubated in collagenase (Sigma, St. Louis, MO, USA) at a concentration of 5 units per mL of suspension buffer (292.5 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 2.5 mmol l^{-1} $MgCl_2$, 3 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} $NaHCO_3$, 2 mmol l^{-1} NaH_2PO_4, 5 mmol l^{-1} glucose and 50 mmol l^{-1} Hepes, [pH 7.8]) for 1 h at -1.5 °C on an orbital shaker. Cells were then strained through a cell sieve, centrifuged at $100 \times g$ for 10 min and then resuspended in suspension buffer. Cells from 6 individuals were then subjected to a range of temperatures in thermally controlled water baths. Temperature exposures were: -2, 0, 2, 6, or 10 °C and at each temperature an aliquot of cells from each individual were removed at either 0, 2, 4, 8, 12 or 24 h of exposure. These temperatures were chosen as control (-2 °C), sub-lethal (0 and 2 °C), a temperature that can cause mortality in some individuals (6 °C) and a temperature that would be lethal over time to many individuals (10 °C). After removal from the water bath, cells were centrifuged at 100 x g for 5 min at 2 °C, resuspended in 100% ethanol and stored at -20 °C to await flow cytometry for cell cycle staging.

A second set of heat exposures was conducted on whole organisms for determining PCNA concentrations via western blotting. Twenty-four individuals were randomly selected from the holding tank and transferred to one of two re-circulating seawater aquaria held at 2.0 °C or 4.0 °C. Twelve control fish remained in the holding tank at ambient temperature (-1.0 ± 0.5 °C). For both of the exposure tanks, partial water changes were conducted daily throughout the experiment. Three fish from each temperature treatment (-1.0 ± 0.5, 2.0 and 4.0 °C) were weighed then killed by cervical transection at 6, 24, 72 and 168 h. Brain, gill, heart, liver, spleen and white muscle tissue was removed and flash-frozen in liquid nitrogen and shipped to Portland State University for analysis.

2.3. Flow cytometry

A protocol for determining cell cycle stages and apoptosis by flow cytometry was adapted from Darzynkiwicz et al. (1992, 1999). Hepatocytes were pelleted by gentle centrifugation, washed twice in ice-cold 1 × PBS and resuspended in staining solution consisting of 0.1% (v/v) Triton X-100 + 0.02% DNase-free RNase A + 0.005% propidium iodide (PI) in ice-cold 1 × PBS. Cells were stained for 30 min on ice and analyzed on a BD Accuri™ C6 Flow Cytometer.

Data were collected and analyzed with C6 software (BD Accuri™). Gates were drawn around populations of cells based upon their DNA content (PI fluorescence) in order to distinguish different cell cycle stages. Doublet discrimination was carried out to minimize the influence of cell clumping. Proportions of total cell counts were recorded for each cell cycle stage and statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) as described below.