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Mitochondrial reactive oxygen species production by fish muscle mitochondria: Potential role in acute heat-induced oxidative stress

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ABSTRACT

Acute heat challenge is known to induce cell-level oxidative stress in fishes. Mitochondria are well known for the capacity to make reactive oxygen species (ROS) and as such are often implicated as a source of the oxidants associated with this thermally-induced oxidative stress. This implication is often asserted, despite little direct data for mitochondrial ROS metabolism in fishes. Here we characterize mitochondrial ROS metabolism in three Actinopterygian fish species at two levels, the capacity for superoxide/H_2O_2 production and the antioxidant thiol-reductase enzyme activities. We find that red muscle mitochondria from all three species have measurable ROS production and respond to different assay conditions consistent with what might be anticipated; assuming similar relative contributions from difference ROS producing sites as found in rat skeletal muscle mitochondria. Although there are species and assay specific exceptions, fish mitochondria may have a greater capacity to produce ROS than that found in the rat when either normalized to respiratory capacity or determined at a common assay temperature. The interspecific differences in ROS production are not correlated with thiol-based antioxidant reductase activities. Moreover, mimicking an acute in vivo heat stress by comparing the impact of increasing assay temperature on these processes in vitro, we find evidence supporting a preferential activation of mitochondrial H_2O_2 production relative to the increase in the capacity of reductase enzymes to supply electrons to the mitochondrial matrix peroxidases. This supports the contention that mitochondria may be, at least in part, responsible for the ROS that lead to oxidative stress in fish tissues exposed to acute heat challenge.

1. Introduction

Fishes, and aquatic ectotherms in general, may face numerous environmental challenges, with acute temperature stress being of particular importance to temperate and subpolar species where seasonal transitions and transient weather anomalies can lead to rapid changes. For most fishes, acute heating is more challenging than a comparable acute bout of cooling. Amongst the various physiological and biochemical responses associated with acute heat challenge, oxidative stress is a common result. For example, in fish exposed to an acute 10–15 °C heat there is an increase in tissue levels of lipid and protein oxidation markers, while the glutathione pool, a major intracellular redox and antioxidant system, becomes more oxidized (Heise et al., 2006; Kaur et al., 2005; Leggatt et al., 2007; Lushchak and Bagnyukova, 2006; Parihar and Dubey, 1995). This indicates that for fishes exposed to an acute heat stress there is a concomitant imbalance between the production of reactive oxygen species (ROS) and the capacity of tissues to detoxify ROS.

Many compartments and membranes in the cell contain enzymes capable of producing ROS, however, mitochondria are often asserted to be the main net source of ROS for the cell. This view has been questioned, especially in light of the very high ROS consumption capacity possessed by mitochondria (Banh and Treberg, 2013; Brown and Borutaite, 2012; Drechsel and Patel, 2010). Nevertheless, since mitochondria possess both ROS producing and consuming pathways it is plausible to suggest mitochondria could be associated with the tissue-level ROS imbalance found with acute heat stress in fishes.

Regardless if formed mitochondrially or extramitochondrially, the enzymes that produce ROS largely do so via the single or dual electron donation from exposed redox reactions to molecular dioxygen, resulting in superoxide ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2) respectively. Although mitochondria have at least 10 different significant ROS contributing sites (Brand, 2010), the majority of them predominately produce superoxide, which does not readily diffuse across membranes. Moreover, topology of mitochondrial production directs the majority of the ROS into the matrix (Brand, 2010). Superoxide is rapidly dismuted to H_2O_2 by superoxide dismutase (SOD) in both the mitochondrial matrix (Mn-SOD) and in the intermembrane space and non-mitochondrial compartments (Cu/Zn-SOD). This production of H_2O_2 is central to cellular oxidant metabolism because H_2O_2 can readily diffuse across

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membranes and thus disperse ROS across cellular compartments. As such, if mitochondria are a major source of ROS that leads to oxidative damage in acute heat stress, then the metabolism of H_2O_2 is a very important aspect to focus on.

There is some evidence that mitochondrial ROS production is a function of temperature. For example, studies using mitochondria from aquatic invertebrates show an increase in the rate of ROS efflux/formation as the assay temperature increases (Abele et al., 2002; Heise et al., 2003; Keller et al., 2004). But clear 'Q_{10}-like' responses over a wide range of temperature are not well established for quantitative assays of mitochondrial ROS production in ectotherms and, to our knowledge, not at all for fish muscle mitochondria. These Q_{10} relationships have been readily demonstrated at the level of single enzymes, as well as some other biochemical processes, with Q_{10} values typically around 2–3 for biological processes (Hochachka and Somero, 2002).

How the H_2O_2 consuming antioxidant pathways may respond to acute temperature change is also unclear. In general, there are three candidate intramitochondrial H_2O_2 consuming pathways comparative mitochondrial studies need to consider; catalase, glutathione-dependent peroxidases and thioredoxin dependent peroxidases (peroxiredoxins). Catalase is the predominant consumer of H_2O_2 by mammalian liver mitochondria, even at low micromolar concentrations of H_2O_2 (Drechsel and Patel, 2010). We in part selected skeletal muscle mitochondria for this study because catalase activity has been reported to be low in fish red muscle (Leary et al., 2003; Moyes et al., 1990) and low in rat muscle mitochondria (Phung et al., 1994). Moreover, respiration independent H_2O_2 decomposition, which would include catalase, is also low in rat muscle mitochondria even at 1–3 μM H_2O_2 (see the 'background' rate reported in Banh and Treberg (2013) which must include catalase activity). We have confirmed similarly low respiration independent H_2O_2 decomposition in trout muscle mitochondria (Zacharias and Treberg unpublished). As such, we assume the influence of catalase on intramitochondrial H_2O_2 metabolism is low in our muscle mitochondria preparations; however, we still caution that comparative investigations of H_2O_2 metabolism by mitochondria from other tissues and taxa should consider the potential influence of catalase. However, the NADPH requiring GSH-dependent pathway is important in rat skeletal muscle (Treberg et al., 2010) and the thioredoxin pathway is likely important in muscle cells (Rohrbach et al., 2006) and is known to be the predominant H_2O_2 consumption pathway in rat brain mitochondria (Drechsel and Patel, 2010). Mitochondrial pathways linked to bioenergetics may be particularly important to acute heat stress-induced oxidative damage if there is a mismatch between the potential to produce ROS and the capacity of detoxification systems with increasing temperature.

Here we characterize superoxide/H_2O_2 production, measured as H_2O_2 efflux, by fish skeletal muscle to test how H_2O_2 metabolism may be influenced by acute heat increase. To do so we developed an improved means of isolating red muscle mitochondria from fish. Using this improved isolation method we compared superoxide/H_2O_2 production by muscle mitochondria from three species of fish as well as the establish rat mitochondrial system. We used four substrate/inhibitor combinations that are well described for rat skeletal muscle mitochondria (Quinlan et al., 2011, 2012a, 2012b, 2013, 2014; Treberg et al., 2010, 2011) and find, intraspecifically, similar relative rates of production between substrates and inhibitor responses; however, compared to rat mitochondria respiring on 5 mM succinate (in the presence of rotenone), fish mitochondria appear to have a greater capacity for superoxide/H_2O_2 production relative to the respiratory activity at physiological temperature. Differences in superoxide/H_2O_2 production are not readily linked to the activities of reductases supplying electrons to the peroxidase systems, or the level of mitochondrial GSH, indicating no clear link between these antioxidant systems and ROS production. Superoxide/H_2O_2 production is temperature sensitive for all conditions tested, although in some cases it deviates from a straightforward exponential Q_{10}-like response indicating underlying complexity between ROS metabolism and temperature. The relative increase in superoxide/H_2O_2 production by fish mitochondria as assay temperatures go from 15 °C to 25 °C is greater than the increase in the activities of the reductases that support peroxidase systems. This differential temperature sensitivity is consistent with mitochondria as a potential source of the oxidants responsible for oxidative damage associated with acute heat increase in fish.

2. Material and methods

2.1. Animals and sampling

Three species of Actinopterygian fishes were used in this study; rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*) and lake sturgeon (*Acipenser fulvescens*). Fish of mixed sex were housed in flow-through tanks maintained at 15 °C and fed chow ad libitum 3 times a week. Sturgeon had been raised from larvae at the University of Manitoba, trout and carp were purchased from commercial suppliers. All fish used in this study were approximately 1.5–4 kg. Fish were killed by a blow to the head and lateral red muscle was dissected immediately for use in isolating mitochondria (see Section 2.2.). Male Sprague–Dawley rats were purchased from the central animal facility at the University of Manitoba and were housed in standard shoe-box style cages with free access to food (standard chow) and water. Rats were approximately 280–380 g when used and skeletal muscle was collected following asphyxiation with CO_2 (and subsequent dual pneumothorax). All animal procedures were approved by the University of Manitoba animal care committee.

2.2. Isolation of mitochondria

2.2.1. Fish

Red muscle mitochondria were initially prepared using typical homogenizing methodologies for fish muscle (Chamberlin et al., 1991; Johnston et al., 1994; Moyes et al., 1988). Early on we found issues with the quality of mitochondria isolated using this approach. We tried several isolation media and modifications of homogenizing techniques. Ultimately we tested two means of isolation for fish muscle mitochondria.

2.2.1.1. Homogenization (Method A). The final methodology used 100 mM KCl, 50 mM Tris, 2 mM EGTA (pH 7.2 at 25 °C) as the isolation medium. Tissue was finely diced on an ice-cold cutting board and rinsed with isolation medium several times to remove lipid and loose connective tissue. Rinsed tissue was transferred to glass homogenizing tubes and mixed with approximately 6–9 volumes of homogenization medium (isolation medium supplemented with 0.3% (w/v) bovine serum albumin). Homogenization was done by three incrementally tighter fitting motor driven PTFE pestles using a Potter–Elvehjem style homogenizer, all of which was done on ice. First, two full passes of a loose-fitting pestle were done, followed by two passes of an intermediate fitting pestle. A final pass with a tight (0.15 mm clearance) pestle was used. Care was taken throughout to prevent cavitation or uneven vertical speed of pestle movement. No improvement in yield or change in quality was observed between one or two passes of the tight fitting pestle. The homogenate was used directly for isolating mitochondria by differential centrifugation. All centrifugation was done at 2–4 °C while resuspensions were done on ice. The centrifugation steps started with 10 min at 900 ×*g* with the supernatant being collected through several layers of cheese cloth. The supernatant was centrifuged at 9000 ×*g* for 10 min to pellet mitochondria. The new supernatant was discarded and any residual lipid was removed from the tube. The pellet was resuspended in a small volume of isolation medium and transferred to a new tube. The tube was filled with isolation medium and centrifuged again at 9000 ×*g* for 10 min. The supernatant was again discarded, the pellet resuspended in isolation medium for a second 'wash' followed by