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Effects of hypoxia on ionic regulation, glycogen utilization and antioxidative ability in the gills and liver of the aquatic air-breathing fish *Trichogaster microlepis*

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ABSTRACT

We examined the hypothesis that *Trichogaster microlepis*, a fish with an accessory air-breathing organ, uses a compensatory strategy involving changes in both behavior and protein levels to enhance its gas exchange ability. This compensatory strategy enables the gill ion-regulatory metabolism to maintain homeostasis during exposure to hypoxia. The present study aimed to determine whether ionic regulation, glycogen utilization and antioxidant activity differ in terms of expression under hypoxic stresses; fish were sampled after being subjected to 3 or 12 h of hypoxia and 12 h of recovery under normoxia. The air-breathing behavior of the fish increased under hypoxia. No morphological modification of the gills was observed. The expression of carbonic anhydrase II did not vary among the treatments. The Na^+/K^+-ATPase enzyme activity did not decrease, but increases in Na^+/K^+-ATPase protein expression and ionocyte levels were observed. The glycogen utilization increased under hypoxia as measured by glycogen phosphorylase protein expression and blood glucose level, whereas the glycogen content decreased. The enzyme activity of several components of the antioxidant system in the gills, including catalase, glutathione peroxidase, and superoxidase dismutase, increased in enzyme activity. Based on the above data, we concluded that *T. microlepis* is a hypoxia-tolerant species that does not exhibit ion-regulatory suppression but uses glycogen to maintain energy utilization in the gills under hypoxic stress. Components of the antioxidant system showed increased expression under the applied experimental treatments.

1. Introduction

Fish gills, which are constantly exposed to the external environment, are multifunctional organs that are important for various homeostatic activities, such as gas exchange and ion regulation (Perry, 1998; Hwang, 2009; Dymowska et al., 2012). Mitochondria-rich cells (MRCs), which are generally distributed in the filaments and inter- and basal-lamellar regions, are the sites of ion uptake and extrusion (Evans et al., 2005; Hwang et al., 2011). Na^+/K^+-ATPase (NKA) in MRCs is the major driving force for ion transport in the fish branchial system. In freshwater, fish gills exhibit up-regulation of NKA in response to salinity changes (Perry et al., 2003; Tresguerres et al., 2007; Huang et al., 2010). This protein up-regulation upon both salt and acid treatments is attributed to an increased expression of mRNA (Scott et al., 2004), protein (Horng et al., 2007) or both (Lin et al., 2006).

Under hypoxia, key pro-survival responses observed in fish include decreasing the energy costs, maintaining major protein expression, and increasing the antioxidant defenses of the gill respiratory surface area (GRSA) (Bickler and Buck, 2007). Fish gills can compensate for changes in ambient oxygen levels by exhibiting potentially widespread morphological variations, as observed in the crucian carp (*Carassius carassius*) (Sollid and Nilsson, 2006; Nilsson, 2007). In normoxic water, this species possesses no protruding lamellae. However, under hypoxic conditions, its gill lamellae become apparent within 14 days due to a reduced interlamellar cell mass (Sollid et al., 2003). When euryhaline sea bass (*Dicentrarchus labrax*) were subjected to different oxygen levels (60, 90 and 140%), the size of their GRSA was found to be negatively correlated with the dissolved oxygen level (Saroglia et al., 2002). Increased GRSA allows the uptake of more ambient oxygen in a hypoxic environment. Hypoxia-inducible factor-1 (HIF-1) is a critical molecular regulator of hypoxic stress in mammalian cells. HIF-1, which is a heterodimeric transcription factor that includes one α and one β subunit, induces the up-regulation of the expression of genes involved in glucose metabolism, angiogenesis and cellular proliferation, among others (Sememza, 2001; Laderoute, 2005; Ruas and Poellinger,

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2005). Sollid and his colleagues found that HIF-1α participates in hypoxia-induced modification of the GRSA in crucian carp (Sollid et al., 2006).

The theme underlying these physiological responses to hypoxia is the maintenance of metabolic efficiency, which involves both the down-regulation of energy consumption and ion-regulatory suppression (Bickler and Buck, 2007). Recently, studies in the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*) have provided evidence supporting this concept. The gills of *A. ocellatus* showed decreased metabolism and ion-regulatory responses under hypoxic conditions (Richard et al., 2007; Wood et al., 2009). A study of the hypoxia-intolerant freshwater rainbow trout (*Oncorhynchus mykiss*) detected different responses to hypoxia (Po_2 ~ 80 mm Hg); NKA activity did not change during 4 h in hypoxic conditions (Iftikar et al., 2010). These differences in ion-regulatory expression may be attributed to species-specific responses that reduce or maintain ion-regulatory ability in periods of decreasing dissolved oxygen in the environment, but these responses are still not clearly understood.

Another key response to hypoxia is the regulation of the energy supply by regulating the glycolysis of storage glycogen, which is a readily available energy resource (Gruetter, 2003; Bickler and Buck, 2007). Previous studies have shown that glucose can be used as fuel for high-energy-consuming tissues when fish require a greater energy supply in stressful environments (Chang et al., 2007; Tseng et al., 2007; Polakof et al., 2012). For example, the euryhaline tilapia (*Oreochromis mossambicus*) showed an increased ion-regulatory ability when it was transferred from freshwater to seawater (Hwang et al., 2011). In this situation, MRCs need a greater energy supply to support the high-energy-requiring primary active ionic transporter (Tseng et al., 2007). Tseng and colleagues found that glycogen phosphorylase (GP) showed increased gene and protein expression in the gills and that glycogen in the glycogen-rich cells (GRCs) in the gills and liver provided the energy resource that supplies MRCs (Chang et al., 2007; Tseng et al., 2009). Therefore, the glycogen in the gills and/or liver represents a short-term energy store that may participate in maintaining the ion-regulatory function of the primary active ionic transporter in fish gills.

Animals usually exhibit 0.1–0.2% reactive oxygen species (ROS) production from their daily oxygen consumption (Gorr et al., 2010). ROS are involved in signal transduction pathways, including those associated with the cell cycle, stress response, and energy metabolism (Gorr et al., 2010). If ROS are overproduced in cells, they damage most types of molecules and result in DNA and protein degradation (Costantini et al., 2010). Therefore, the antioxidant system is necessary to protect organisms against hypoxic stress. There are a number of cellular defense systems for combating oxidative stresses. Several different antioxidant enzymes, such as catalase (CAT), glutathione peroxidase (GPx), and superoxidase dismutase (SOD), are involved, and the non-enzymatic element glutathione also plays a critical role in removing free radicals from cells (Sampath et al., 1994; Lee et al., 2000).

Aquatic air-breathing fish have the ability to exchange gases directly with the aerial environment, and these fish all exhibit an accessory air-breathing organ (Graham, 1997). Anabantoidei species possess a labyrinth organ (LO), an accessory air-breathing organ that consists of branchial and systemic circuits similar to a double-circuit circulatory system (Olson et al., 1986; Munshi et al., 2001). Carbonic anhydrase II (CAII) is an important enzyme for gas exchange and is widely distributed in the labyrinth organ and gills of *Trichogaster trichopterus* (Burggren and Haswell, 1979). This enzyme catalyzes the reversible hydration/dehydration reactions of CO_2 and is responsible for aerial CO_2 excretion (Henry and Swenson, 2000). These species are found not only in the well-oxygenated littoral zone but also in hypoxic rivers and lakes (Randle and Chapman, 2005). *Trichogaster microlepis*, an aquatic air-breathing fish species in Anabantoidei, exhibits significant morphological variation in both the length of the filaments and lamellae between the anterior (1st and 2nd gills) and posterior (3rd and 4th gills) gills. This species used only the anterior gills as the major site to respond to environmental variation (Huang et al., 2011). In this study, ion regulation (energy consumption), glycogen utilization (energy production) and antioxidative ability (detoxification) were examined to provide an integrated view of energy homeostasis in aquatic air-breathing fish.

We examined the hypothesis that *T. microlepis* uses a compensatory strategy involving changes in behavior and protein levels to increase the ability for gas exchange and to enable the gill ion regulatory metabolism to maintain homeostasis during exposure to hypoxia. In the first experiment, *T. microlepis* were sampled at 0, 1.5, 3, 6, 12, and 72 h of exposure to hypoxic treatment conditions. The profiles of the frequency of air breathing, the expression of HIF-1α and NKA mRNA and the relative expression of CAII and NKA proteins were recorded. The effects of hypoxic stress on *T. microlepis* were further examined at 3 and 12 h of hypoxia and 12 h of normoxic recovery for the following: (1) blood glucose and glycogen contents; (2) relative GP protein expression; (3) gill morphology based on the examination of histological sections, the number of MRCs and GRCs in the lamellar region and the specific enzyme activity of NKA; and (4) the antioxidant enzyme activity of CAT, GPx, and SOD, as the antioxidant system is necessary to protect organisms against hypoxic stress. These parameters are important for describing the short-term adjustment to hypoxic stress.

2. Materials and methods

2.1. Animals and experimental tanks

We purchased *T. microlepis* (either sex, 4–6 cm standard length) from a local fish shop and maintained 40 individuals in one plastic tank (45 × 25 × 30 cm) with aerated, circulating local tap water filled to a height of 20 cm. One-fifth of the water was replaced every 7 days. The fish were acclimated at 28 ± 1 °C under a 12 h:12 h light:dark cycle and fed with commercial fish food (NOVO Bits, JBL, Germany) once daily for at least a week before the experiment. The fish were not fed during the experiments. The pH (Jenco, pH vision 6071, HK) and dissolved oxygen (DO) levels (Orion model 810, UK) in the experimental tanks were monitored. The experiments and handling of the animals complied with the current laws of Taiwan.

The experimental tanks consisted of plastic tanks (26 × 15 × 15 cm) filled to a height of 14 cm. Aerated and filtered local tap water was used as freshwater in each experiment. Normoxia-acclimated fish were subjected to the following treatments: (1) control (aquatic normoxia) and (2) hypoxic (aquatic hypoxia). In the hypoxic group, nitrogen was bubbled continuously, and DO was maintained at a concentration of approximately 1.0 mg/L. No bottom sand was provided. There were 8 fish in each of the treatments, and one individual in each chamber was treated in the experiment. The water chemistry is summarized in Tables 1 and 2.

2.2. Methodology

Most of the procedures performed in this study were the same as in previous studies by our group (Huang et al., 2010, 2011), unless otherwise noted. These procedures included protein extraction, immunoblotting analysis of relative protein abundance, detection of NKA enzyme activity, and examination of histological sections.

2.3. Air-breathing frequency

After a two-day pre-acclimation period under normoxic conditions, the fish were transferred to hypoxic conditions for 72 h. A standardized 60-min video recording (DCR-HC 46; Sony, Japan) was made at 0 (before transfer), 1.5, 3, 6, 12, and 72 h ($N = 8$). The video recordings were always made between 08:00 and 21:00 h. Air breathing was recorded when fish directly swallowed air at the water's surface. The