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Expression levels of myoglobin in muscle and non-muscle tissues of rainbow trout *Oncorhynchus mykiss,* a hypoxia intolerant species

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ABSTRACT

Myoglobin (Mb) is one of the most intensively studied intracellular respiratory muscle proteins. Since the discovery of the fascinating fact that Mb is not confined only to oxidative muscle tissues but also is co-localized in different non-muscle tissues of cyprinids, hypoxia tolerant cyprinids have been established as the model teleost. Mb both at mRNA and protein levels have been reported in this study for the first time from a number of muscle and non-muscle tissues of rainbow trout *Oncorhynchus mykiss*, a hypoxia intolerant species. Mb transcript levels were high in the heart and slow skeletal muscle, and were comparatively high in the gonad and gill among the non-muscle tissues. Western-blotting by using anti-rainbow trout Mb peptide rabbit antibody detected Mb protein in the muscles and several non-muscle tissues. By both RNA *in situ* hybridization and immunofluorescence, Mb was localized in the cardiomyocytes and oxidative muscle fibers. On the other hand, Mb both at mRNA and protein levels was restricted to the lamellar epithelial cells of the gill, epithelial layers of hepatobiliary duct, neurons and endothelial cells of brain, ooplasm of gonad, kidney tubules, endothelial cells, and epithelial layer of intestine. Neuroglobin isoform 1 and 2 mRNAs along with Mb mRNA were localized in the granular layer of cerebellum. Considering the previous data reported for cyprinids, the expression sites of Mb in the muscle and non-muscle tissues of teleost could be universal, where Mb concerted with the other globins might play meaningful physiological roles.

1. Introduction

Myoglobin (Mb), the first protein for which its three-dimensional structure was solved (Kendrew et al., 1960), is a small, monomeric intracellular hemoprotein that has captivated the interests of researchers for a long time in protein structures and functions relationship (Ordway and Garry, 2004). Mb is widely demonstrated to be expressed in the cardio-myocytes and oxidative skeletal (slow) muscle fibers of vertebrates (Kanatous and Mammen, 2010). Owing to this oxygen-repository protein, diving mammals like seals and whales are endowed with extended breath-holding ability (Kooyman and Ponganis, 1998), while reptiles (Helbo et al., 2015) and teleosts (Sidell, 1998) have acquired reversible oxygen (O_2) binding ability. Due to the location of this protein, *i.e.*, the cytoplasm of the cell, the O_2 bound to Mb is able to diffuse to locations in the cell where O_2 is necessary (Wittenberg et al., 1975; Garry et al., 2003; Wittenberg and Wittenberg, 2003). The 'classical' functional roles of Mb elucidated by previous biophysical, cellular, molecular, and physiological investigations have been reassessed (Brunori, 2001; Fraser et al., 2006; Hendgen-Cotta et al., 2008; Helbo and Fago, 2011). Being one of the most-studied proteins in history, it was astonishing to find that Mb still has hitherto unidentified functions (Cossins and Berenbrink, 2008). As a multifunctional heme binding protein, the physiological significances of Mb have been considered to play key roles in oxygen transport (Wittenberg and Wittenberg, 2003), short and long term oxygen storage (Hochachka and Somero, 2002; Enoki et al., 2008), partial oxygen buffering, facilitated oxygen diffusion (Flonta et al., 2009) and scavenging of free radicals like nitric oxide (NO) (Brunori, 2001). Recently, Mb has also been localized in the mitochondrion, where it enhances the activity of cytochrome *c* oxidase (Yamada et al., 2016).

Mb has been employed in the model systems to clarify relevant biochemical processes such as ligand binding (Adachi et al., 1992) and protein folding (Peterson et al., 2008). Recently, Mb has become the object of renewed interest regarding its potential roles beyond those previously characterized. With the recognition of alternative functions, such as NO and reactive oxygen species (ROS) metabolism and

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scavenging activities (Brunori, 2001), the year old concept of restriction of Mb to muscle has been challenged. As NO and ROS reactivities take place beyond the oxidative myocytes, succeeding investigations revealed Mb transcripts expression in several non-muscle tissues of zebrafish *Danio rerio* and common carp *Cyprinus carpio* (van der Meer et al., 2005; Fraser et al., 2006), namely, in the gills of adult zebrafish as well as in the liver (van der Meer et al., 2005), the gills and brain of adult carp (Fraser et al., 2006; Cossins et al., 2009), in the brain, gill, heart, liver, kidney and swim bladder of goldfish *Carassius auratus* (Roesner et al., 2008), in the gizzard of avian species (Enoki et al., 2008), certain human epithelial cancers and tumors (Flonta et al., 2009), in the heart, gill, brain, kidney, muscle, spleen, skin, liver and intestine of channel catfish *Ictalurus punctatus* (Feng et al., 2015), in the heart, slow muscle, brain, spinal cord, kidney and electric organs of electric eel *Electrophorus electricus* (Tian et al., 2017), in the heart, liver, muscle, kidney, brain, eye and skin of a highly specialized schizothoracine fish *Schizopygopsis pylzovi* (Qi et al., 2017). Moreover, cyprinids generate two Mbs from distinct genes (Mb-1 and Mb-2). Mb-1 was ubiquitously expressed in several non-muscle tissues, whereas, Mb-2 was restricted to brain (Fraser et al., 2006; Roesner et al., 2008; Cossins et al., 2009). Concurrent expansion of globin superfamily by neuroglobin (Ngb), cytoglobin (Cygb), globin E (GbE), globin X (GbX) and globin Y (GbY), which are also thought to play protective roles against ROS, and in NO metabolism as well as cytoprotection from ischaemia (Brunori, 2006) in non-muscle tissues, substantially signified the wider distribution of Mb. Although the exact facts behind the fascinating features of non-muscle expression of Mb are still to be unveiled completely, recent findings have challenged the early concepts about Mb indicating that Mb might play much wider roles than previously understood. Thus, it is essential to evaluate the influences of life style and living habitat on Mbs expression pattern.

The prevalent understanding of Mb has been gradually, but definitely, changed (Wittenberg and Wittenberg, 2003; Enoki et al., 2008) in terms of distribution patterns and functional diversity. It has been speculated that Mb distribution and expression pattern might be influenced by the life style and inhabiting environment of organisms (Fraser et al., 2006). Since the discovery, investigations on non-muscle expression sites of Mb have so far been limited as described above, although teleost habitat is much more diversified than terrestrial animals, from the viewpoint of available oxygen concentration. Rainbow trout *Oncorhynchus mykiss* is one of the most extensively studied salmonid species, and has been used as a model organism from the limited aspects such as toxicology, immunology, physiology, *etc.* (Thogaard et al., 2002). Rainbow trout has been well characterized as hypoxia-intolerant species with typical response to hypoxia like hyperventilation, increasing hemoglobin's affinity to oxygen, redistributing blood flow, and tachycardia (Dunn and Hochachka, 1982; Omlin and Weber, 2010). Previous investigations on rainbow trout Mb mostly highlighted the biochemical and biophysical aspects of this protein (Helbo and Fago, 2011; Pedersen et al., 2010). It has been hypothesized that Mb in this species of interest could have different expression patterns at tissue and cellular levels from those of cyprinid Mbs. In the present study, Mb expression patterns (both mRNA and protein levels) in rainbow trout were examined by biochemical and histochemical analyses.

2. Materials and methods

2.1. Animals

Three live rainbow trout specimens (total length average 22 cm, body weight average 580 g) were collected from Tokyo Metropolitan Fisheries Station. Just after sacrificing by decapitation, the tissue samples were excised from all over the body, namely, slow skeletal muscle, fast skeletal muscle, gill, brain, liver, heart, spleen, intestine, kidney, gonad and caudal fin, which were immediately preserved in RNA*later*® (Ambion, Austin, TX, USA), and stored at −80 °C until used for further experiments.

2.2. Total RNA extraction and first strand cDNA synthesis

Total RNAs were extracted from the slow muscles of rainbow trout using ISOGEN (Nippon Gene, Tokyo, Japan) followed by treatment with recombinant DNase I (Takara, Shiga, Japan) to degrade DNA contaminants in the RNA preparation. First strand cDNAs were synthesized using an aliquot of 5 μg of the total RNA. The AP primer (5′-GGCCAC GCGTCGACTAGT AC(T)16-3′) (adapter primer) was used to initiate the synthesis with SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) in 50 mM Tris-HCl (pH 8.3), containing 75 mM KCl, 3 mM $MgCl_2$, 500 μM dNTP, and 10 mM dithiothreitol. The reaction was carried out at 48 °C for 1.5 h and stopped by heat inactivation at 70 °C for 15 min. In order to eliminate the RNA templates from the cDNA:RNA hybrid molecule, RNase H (Thermo Fisher Scientific) was used just after the first strand cDNA synthesis. The reaction was carried out at 37 °C for 1 h.

2.3. Semi-quantitative RT-PCR

The levels of Mb transcripts in different tissues of rainbow trout were examined by semi-quantitative RT-PCR. Nucleotide sequences of PCR primers were designed based on the coding region of the respective species. PCR was initiated by adding 1 μl of 10 times diluted first strand cDNA as a template to 20 μl of the total reaction volume containing 2 μl of 10 × Ex *Taq* buffer (Takara, Shiga, Japan), 1 μl of each 10 pmol forward and reverse primers, 1.6 μl of 2.5 mM dNTP, 0.2 μl of Ex *Taq* DNA polymerase (Takara), and 13.2 μl of sterilized distilled water. Thermocycling included one cycle at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s with appropriate annealing for 30 s and extension at 72 °C for 1 min, and finally, a single extended polymerization step at 72 °C for 5 min.

In order to validate RT-PCR data, primers were also designed from EF1α of rainbow trout and used as a house keeping gene. Nucleotide sequences of primers have been presented in Table 1. DDBJ/EMBL/GenBank accession numbers of EF1α sequences for rainbow trout is **AF498320**.

2.4. Quantitative real-time RT-PCR (qRT-PCR)

cDNAs were diluted 100 times with DNase-free water. Primers for qRT-PCR were designed with Primer Express ver. 3.0 (Thermo Fisher Scientific) using cDNA sequence of rainbow trout Mb (DDBJ/EMBL/GenBank accession number is **AB526463**) and rainbow trout EF1α (DDBJ/EMBL/GenBank accession number is **AF498320**). The list of nucleotide primers used for qRT-PCR is presented in Table 2. qRT-PCR experiments were carried out on real-time PCR system (ABI PRISM 7300, Thermo Fisher Scientific). The reaction mixture (20 μl) contained 1 μl of 100-fold diluted cDNA, 10 pmol of forward and reverse primers, 10 μl of 2 × SYBR Premix Ex *Taq* (Takara, Shiga, Japan), and 0.3 μl of 50 × ROX reference dye (Takara, Shiga, Japan). The subsequent PCR reactions were carried out as follows: 30 s at 95 °C, 40 cycles of 95 °C for 5 s, and 60 °C for 31 s in 96-well optical reaction plates (Thermo Fisher Scientific). The absence of non-specific PCR product was confirmed by running the dissociation protocol (95 °C for 15 s, 60 °C for 30 s, and

Table 1
Nucleotide sequences of primers used for semi-quantitative RT-PCR.

Primer name	Sequence 5′–3′
Rtrout_Tissue_F	TTTGACATGGTTCTGAAGTGC
Rtrout_Tissue_R	CACGTTGCAGATGATCTCAGT
Rtrout_EF1_F	GGGTGAGTTTGAGGCTGGTA
Rtrout_EF1_R	CCATCCTTACGTTCGACCTTCC