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Spatiotemporal expression and transcriptional regulation of heme oxygenase and biliverdin reductase genes in zebrafish (*Danio rerio*) suggest novel roles during early developmental periods of heightened oxidative stress

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ABSTRACT

Heme oxygenase 1 (HMOX1) degrades heme into biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (BVRa or BVRb) in a manner analogous to the classic *anti*-oxidant glutathione-recycling pathway. To gain a better understanding of the potential antioxidant roles the BVR enzymes may play during development, the spatiotemporal expression and transcriptional regulation of zebrafish *hmox1a*, *bvra* and *bvrb* were characterized under basal conditions and in response to pro-oxidant exposure. All three genes displayed spatiotemporal expression patterns consistent with classic hematopoietic progenitors during development. Transient knockdown of Nrf2a did not attenuate the ability to detect *bvra* or *bvrb* by ISH, or alter spatial expression patterns in response to cadmium exposure. While *hmox1a*:mCherry fluorescence was documented within the intermediate cell mass, a transient location of primitive erythrocyte differentiation, expression was not fully attenuated in Nrf2a morphants, but real-time RT-PCR demonstrated a significant reduction in *hmox1a* expression. Furthermore, Gata-1 knockdown did not attenuate *hmox1a*:mCherry fluorescence. However, while there was a complete loss of detection of *bvrb* expression by ISH at 24 hpf, *bvra* expression was greatly attenuated but still detectable in Gata-1 morphants. In contrast, 96 hpf Gata-1 morphants displayed increased *bvra* and *bvrb* expression within hematopoietic tissues. Finally, temporal expression patterns of enzymes involved in the generation and maintenance of NADPH were consistent with known changes in the cellular redox state during early zebrafish development. Together, these data suggest that Gata-1 and Nrf2a play differential roles in regulating the heme degradation enzymes during an early developmental period of heightened cellular stress.

1. Introduction

Oxidative stress results from an over-abundance of reactive oxygen species (ROS) which can damage major cellular macromolecules and ultimately lead to a disruption in cellular homeostasis. Efficient cellular responses to oxidative stress are crucial for proper development, survival, and proliferation. Accordingly, multiple mechanisms exist to protect cells from oxidative stress. Glutathione (GSH), a potent *anti*-oxidant that effectively quenches ROS, is often considered to be the frontline defense against cellular oxidative stress. Reduced GSH is maintained by the classic GSH recycling pathway via the action of NADPH-dependent glutathione reductase (GRX). However, in more recent years an analogous enzyme pathway, the biliverdin/bilirubin-recycling pathway, has been suggested to be an important *anti*-oxidant defense mechanism that plays an important role in protecting lipid-rich regions of the cell (Barañano et al., 2002; Stocker et al., 1987a, 1987b). Either of two isoforms of the heme-degrading enzyme, heme oxygenase (HMOX1 or HMOX2), is responsible for the first step in the catabolism of heme into carbon monoxide (CO), free iron, and the bile pigment biliverdin (Abraham and Kappas, 2008; Tenhunen et al., 1969). Biliverdin reductase (BVR) enzymes mediate the second step in which biliverdin is further reduced to bilirubin, which can function as an *anti*-oxidant through a recycling reaction mediated by BVR (Barañano et al., 2002; Stocker et al., 1987a, 1987b). Continuous regeneration of reduced GSH or bilirubin is dependent on the availability of reducing power in the form of NADPH, which is generated via the pentose phosphate pathway (PPP) (Wu et al., 2011) as well as by other cytosolic and mitochondrial enzymes including isocitrate dehydrogenase (IDH) (Jennings and Stevenson, 1991; Jo et al., 2001), malate dehydrogenase (ME) (Pongratz et al., 2007), and the membrane bound nicotinamide nucleotide transhydrogenase (NNT) (Kirsch and De Groot, 2001; Rydstrom, 2006; Yin et al., 2012).

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While BVRb also utilizes NADPH as a cofactor, BVRa's reductase domain has dual cofactor (NADH and NADPH) dual pH specificity (Kutty and Maines, 1981). BVRa is also further differentiated by the presence of multiple functional domains, such as a leucine zipper DNA-binding domain (Ahmad et al., 2002) that allows it to function as a transcription factor, as well as a serine/threonine/tyrosine kinase domain by which it has been demonstrated to participate in cellular signal transduction cascades via interaction with MAP kinases (Kutty and Maines, 1981; Lerner-Marmarosh et al., 2005; Salim et al., 2001). Furthermore, both CO and biliverdin can serve as signaling molecules and have been demonstrated to mediate anti-apoptotic and anti-inflammatory signaling processes (Bellner et al., 2011; Brouard et al., 2000; Otterbein et al., 2000; Petrache et al., 2000). Thus the various components of the HMOX/BVR enzymatic pathway have the potential to participate in cellular stress responses through a variety of different mechanisms.

We have previously characterized the sex-specific differences in basal expression of all four *hmox* zebrafish paralogs and both *bvr* genes, as well as differences in induction by cadmium (Cd) exposure in adult zebrafish tissues (Holowiecki et al., 2016). Furthermore, we have also characterized the basal expression of all six *hmox* and *bvr* genes during zebrafish development and in response to multiple pro-oxidant exposures (Cd, tert-butylhydroquinone, and hemin) (Holowiecki et al., 2016). To expand this characterization, we sought to investigate any changes in the spatial and temporal expressions of *bvra* and *bvrb* during early zebrafish development under both basal conditions and in response to pro-oxidant exposure. Cd was chosen as the pro-oxidant since we have previously demonstrated that the Cd-inducibility of *hmox1a* and *bvrb* during zebrafish development is regulated in part by Nrf2a (Holowiecki et al., 2016). *hmox1a* expression was also characterized to add greater context to the observed changes in *bvra* and *bvrb* expression.

We also wanted to investigate the transcriptional regulation of these genes during development and in response to Cd exposure to determine how transcriptional regulation may influence spatial and temporal expression patterns. A previous study has demonstrated that *hmox1a* is expressed within the posterior blood island (PBI), an intermediate location of blood cell differentiation, at ~36 h post fertilization (hpf) (Craven et al., 2005). Similarly, *bvrb* has been shown to be expressed within the intermediate cell mass (ICM), the site of embryonic erythrocyte formation during early zebrafish development, and this expression appears to be dependent on the erythroid specific transcription factor GATA-binding factor 1 (Gata-1) (Galloway et al., 2005), which is also known as nuclear factor erythroid 1 (Nf-e1). Furthermore, it is well established that HMOX1 is regulated by nuclear factor erythroid 2-related factor 2 (NRF2), the master regulator of the oxidative stress response (Alam et al., 1999), and previous studies have provided evidence of a role for NRF2 in regulating BVR expression (Moon et al., 2012; Wu et al., 2011; Holowiecki et al., 2016). Interestingly, recent studies have suggested a novel developmental role for NRF2 as a regulator of hematopoietic stem cell (HSC) homeostasis (Merchant et al., 2011; Tsai et al., 2013). Therefore, we also characterized the changes in spatial expression of *hmox1a*, *bvra*, and *bvrb* during early zebrafish development in response to Cd exposure and after transient knockdown of Nrf2a via morpholino, as well as basal changes in spatial expression after Gata-1 knockdown. Finally, we also documented the developmental expression profiles of several cytoplasmic and mitochondrial enzymes that participate in the generation of NADPH, which is required for BVR function. These assessments were accomplished by using a combination of whole mount in situ hybridization (WISH), in vivo promoter analysis and quantitative polymerase chain reaction (qPCR). The results of these experiments provide novel insights regarding the spatial and temporal expression patterns and the differential regulation of these heme degradation genes during zebrafish development. Collectively, these results suggest novel roles for the heme degradation genes in mediating erythropoiesis during developmental periods of heightened oxidative stress and raise new questions regarding the potential in vivo antioxidant function of this enzymatic pathway.

2. Materials and methods

2.1. Fish husbandry

The TL (Tupfel/Long fin mutations) wild-type strain of zebrafish was used for all experiments. Fertilized eggs were obtained from multiple group breedings from a Mass Embryo Production System (MEPS; Aquatic Habitats, Apopka, FL) with ~200 fish at a ratio of 2 females per 1 male fish. Procedures used in these experiments were approved by the Institutional Animal Care and Use Committee of the University of Alabama, Tuscaloosa, Alabama, USA.

2.2. hmox1a in vivo promoter construct cloning

To create a *hmox1a* promoter construct, a 5.4 kb DNA region upstream of HO-1a exon 2 was amplified from zebrafish bacterial artificial chromosome (BAC) DKEY-69E1 using Kapa HiFi DNA Polymerase (Kapa Biosystems, Inc., Boston, MA) and gene specific primers flanked by attB4 and attB1 sites. Entry clones and destination vectors for Tol2 transgenesis were kindly provided by the Chien lab (Kwan et al., 2007). To create a 5′ entry vector, the PCR product was recombined with donor vector #219 pDONRP4-P1R using Gateway® BP Clonase™. The transgenic construct was created by performing a 3-way recombination reaction using Gateway® LR Clonase II ™ Enzyme to combine the HO-1a promoter 5′ entry vector with middle vector #386 (containing mCherry) and 3′ entry vector #302 (containing the SV40 late polyA signal) into destination vector #395 (containing Tol2 inverted repeats and the cardiac myosin light-chain (*cmlc2*:eGFP) "heart marker" gene) to create pDestTol2CG2;*hmox1a*:pME-mCherry-p3EpolyA (Supplementary Fig. 1). All Gateway® reactions were performed according to manufacturer's instructions. One Shot® Top 10 cells (Invitrogen, Life Technologies™ Corporation, Carlsbad, CA) were used for transformation of the transgenic DNA construct and an EndoFree Plasmid Maxi Kit (QIAGEN Inc., Valencia, CA) was used for purification and removal of any potential endotoxins.

2.3. Generation of hmox1a stable transgenic lines and in vivo promoter analysis

To generate the stable transgenic line *Tg(hmox1a:mCherry;cmlc2-eGFP)mjj1*, embryos at the 1–2 cell stage were co-injected with 50 pg of the HO-1a promoter construct (pDestTol2CG2; *hmox1a*:pME-mCherry-p3EpolyA) and 5 pg of in vitro transcribed transposase mRNA in a 2.1 nL volume using a Narishige IM-300 microinjector. Injection volumes were calibrated by injecting solutions into mineral oil and measuring the diameter of the sphere with a stage micrometer (volume $= 4/3\pi r^3$; 160 μm diameter is equivalent to 2.1 nl). Embryos Q4 were screened for expression of the *cmlc2*:eGFP heart marker at 24–48 hpf and subsequently screened for mCherry expression daily from 1 to 5 days past fertilization (dpf). Embryos strongly expressing the mCherry transgene were raised to adulthood and crossed with wild-type TL fish to create *F*1 heterozygous progeny as described by (Kawakami, 2007; Kawakami et al., 2000). *F*1 progeny were subsequently raised to adulthood and crossed with wild-type TL fish to create F2 *Tg(hmox1a:mCherry;cmlc2-eGFP)mjj1*.

We initially documented *hmox1a*:mCherry fluorescence in *Tg(hmox1a:mCherry;cmlc2-eGFP)mjj1* embryos starting at ~20–24 hpf, and continued to do so every 24 h for up to 14 days. To more specifically identify timepoints of expression during early development, embryos were collected from a mating between one F2 male HO-1a transgenic fish and two wild type TL females. Changes in *hmox1a* driven mCherry fluorescence were documented in embryos every 3 h starting at

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