Aquatic Toxicology 180 (2016) 141–154

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

The transcription factor, Nuclear factor, erythroid 2 (Nfe2), is a regulator of the oxidative stress response during *Danio rerio* development

Larissa M. Williams[a,b,*], Briony A. Lago[c], Andrew G. McArthur[c], Amogelang R. Raphenya[c], Nicholas Pray[a], Nabil Saleem[a,b], Sophia Salas[a,b], Katherine Paulson[a,b], Roshni S. Mangar[b,d], Yang Liu[e], Andy H. Vo[e], Jordan A. Shavit[e]

[a] *Biology Department, Bates College, 44 Campus Avenue, Lewiston, ME 04240, USA*
[b] *The MDI Biological Laboratory, 159 Old Bar Harbor Road, Bar Harbor, ME 04609 USA, USA*
[c] *M.G. DeGroote Institute for Infectious Disease Research, Department of Biochemistry and Biomedical Sciences, DeGroote School of Medicine, McMaster University, Hamilton, ON L8S 4K1, Canada*
[d] *College of the Atlantic, 105 Eden Street, Bar Harbor, ME 04609, USA*
[e] *Department of Pediatrics and Communicable Diseases, University of Michigan, 8200 MSRB III 1150 West Medical Center Drive, Ann Arbor, MI 48109, USA*

ARTICLE INFO

Article history:
Received 4 July 2016
Received in revised form
28 September 2016
Accepted 30 September 2016
Available online 1 October 2016

Keywords:
Nfe2
Nrf
oxidative stress
development
zebrafish
RNA-Seq

ABSTRACT

Development is a complex and well-defined process characterized by rapid cell proliferation and apoptosis. At this stage in life, a developmentally young organism is more sensitive to toxicants as compared to an adult. In response to pro-oxidant exposure, members of the Cap'n'Collar (CNC) basic leucine zipper (b-ZIP) transcription factor family (including Nfe2 and Nfe2-related factors, Nrfs) activate the expression of genes whose protein products contribute to reduced toxicity. Here, we studied the role of the CNC protein, Nfe2, in the developmental response to pro-oxidant exposure in the zebrafish (*Danio rerio*). Following acute waterborne exposures to diquat or *tert*-buytlhydroperoxide (tBOOH) at one of three developmental stages, wildtype (WT) and *nfe2* knockout (KO) embryos and larvae were morphologically scored and their transcriptomes sequenced. Early in development, KO animals suffered from hypochromia that was made more severe through exposure to pro-oxidants; this phenotype in the KO may be linked to decreased expression of *alas2*, a gene involved in heme synthesis. WT and KO eleutheroembryos and larvae were phenotypically equally affected by exposure to pro-oxidants, where tBOOH caused more pronounced phenotypes as compared to diquat. Comparing diquat and tBOOH exposed embryos relative to the WT untreated control, a greater number of genes were up-regulated in the tBOOH condition as compared to diquat (tBOOH: 304 vs diquat: 148), including those commonly found to be differentially regulated in the vertebrate oxidative stress response (OSR) (e.g. *hsp70.2*, *txn1*, and *gsr*). When comparing WT and KO across all treatments and times, there were 1170 genes that were differentially expressed, of which 33 are known targets of the Nrf proteins Nrf1 and Nrf2. More specifically, in animals exposed to pro-oxidants a total of 968 genes were differentially expressed between WT and KO across developmental time, representing pathways involved in coagulation, embryonic organ development, body fluid level regulation, erythrocyte differentiation, and oxidation-reduction, amongst others. The greatest number of genes that changed in expression between WT and KO occurred in animals exposed to diquat at 2 h post fertilization (hpf). Across time and treatment, there were six genes (*dhx40*, *cfap70*, *dnajb9b*, *slc35f4*, *spi-c*, and *gpr19*) that

Abbreviations: ARE, antioxidant response element; bp, base pair; bZIP, basic leucine zipper; ChIP, chromatin immunoprecipitation; CNC, Cap'n'Collar (CNC); hpe, hours post exposure; hpf, hours post fertilization; KO, knockout; NFE2, Nuclear Factor (Erythroid-Derived 2); NRF1, Nuclear Factor (Erythroid-Derived 2)-like 1; NRF2, Nuclear Factor (Erythroid-Derived 2)-like 2; NRF3, Nuclear Factor (Erythroid-Derived 2)-like 3; OSR, oxidative stress response; PBS, phosphate buffered saline; ROS, reactive oxygen species; tBOOH, *tert*-butylhydroperoxide; tBHQ, *tert*-butylhydroquinone; tRNA-IPT, RNA-isopentenyltransferase; TSS, transcriptional start site; WT, wildtype.

* Corresponding author at: 44 Campus Avenue, Lewiston, ME 04240, USA.
E-mail addresses: lwillia2@bates.edu (L.M. Williams), lagoba@mcmaster.ca (B.A. Lago), mcarthua@mcmaster.ca (A.G. McArthur), raphenar@mcmaster.ca (A.R. Raphenya), pray.nicholas@gmail.com (N. Pray), nabilsaleem@gmail.com (N. Saleem), sophia.salas2@gmail.com (S. Salas), krpaulson@gmail.com (K. Paulson), rmangar@coa.edu (R.S. Mangar), yangliu0302@gmail.com (Y. Liu), Andy.Vo@northwestern.edu (A.H. Vo), jshavit@umich.edu (J.A. Shavit).

http://dx.doi.org/10.1016/j.aquatox.2016.09.019
0166-445X/

were significantly up-regulated in KO compared to WT and four genes (*fhad1*, *cyp4v7*, *nlrp12*, and *slc16a6a*) that were significantly down-regulated. None of these genes have been previously identified as targets of Nfe2 or the Nrf family. These results demonstrate that the zebrafish Nfe2 may be a regulator of both primitive erythropoiesis and the OSR during development.

1. Introduction

Development is a multifaceted process that depends on the delicate balance and timing of cellular proliferation, differentiation, and apoptosis. Reactive Oxygen Species (ROS), produced endogenously via respiration and oxygenating enzymes, play an important role in normal development by functioning as messengers in cell signal transduction and differentiation (Hitchler and Domann, 2007). In excess, ROS create a disruption of redox signaling and control (Jones, 2006), and consequently may damage lipids as well as proteins (Livingstone, 2001; Valavanidis et al., 2006), leading to premature cell cycle arrest or differentiation (Li et al., 2007; Smith et al., 2000).

To combat oxidative stress, organisms have both basal antioxidant molecules (Mandal et al., 2009) and inducible antioxidant proteins (Hu et al., 2006; Mathers et al., 2004; McMahon et al., 2001; Nair et al., 2007; Rangasamy et al., 2004; Timme-Laragy et al., 2013). Despite the fact that embryos create and need ROS for cellular signaling, they have a reduced although ontogenetically dynamic antioxidant capacity as compared to adults (Juchau, 2003; Timme-Laragy et al., 2013; Wells and Winn, 1996). This leaves embryos susceptible to toxicity associated with increases in oxidative stress, whether that arises from endogenous or exogenous sources. The essential mediators of the basal and inducible antioxidant response are known as the oxidative stress response (OSR). The OSR has been identified in adult vertebrates and cells in culture, including the NRF (Nfe2-related factor) signaling pathway (Kensler et al., 2007; Lee et al., 2005; Motohashi and Yamamoto, 2004; Osburn et al., 2006b), but only recently has embryonic sensitivity to chemically-induced oxidative stress and the role of NRF signaling become known.

The inducible response to ROS, electrophiles and pro-oxidant chemicals proceeds through a family of Cap'n'collar (CNC) basic leucine zipper (bZIP) transcription factors (Andrews et al., 1993). In mammals, there are four NFE2-related CNC-bZIP proteins: Nuclear Factor Erythroid, 2 (NFE2), Nuclear Factor Erythroid, 2 like 1 (NRF1), Nuclear Factor Erythroid, 2 like 2 (NRF2), and Nuclear Factor Erythroid, 2 like 3 (NRF3) (Motohashi et al., 2002) and two distantly related proteins BACH1 and BACH2 (Oyake et al., 1996). All vertebrate CNC members regulate transcription by binding to MAF recognition elements, also known as Antioxidant Response Elements (ARE), upon heterodimerizing with one of three small MAF proteins. (Motohashi et al., 2002, 1997). NRF2 is the most well-characterized of the family, especially as it relates to the OSR (Kensler et al., 2007; Nguyen et al., 2003). The OSR is not solely regulated by NRF2; distinct and non-overlapping functions of the various NRFs during the response have been identified (Motohashi et al., 2010; Ohtsuji et al., 2008), despite sharing binding affinity to the same *cis*-acting ARE motifs.

Antioxidant defenses of fish (Di Giulio et al., 1989; Hahn et al., 2014; Kelly et al., 1998; Timme-Laragy et al., 2013, 2012; Valavanidis et al., 2006; Williams et al., 2013; Winston and Di Giulio, 1991) include enzyme systems and low molecular weight antioxidants are similar to those found in mammals (George, 1994; Richard and Joel, 2008; Stegeman et al., 1992; Timme-Laragy et al., 2013). However, as a result of fish-specific whole genome duplication (Amores et al., 1998; Postlethwait et al., 2004; Taylor et al., 2001), there are several OSR genes in zebrafish that are co-orthologous to mammalian genes that exist as paralogs in fish. Thus, there are six *nrf* family genes: *nfe2*, *nrf1a*, *nrf1b*, *nrf2a*, *nrf2b*, and *nrf3*, all of which all share strong relationships with *NRF* orthologs in vertebrates (Timme-Laragy et al., 2012). Unlike *nrf1* and *nrf2* which are found as paralogs in zebrafish, *nfe2* is found as a single ortholog, with little known about its function, although its expression has been documented (Pratt et al., 2002). *nfe2* is expressed throughout development, with the highest concentration of transcript found in the unfertilized egg (Williams et al., 2013). Spatially, it is concentrated in erythroid cells from 10 somites (~12hpf) to 36 hpf and in the developing ear at 48 hpf (Pratt et al., 2002). Given its sequence similarity to human NFE2, spatial expression, and lack of expression in *cloche* mutants, it has been hypothesized that Nfe2 function is similar to its mammalian ortholog and is involved in hematopoiesis (Pratt et al., 2002). Phenotypic outcomes of transient Nfe2 knockdown in zebrafish and knockout in mice have provided some insight into the potential molecular targets of Nfe2.

In the mouse model, *Nfe2* null mice lack circulating platelets due to a late block in megakaryocyte maturation, and most die of hemorrhage in the neonatal period (Shivdasani et al., 1995). Further examination of megakaryocytes from null embryonic mice indicate the novelty of NFE2 in regulating ROS signaling, a crucial step in the maturation of these cells. NFE2 competes with NRF2 to regulate cytoprotective genes such as heme oxygenase 1(*Ho-1*) and NADP(H):quinine oxidoreductase (*Nqo1*) (Motohashi et al., 2010). However, since the *Nfe2* knockout is neonatally lethal in most mice, the role of NFE2 could be examined only in the few surviving adults from these litters. In mice that survive the knockout, it has been found that NFE2 is involved in the production of proplatelets (Lecine et al., 1998). Using the zebrafish model and transient morpholino knockdown of Nfe2, additional biological roles of Nfe2 have been elucidated including roles in swimbladder inflation and otic vesicle formation (Williams et al., 2013). However, the role of Nfe2 in responding to and regulating the OSR during development has not been explored in zebrafish.

In this study, we used a zebrafish *nfe2* knockout model, which is not developmentally lethal, to examine the role of Nfe2 in regulating the response to oxidative stress. Zebrafish at three distinct developmental periods (blastula/gastrula, hatching, and larval) were acutely exposed to two model pro-oxidants: diquat (Sandy et al., 1987; Stancliffe and Pirie, 1971) and *tert*-butylhydroperoxide (Ahmed-Choudhury et al., 1998). Following the waterborne exposure, phenotypic outcomes were compared between wildtype and *nfe2* knockout fish. In addition, transcriptome analyses were completed to identify differential expression between treatment groups and strains to ascertain the transcriptional regulatory role of Nfe2.

2. Methods

2.1. Chemicals

Diquat dibromide monohydrate was purchased from Sigma-Alrich (St. Louis, MO, USA), and freshly dissolved in 0.3X Danieau's. Luperox® TBH70X *tert*-butylhydroperoxide (tBOOH) solution was purchased from Sigma-Aldrich (St. Louis, MO, USA) and freshly