Comparative Biochemistry and Physiology, Part A 201 (2016) 156–161

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

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

Acute hyperthermic responses of heat shock protein and estrogen receptor mRNAs in rainbow trout hepatocytes

Yudong Jia [a,b], Timothy D. Cavileer [a], James J. Nagler [a,*]

[a] Department of Biological Sciences and Center for Reproductive Biology, University of Idaho, Moscow, ID, USA
[b] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

ARTICLE INFO

Article history:
Received 9 October 2015
Received in revised form 15 April 2016
Accepted 22 April 2016
Available online 13 July 2016

Keywords:
Heat shock protein
Estrogen receptor
Vitellogenin
Hepatocyte
Fish

ABSTRACT

Heat shock proteins (HSPs) are induced upon elevated temperature in fishes. HSPs also function as molecular chaperones for cellular proteins, including steroid hormone receptors. Estrogen receptors (ERs) are critical for the hormone signaling necessary during the liver production of the yolk precursor protein vitellogenin in oviparous vertebrates. Considering the possible regulatory role of HSPs on the ER signaling pathway, the present study characterized the mRNA expression of all known isoforms of HSP70 (*hsp70a*, *hsp70b*), HSP90 (*hsp90a1a*, *hsp90a1b*, *hsp90a2a*, *hsp90a2b*, *hsp90b1*, *hsp90b2*), and ERs (*erα1*, *erα2*, *erβ1*, *erβ2*) in Rainbow Trout hepatocytes following an acute heat shock (1 h at 25 °C) compared to a control treatment (12 °C). The results showed that the mRNA levels of *hsp70a*, *hsp70b*, *hsp90a1b*, *hsp90a2a*, and *hsp90b2* were significantly increased after heat shock, while *erα1* mRNA levels were significantly reduced by this treatment. *hsp90a1a*, *hsp90a2b*, *hsp90b1*, *erα2*, *erβ1* and *erβ2* were unaffected by this acute hyperthermic treatment. Comparatively, the responses of the two *hsp70* isoforms were much greater than the *hsp90* isoforms. Acute heat shock treatment of hepatocytes followed by a 24 h exposure to 17β-estradiol (E2) exposure also resulted in decreased expression of *erα1* mRNA, but not vitellogenin (*vtg*) mRNA. This study showed that some *hsp70* and *hsp90* isoforms display a robust response to an acute hyperthermic treatment in Rainbow Trout hepatocytes. Among the transcripts measured here, the *erα1* isoform uniquely showed significantly decreased mRNA levels upon acute heat treatment.

1. Introduction

Heat shock proteins (HSPs) have been demonstrated to be induced in all organisms by a variety of stressors such as heat, osmotic shock, heavy metals, starvation or oxidative stress (Kregel, 2002; Basu et al., 2002; Cara et al., 2005; Roberts et al., 2010). Based on their molecular weights, HSPs are traditionally classified into several families and each family plays various roles at the cellular level. The high molecular weight HSPs, HSP70 and HSP90, are particularly well known for their interaction with other intracellular proteins that are involved in many cell functions (Ellis, 1987; Welch, 1993; Hartl, 1996; Wegele et al., 2004; Pratt et al., 2010; Richter et al., 2010; Mashaghi et al., 2014). It has emerged through DNA sequencing efforts that most animals have multiple HSP70 and HSP90 isoforms (Gupta, 1995; Kregel, 2002; Daugaard et al., 2007). This is very complex in fishes, due to a past genome duplication event in this lineage that has resulted in many HSP isoforms (Iwama et al., 1998, 1999; Manchado et al., 2008; Deane and Woo, 2011; Garcia de la serrana and Johnston, 2013). In most cases it is not known which isoforms are stress responsive. Additionally, amongst HSPs that demonstrate a stress response there are differences in the magnitude of those responses. This has been shown by the work of Boone and Vijayan (2002) and Ojima et al. (2005) that found differences in HSP70 mRNA isoform expression after heat shock in Rainbow Trout hepatocytes and RTG-2 cells, respectively. Because of the numerous HSP70 and HSP90 isoforms existing within a species it becomes important to know which exhibit a stress response in order to interpret how HSP induction might affect their cellular function.

HSPs are well known to be necessary for the normal functioning of steroid hormone receptors (Picard, 2006). The HSP90 family work as chaperone proteins for steroid hormone receptors (Chambraud et al., 1990; Pratt and Toft, 1997) and the HSP70 family is necessary for translocation and protein folding (Gething and Sambrook, 1992; Kohno et al., 2010). Estrogen receptors (ERs) are a steroid hormone group that have interactions with HSPs (Cheung and Smith, 2000; Pratt and Toft, 1997, 2003; Pratt et al., 2004). Briefly, unliganded ERs are assembled into hetero-complexes with HSP90, HSP70, and other proteins by a multi-protein chaperone machinery. The ligand binding of ERs cause the dissociation of the receptor from these hetero-complexes, allowing the receptor-ligand complex to bind estrogen response elements, which can trigger an ER-mediated signal cascade reaction (Pratt et al., 2004). By forming hetero-complexes with HSPs, the chaperone machinery stabilizes the ER for proper folding, trafficking, nuclear cycling and

* Corresponding author at: Department of Biological Sciences, University of Idaho, 875 Perimeter MS3051, Moscow, ID 83844-3051, USA.
E-mail address: jamesn@uidaho.edu (J.J. Nagler).

http://dx.doi.org/10.1016/j.cbpa.2016.04.023
1095-6433/

turnover. Estrogens are important endocrine hormones and involved in the regulation of numerous physiological processes. The free steroid binds to ERs in order to initiate the transcription of estrogen responsive genes (Berg et al., 2004; Menuet et al., 2004; Guiguen et al., 2010; Hao et al., 2013; Bondesson et al., 2015). In most vertebrates, the ER family consists of two ERs, ERα and ERβ, (Eick and Thornton, 2011). However, in fishes due to the genome duplication described above there are additional ER isoforms similar to the situation for HSPs. How these numerous HSP and ER isoforms interact within the cells of fishes is not known.

Fishes, as aquatic ectotherms, typically experience variable water temperatures throughout their lives. Temperature has a modifying role, particularly in cueing the precise timing of gamete maturation and spawning, providing the capacity for reproductive cycles to be locally tuned to shorter term and less predictable variations in thermal condition (Pankhurst and Porter, 2003). Increasing temperatures will at some level have adverse effects on reproduction in fishes (Van Der Kraak and Pankhurst, 1997). Studies on salmonids indicated that elevated temperature retards final oocyte maturation and ovulation, and impairs reproductive success (Pankhurst and Thomas, 1998; Pankhurst et al., 1996; King et al., 2007; Pankhurst and King, 2010). Most fishes exhibit a robust heat shock response by increasing the transcription of HSP mRNA and protein (Dietz and Somero, 1993; Palmisano et al., 1999; Boone and Vijayan, 2002; Yamashita et al., 2004; Ojima et al., 2005; Manchado et al., 2008). Induction of HSPs is presumably adaptive but few physiological responses have been investigated in detail and a direct connection between heat responsive proteins and reproduction has yet to be made.

It has been reported that elevated temperature affects reproduction in Rainbow Trout (*Oncorhynchus mykiss*) by impairing estrogen synthesis (Pankhurst and King, 2010). One mechanism by which temperature could impact estrogen function would be through the role HSPs have as molecular chaperones of ERs. The goal of this study was to measure the mRNA expression patterns of all known *hsp70*, *hsp90*, and *er* isoforms in Rainbow Trout (*Oncorhynchus mykiss*) hepatocytes following an acute hyperthermic shock to identify which isoforms are temperature responsive. In addition, an experiment was conducted to examine the effect of elevated temperature of the induction pathway of an estrogen-responsive gene, vitellogenin (*vtg*). This information will be important to guide future studies to establish the validity of the mechanism described above.

2. Materials and methods

2.1. Animals

Mixed sexes of juvenile rainbow trout (*Oncorhynchus mykiss*), body weight = 117 ± 8.5 g, were obtained from the University of Idaho Aquaculture Research Institute Moscow Facility. Fish were maintained in re-circulating tanks with a photoperiod of 12 h Light: 12 h Dark, a water temperature of 12 °C, and were fed a commercial fish food (Rangen, Inc., Buhl, ID). All live fish procedures were conducted according to the guidelines established by the Institutional Animal Care and Use Committee at the University of Idaho.

2.2. Hepatocyte isolation and treatment

Fish were killed by an overdose of buffered tricaine methanesulfonate (200 mg/L). Hepatocytes were isolated following the perfusion and dissociation protocols of Mommsen et al. (1994). The cell suspension was seeded in Primaria 24-well culture plates (Corning) at a density of 5.0×10^5/mL in 0.5 mL modified RPMI-1640 medium (Sigma-Aldrich), containing 50 mg/mL gentimicin and 2.5 mg/mL fungizone, pH 7.6. Cells were plated for 24 h at 12 °C prior to treatment to allow settling and attachment. The heat shock was accomplished by placing the cells at 25 °C for 1 h. The ultimate incipient lethal temperature for Rainbow Trout is 26.2 °C (Kaya, 1978). Hepatocyte viability following the one-hour heat shock treatment was determined using the CytoSelectTM Cell Viability and Cytoxicity Assay Kit (Cell Biolabs Inc.). In one experiment, immediately after the heat shock treatment, the media was replaced with media containing 17β-estradiol (E2) (2 μg/mL in RPMI-1640) and incubated at 12 °C for an additional 24 h was conducted. This was performed to examine the effect of HSP induction before estrogen stimulation. Triplicate wells were used for each treatment within an experiment which utilized a single fish. Experiments were replicated using a total of three fish. At the end of all experiments, media was removed from the wells and 350 μL of TRIzol Reagent (Invitrogen, CA, USA) was added. Hepatocytes were stored frozen at −80 °C until used for total RNA extraction.

2.3. RNA isolation and PCR

Total RNA was extracted from freshly thawed hepatocytes using TRIzol. The quantity of the total RNA was determined using the Quant-iT RiboGreen RNA Reagent Kit (Invitrogen) and Modulus Luminometer (Turner Biosystems) as described in Nagler et al. (2012). Total RNA (1 μg) was DNase treated using the TURBO DNA-free Kit (Life Technology). Because an endogenous gene for PCR normalization could not be found that consistently amplified in hepatocyte samples a transcribed RNA (cRNA) internal standard was used. Enhanced green fluorescent protein (*eGFP*) cRNA generated by in vitro transcription was added at this step to account for amplification differences between samples as described in Nagler et al. (2007). The RNA mixture was then reverse transcribed via Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). The resulting cDNA was diluted (1:5) with sterile deionized water and stored at −20 °C until used in real time polymerase chain reaction PCR (RT-PCR) using an ABI 7900HT thermocycler (Applied Biosystems).

The mRNAs for HSP isoforms (*hsp70a*, *hsp70b*, *hsp90a1a*, *hsp90a1b*, *hsp90a2a*, *hsp90a2b*, *hsp90b1*, *hsp90b2*), ER isoforms (*erα1*, *erα2*, *erβ1*, *erβ2*), vitellogenin (*vtg*), and *eGFP* were quantified by RT-PCR. Briefly, a final volume of 20 μL, which contained 10 μL SYBR Green PCR mix, 6.0 μL sterile distilled water, 2.0 μL cDNA template, and 1.0 μL each of forward and reverse primers at 10 pmol was used for amplification. A no-primer control treatment for each template used 2.0 μL TE in the reaction mixture instead of primers. The thermal program used was 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Disassociation curves for each sample were analyzed on all plates. The relative mRNA expression was normalized to the control levels at 12 °C in all experiments. The primers for the *hsp70* and *er* isoforms were obtained from Ojima et al. (2005) and Nagler et al. (2012), respectively. The *hsp90* isoform primers were designed based upon Rainbow Trout cDNA sequences reported in NCBI that were located using the Atlantic Salmon HSP DNA sequences reported in Garcia de la serrana and Johnston (2013). The *vtg* primers were designed based on the Rainbow Trout DNA sequence (Accession no. X92804) in GenBank. The *eGFP* primers were derived from Nagler et al. (2007). All primer sequences and their PCR product lengths are listed in Table 1.

2.4. DNA sequencing

Heat shock protein 90 amplicons from completed reactions (i.e., *hsp90a1a*, *hsp90a1b*, *hsp90a2a*, *hsp90a2b*, *hsp90b1*, *hsp90b*) were run on a 3% high resolution buffered agarose gel with a 50 bp ladder and visualized with ethidium bromide (1XTAE buffer at 90 V for 1 h). Individual fragments were excised and eluted separately through a 200 μL filtered Rainin pipet tip (Mettler Toledo, Oakland, CA) at 4000 rpm for 4 min in a benchtop microcentrifuge. The elute was directly used in BigDye v3.1 (Thermo Scientific, Waltham, MA) sequencing reactions according to the manufacturer's protocol. Sequencing reactions were run on an ABI-3730 instrument at the Molecular Biology and Genomics Core (Washington State University,