Potential osmoprotective roles of branchial heat shock proteins towards Na⁺, K⁺-ATPase in milkfish (Chanos chanos) exposed to hypotonic stress

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In euryhaline teleosts, osmoregulatory mechanisms vary with osmotic stresses, and heat shock proteins (HSPs) play a central role in maintaining cellular homeostasis. The present study aimed to investigate the expression and potential roles of HSP70 and HSP90 in the gills of seawater (SW)- and freshwater (FW)-acclimated milkfish (Chanos chanos). Four HSP genes, including Cchsp70 (heat shock cognate 70), Cchsp70, Cchsp90α, and Cchsp90β, were analyzed in milkfish gills. Among these genes, only the mRNA abundance of branchial Cchsp90α was significantly lower in the SW-acclimated than in the FW-acclimated milkfish. Immunoblotting showed no significant difference in the relative protein abundance of branchial HSP70 and HSP90 between the two groups.

The time-course experiments (from SW to FW) showed that the protein abundance of HSP70 and HSP90 at the 3 h and 6 h post-transfer and then declined gradually. To further illustrate the potential osmoprotective roles of HSP70 and HSP90, their interaction with Na⁺, K⁺-ATPase (NKA, the primary driving force for osmoregulation) was analyzed using co-immunoprecipitation. The results showed the interaction between HSP70, HSP90 and NKA after acclimation to SW or FW increased within 3 h; and then returned to normal levels within 7 days.

To our knowledge, the present study was the first to demonstrate that the interaction between HSP70, HSP90 and NKA changes with hypotonic stress in euryhaline teleosts. Before the transfer, no interaction was detected. When transferred to FW from SW, the interaction of HSP70 and HSP90 with NKA were detected.

Besides, some compensatory mechanisms might allow an individual to deal with environmental stresses by producing various metabolic or structural components that could maintain basic cellular functions (Palmisano et al., 2000). Among these mechanisms, stress proteins play a central role in maintaining cellular homeostasis and, thus, minimize acute stress damage (Welch, 1993). Moreover, previous studies have demonstrated that stressors induce the production of chaperone proteins, especially heat shock proteins (HSPs), which are involved in protein biogenesis and prevent protein misfolding (Roberts et al., 2010).

1. Introduction

Living organisms interact with their environments throughout their life spans. Some unfavorable environments containing biotic or abiotic stressors threaten or disturb the dynamic equilibrium (homeostasis) of individuals (Wendelaar Bonga, 1997). Environmental stresses are caused by a combination of abiotic factors, such as salinity, temperature extremes, pollutants, and anoxia, and biotic factors, such as parasitism and predation (Fishelson et al., 2001; Iwama et al., 2004; Padmini and Rani, 2009; Roberts et al., 2010; Taleb et al., 2008; Tine et al., 2010).

Abbreviations: BW, brackish water; Cchsp/Chsp, Chanos chanos heat shock protein; EF-1α/ef-1α, elongation factor-1α; FW, fresh water; HSC, heat shock cognate; HSE, heat shock element; HSF, heat shock transcription factor; NKA, Na⁺ K⁺-ATPase; S.E.M., standard error of the mean; SW, seawater.

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The HSP family is an important group of chaperone proteins that were originally identified as proteins whose expression was induced by heat stress (Basu et al., 2002; Poumpoung et al., 2014; Roberts et al., 2010). An environmental stressor could disrupt the three-dimensional structure of a protein (Ellis and Minton, 2006; Goldberg, 2003); thus, HSPs are indispensable for maintaining normal cell function under stressful conditions. They are highly conserved in diverse organisms (Iwama et al., 1998, 1999; Lindquist and Craig, 1988; Metzger et al., 2016; Srivastava, 2002). Moreover, some HSP family members, which play a role in various aspects of protein metabolism, are expressed under normal conditions (Poumpoung, 2014) to maintain cellular integrity (Iwama et al., 1998). Previous studies have demonstrated that the HSP family proteins are rapidly induced by various stressors and exhibit cytoprotective functions (Ali et al., 1996; Gething and Sambrook, 1992; Hartl et al., 2011; Hightower, 1991). So far, HSPs have been classified into several distinct groups according to their molecular weights, amino acid sequences, and functions (Freeman and Morimoto, 1996; Lindquist, 1986). Among them, there are three major HSP families: HSP90 (85–90 kDa), HSP70 (68–73 kDa), and HSP60 (58–62 kDa) (Buchner, 1996). HSP90 proteins include hsp90α (i.e., Hsp90αA or inducible form) and hsp90β (i.e., Hsp90AB or constitutive form) have been identified in vertebrates. Both of them are majorly cytosolic HSP90 and are important chaperone proteins that suppress intracellular aggregation in general (Buchner, 1999). In the cellular context, hsp90α emerges as a fast-response isoform, while hsp90β seems to be associated with long-term cellular adaptation and is more specifically responsible for germl cell maturation, cytoskeletal stabilization, cellular transformation and signal transduction (Sreedhar et al., 2004). Furthermore, HSP90s can influence the function of glucocorticoid receptor to regulate the transcription induced by a steroid hormone (Iwama and Didier, 2007). On the other hand, the HSP70 family which is the primary group of HSPs is composed of both environmentally induced (HSP70) and constitutively expressed members (HSC70). By consuming ATP, HSP70 tightly binds to hydrophobic amino acids in order to prevent protein aggregation that renders proteins non-functional (Mashaghi et al., 2016). When facing stressful situation, HSP70 is highly induced from low basal levels, with transcriptional regulation via heat shock factor 1 (Hsf 1) (Deane and Woo, 2005; Westwood et al., 1991), while HSC70 is often considered to be part of constitutive cell functions in “non-stress” situation (Yeh and Hsu, 2002; Yamashita et al., 2004; López-Maury et al., 2008). HSP60 is a mitochondrial chaperone responsible for protein refolding and transportation from the cytoplasm into the mitochondrial matrix (Koll et al., 1992). Among these HSPs, HSP70 and HSP90 have been shown to be an integral part of the cellular stress response pathways in several fishes (Ali et al., 2003; Basu et al., 2002; Boone and Vijayan, 2002; Deane and Woo, 2006, 2011; Jesus et al., 2013; Liu et al., 2012; Molina et al., 2000; Padmini and Rani, 2008; Padmini and Tharani, 2015; Wang et al., 2014; Wu et al., 2013; Zhang et al., 2014).

Fish are aquatic organisms that dwell in various habitats and undergo long-term exposure to various stressors (Bartoun, 2002). To acclimate to external environments, it is important for fish to maintain their internal homeostasis. An exposure to environmental stressors might reestablish the system of behavior, physiology, ecology, and even evolution in fishes (Iwama et al., 1999; Padmini, 2010; Sørensen and Loeschcke, 2007). Among teleosts, approximately 5% are euryhaline and can survive in fresh water (FW), brackish water (BW), and seawater (SW). These groups of fishes are commonly found in habitats, such as estuaries and tide pools, where salinity usually changes dramatically (Evans et al., 2005), making euryhaline fishes an excellent model to study osmotic stress in vivo.

In euryhaline teleosts, Na+, K+-ATPase (NKA) is an indispensable protein that plays an important role in osmoregulation. It provides an ion gradient to promote the activity of other transporters in gill ionocytes by consuming energy (Hwang et al., 2011; Lee et al., 2000; Yang et al., 2019). In the mammalian kidney, NKA plays similar roles as in teleost gills (Gagnon et al., 1999). Furthermore, it was demonstrated that under renal injury, the regulated HSP70 was bound to NKA in porcine kidney epithelial cell line LLC-PK1 (Riordan et al., 2005), and the interaction between HSP70 and NKA increased in rats recovering from a low-protein diet (Ruje et al., 2008). These studies indicated that when mammals were exposed to nutritional stress, HSP70 was activated to maintain the function of NKA. However, it is unknown whether HSPs interact with NKA in euryhaline teleosts, and if milkfish respond to osmotic stress through an NKA-HSP interaction to avoid unfolding. The roles of HSP70 and HSP90 in the osmoregulatory processes of milkfish also remain undetermined. In this study, we aimed to (1) investigate the mRNA and protein expression of branchial HSP70 and HSP90; (2) demonstrate the interaction between HSP70 or HSP90 and NKA by Co-immunoprecipitation (Co-IP) to identify their potential osmoprotective roles in the osmoregulatory mechanisms of euryhaline milkfish reared in SW and FW.

2. Material and methods

2.1. Experimental fish and environments

Juvenile milkfish (C. chanos) with a weight of 20.0 ± 7.0 g and a standard length of 13.5 ± 1.3 cm were obtained from a local fish farm (Tainan, Taiwan). After acclimation to BW (15%) prepared from dechlorinated local tap water (FW) with proper amounts of Blue Treasure Sea Salts (New South Wales, Australia) for at least 2 weeks, the milkfish were transferred directly to either FW or SW (35%) with 28 ± 1 °C for at least 4 weeks before sampling for the long-term FW and SW acclimation groups and performing time-course FW-transfer experiments. For the acclimation experiments, six individuals in each group were used for the following analyses. The water was continuously circulated through fabric-floss filters that were partially and periodically re-freshed. The photoperiod cycle was 12 h of light and 12 h of darkness (Chang et al., 2016a, 2016b; Hu et al., 2017; Kang et al., 2015). The fish was fed twice a day (the first diet was before 11 a.m. and the second diet was after 4 p.m.) with diets of commercial pellets. Before sacrifice, the fish were fasted for one day to avoid the effect of electrolytes in the feed. All experiments were conducted according to the principle and procedures approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 102–114 to T.H. Lee).

In the time-course experiment, the SW-acclimated milkfish were transferred directly to FW (study group; n = 5 to each point) or SW (control group; n = 3 to each point) for the induction of abrupt osmotic stress. The gills were sampled at 0, 3, 6, 24, and 168 h post-transfer, immediately immersed into liquid nitrogen, and then stored at −80 °C for subsequent analyses.

2.2. RNA extraction and reverse transcription

The methods used in this study were modified from our previous study (Hu et al., 2017). The total RNA was extracted using the TriPure Isolation Reagents (#11667165001; Roche, Mannheim, Germany) and homogenized by POLYTRON (PT1200E; Kinematica, Lucerne, Switzerland) at maximal speed (14 m/s) for 10 s per stroke. After adding 1-μl bromo-3-chloropropane [BCP; 1:5 (v/v) with 28 ± 1 °C for at least 4 weeks before sampling for the long-term FW and SW acclimation groups and performing time-course FW-transfer experiments. For the acclimation experiments, six individuals in each group were used for the following analyses. The water was continuously circulated through fabric-floss filters that were partially and periodically re-freshed. The photoperiod cycle was 12 h of light and 12 h of darkness (Chang et al., 2016a, 2016b; Hu et al., 2017; Kang et al., 2015). The fish was fed twice a day (the first diet was before 11 a.m. and the second diet was after 4 p.m.) with diets of commercial pellets. Before sacrifice, the fish were fasted for one day to avoid the effect of electrolytes in the feed. All experiments were conducted according to the principle and procedures approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University (IACUC approval no. 102–114 to T.H. Lee).

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Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′ to 3′)</th>
<th>Amplicon size (bp)</th>
<th>Gene sequence reference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cchsp70</td>
<td>F: AGGTCGACTAAGGGACTACA</td>
<td>149</td>
<td>KX601162</td>
</tr>
<tr>
<td>(Ei: 0.99)</td>
<td>R: TGCAATGGTCATCTGATGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cchsp70</td>
<td>F: GAGTCGACCTGGCTGAAAGGG</td>
<td>92</td>
<td>KX601163</td>
</tr>
<tr>
<td>(Ei: 1.01)</td>
<td>R: GAAAATTCACACTGGACGCTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cchsp90a</td>
<td>F: GCGAGGGAAGGGACTGACCA</td>
<td>96</td>
<td>KX601164</td>
</tr>
<tr>
<td>(Ei: 1.02)</td>
<td>R: GGCAAGTACAGCAGTCACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cchsp90b</td>
<td>F: TCCGATGGAGAAATGGATT</td>
<td>112</td>
<td>KX601165</td>
</tr>
<tr>
<td>(Ei: 0.99)</td>
<td>R: GAAGAACCTAGACCGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ef-1α</td>
<td>F: TGTCAAGGACATCGCGGTCG</td>
<td>102</td>
<td>MK817356</td>
</tr>
<tr>
<td>(Ei: 1.00)</td>
<td>R: GGGTTGTCAGGATGACTAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†, the accession numbers of target gene sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

2.3. Quantitative PCR

The methods used in this study were as previously described (Hu et al., 2017; Yang et al., 2019). The expression levels of the Cchsp genes were quantified by using the MiniOpticon Real-Time PCR System (Bio-Rad). The PCR amplification was carried out in a 20 μL reaction volume comprising 10 μL of 2× KAPA SYBR®FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA), 8 μL of sterilized DDW (i.e., distilled and deionized water), 1 μL of cDNA, and 0.2 μM of each primer pair specific to the studied Cchsp genes. The ef-1α (elongation factor-1α) gene was used as the housekeeping gene to normalize the expression of Cchsp genes (Hu et al., 2015, 2017). The sequence of Cchsp genes were obtained from the transcriptome database of milkfish (Hu et al., 2015). The primer pairs were designed using Perl Primer (Geeknet, Fairfox, VA, USA) and the amplicons were sequenced to check the correctness of predictive targeting sequences. The information of primers is shown in Table 1, and the efficiency of amplification ranged between 99% and 102%. To quantify the expression of the studied Cchsp genes, the comparative Ct method with the eq. 2^(-ΔΔCt) was used to obtain the corresponding values of the target genes, where Ct corresponded to the threshold cycle number (Livak and Schmittgen, 2001).

2.4. Antibodies

The primary antibodies used in the present study included: (1) anti-HSP70: a mouse monoclonal antibody (HS147; Sigma, St. Louis, MO, USA; diluted 1:10000 for immunoblotting) generated by immunization with purified bovine brain HSP70 and BRM-22; (2) anti-HSP90: a rabbit polyclonal antibody (#4874; Cell Signaling Technology, Beverly, MA, USA; diluted 1:4000 for immunoblotting) corresponding to human HSP90α; (3) anti-β-actin: a mouse monoclonal antibody (MAB1501; Millipore, Bedford, MA, USA; diluted 1:5000 for immunoblotting) used as a loading control; (4) anti-NKA α-subunit: a mouse monoclonal antibody (a5: Development Studies Hybridoma Bank, Iowa City, IA, USA; diluted 1:2000 for immunoblotting) raised against the α subunit of avian NKA. The secondary antibodies for immunoblotting analyses were horseradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (Chemicon, Temecula, CA, USA).

2.5. Gill homogenates

The first pair of milkfish gills were excised and immediately immersed into liquid nitrogen. The tissues were rapidly homogenized in 500 μL SEID buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.4) and protease inhibitor (PIR-100, Roche, Mannheim, Germany). Homogenization was performed with a POLYTRON PT1200E (Kinematica) at maximum speed for 10 s. The homogenates were then centrifuged at 5500 ×g, 4 °C for 20 min. Subsequently, the supernatants were collected and used for the determination of protein concentrations. The BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to measure the protein concentrations with bovine serum albumin (BSA) as the standard.

2.6. Immunoblotting

The procedures of immunoblotting were performed as described by Lee et al. (2000) with some modification. Briefly, the protein lysate mixture was heated with SDS sample buffer (v/v: 1/5) to denature the proteins at 95 °C for 5 min for HSP70 and HSP90 or at 37 °C for 30 min for NKA and then separated by electrophoresis on 7.7% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (15 μg protein per lane) on a Mini-Protein II Electrophoresis cell (Bio-Rad). The pre-stained protein ladder was purchased from Thermo Scientific (#26616). The separated proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) using a transfer system (Bio-Rad Mini Protein 3) by electroblotting at 100 V for 60 min. The blots were pre-incubated with 5% (w/v) non-fat dried milk in PBST buffer (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, 0.2% (v/v) Tween 20, pH 7.4) at room temperature for 2 h to minimize non-specific binding and then incubated with the primary antibodies diluted in 1% BSA and 0.05 sodium azide in PBST overnight at 4 °C. After washing several times with PBST, the membranes were incubated with diluted secondary antibodies (120000) at room temperature for 1 h. The signals of immunoproteins were detected using the enhanced chemiluminescent assay (Millipore) and a cooling-charge-coupled-device camera (ChemiDoc XRS®; Bio-Rad). For quantitative analyses, the immune-reactive bands were converted to values by using the Image Lab software version 3.0 (Bio-Rad).

2.7. Co-immunoprecipitation (co-IP)

To show the interaction between proteins, the SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.5), instead of SEID, was used to homogenize the samples (n = 1 for each group or time point). Gill lysates or PBS (phosphate-buffered saline; negative control) were used for immunoprecipitation performed with the Catch and Release® v2.0 Reversible Immunoprecipitation System (17–500; Millipore) according to the manufacturer’s manual. Gill lysates (500 μg), NKA α5 antibody (4 μg), and antibody capture affinity ligand were mixed and incubated in spin columns at 4 °C overnight. Subsequently, unbound proteins were removed by washing and spinning, and the captured complex was then eluted from the resin by 2× elution buffer, and the eluate was used for immunoblotting. To demonstrate the correctness of the immunoprecipitation of α5, the negative control (NC; IP with antibody only) was prepared by replacing the gill lysate with the same volume of PBS buffer (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). The dilution of the primary antibody for immunoblotting was 1:500 for NKA α5, 1: 2000 for anti-HSP70, and 1:1000 for anti-HSP90. The immunoreactive bands were quantified as described above. The level of protein-protein interaction was calculated using the formula HSP70/NKA or HSP90/NKA, and the numerical value of the SW sample was set at 1.0 for normalization (Chang et al., 2016a, 2016b).

2.8. Statistical analyses

The results were analyzed via SPSS 20.0 (SPSS, Chicago, IL, USA). To compare the differences between the SW and FW groups, the results were analyzed by using the Student’s t-test. For the time-course experiment, the statistical significance was determined by one-way analysis of variance (ANOVA) using the Dunnett’s test that compared the
time-course data with 0 h (SW group) as control. The equality of variance was assessed by using Leven’s test in the SPSS 20.0. The p value < .05 was set as the significance level, and the data were expressed as means ± S.E.M. (standard error of the mean).

3. Results

3.1. mRNA and protein expression of CcHSPs in seawater (SW)- and fresh water (FW)-acclimated milkfish gills

Branchial Cchsp90α was significantly decreased in the FW-acclimated milkfish (approximately 0.5-fold) compared to the SW-acclimated individuals (Fig. 1C). Meanwhile, the quantitative PCR results showed no significant difference in the expressions of Cchsc70, Cchsp70, and Cchsp90β between SW- and FW-acclimated milkfish gills (Fig. 1A, B, D). On the other hand, at the protein level, the representative immunoblots showed single bands at approximately 70 and 90 kDa for HSP70 and HSP90, respectively (Fig. 2). According to the immunoblotting results, the relative protein abundances of branchial HSP70 and HSP90 were not significantly different between SW and FW groups (Fig. 2).

3.2. Time-course changes in branchial Cchsps and CcHSPs of milkfish under hypotonic challenge

The quantitative PCR analysis of four Cchsp genes revealed the mRNA expression profiles of Cchsps in the gills of milkfish within 168 h (7 days) after transfer from SW to FW. The results showed no significant difference in the relative mRNA expression of Cchsc70 at all time points compared to 0 h (Fig. 3A). Meanwhile, the mRNA expression of Cchsp70 increased significantly at 6 and 12 h, and the expression reached the highest level at 6 h. The mRNA expression pattern of Cchsp90α was similar to Cchsp70, yet it was significantly higher only at 12 h post transfer (Fig. 3B, C). The expression levels of both Cchsp70 and Cchsp90α returned to the SW levels after FW acclimation for 24 h. However, in the case of Cchsp90β, no significant difference was found.
within 168 h after transfer (Fig. 3D). The control groups (transferred from SW to SW) of these four branchial Chsps showed no significant change in their mRNA expression levels after transfer to FW compared to their expressions at 0 h.

Similar to mRNA expression, the protein abundances of both HSP70 and HSP90 were elevated in milkfish gills after transfer to FW from SW. Branchial HSP70 showed significantly increased abundance at 3 and 6 h, followed by a decrease to the pre-transfer levels (Fig. 4C). The protein abundance of HSP90 increased after a salinity challenge and was significantly higher at 6 h compared to 0 h and then returned to the pre-transfer levels (Fig. 4D).

3.3. Interaction between HSPs and NKA α-subunit in milkfish gills under hypotonic stress

Quantification of protein levels revealed the induction of CcHSP70 and CcHSP90 expression in the gills of milkfish upon exposure to hypotonic challenge. To further verify the protective roles of branchial CcHSP70/90 to NKA under hypoopsmotic stress, the interactions of HSP70 or HSP90 with NKA α-subunit (NKA α) were examined by co-immunoprecipitation (co-IP) using the lysate of milkfish gills. These results depicted two immunoreactive bands of NKA α at approximately 100 kDa (Fig. 5A). The immunoreactive signals of HSP70 were weak and those of HSP90 were even undetected in both SW- and FW-acclimated milkfish gills (Fig. 5B, C). The bands appeared at approximately 55 kDa in the negative control, SW group and FW group indicated the heavy chain of the antibody (Fig. 5B).

Although no obvious interactive expression of HSP70 and HSP90 with NKA was detected in the long-term acclimated groups, the results of the time-course experiments were different. The analysis used to reveal the interaction between CcHSP70 or CcHSP90 with NKA was done following Chang et al. (2016a, 2016b). To obtain relative abundances of CcHSP70 or CcHSP90 binding with a certain amount of NKA α subunit, the immunoreactive signal of NKA α subunit was used as the loading control to assess the binding of CcHSP70 or CcHSP90 on NKA α subunit. In addition, all ratios of CcHSPs to NKA α subunit were normalized by 0 h. The results showed that the interaction of both CcHSP70 and CcHSP90 with branchial NKA increased in 3 h post-transfer (approximately 6.2-fold for CcHSP70 and 8.2-fold for CcHSP90). The interaction lasted until 24 h and then recovered to the state of the long-term SW- and FW-acclimated groups at 168 h (Fig. 6).

4. Discussion

Euryhaline teleosts have the capacity to maintain individual homeostasis under changing environmental salinities (Burnett et al., 2007; Choi and An, 2008; Deane et al., 2002; Deane and Woo, 2004; Fiol and Kültz, 2007; Hiroi and McCormick, 2007; Kang et al., 2010; Marshall et al., 1999; McCormick et al., 2003; Sangiao-Alvarellos et al., 2003; Tang and Lee, 2013a, 2013b; Yang et al., 2009). In SW, the internal environments of fish are hypotonic compared to the external media so that they encounter water crisis and excessive diffusion of ions towards the body. The salinity gradient is inversed in FW, which has low ionic concentrations. The hypotonic environment, thus, makes the fish lose ions and gain water mainly through the gills (Edwards and Marshall, 2013; Evans et al., 2005). To cope with extreme changes in environmental salinities, diverse mechanisms of cellular homeostasis have been observed in euryhaline teleosts (Choi and An, 2008; Deane and Woo, 2004; Deane et al., 2002; Kültz, 2015; Marshall, 2012).

Previous studies have demonstrated the importance of HSPs in the responses induced by different stressors in fishes. Few studies have considered the roles of HSPs following salinity changes in euryhaline
fish. Most salinity stress studies on HSPs in euryhaline teleosts have focused on hyperosmotic stress rather than hypoosmotic stress (Deane et al., 2002; Palmisano et al., 2000; Pan et al., 2000; Tang and Lee, 2002, 2003). Milkfish is one of the euryhaline teleosts that spend most of their life in SW (Bagarinao, 1994). In addition, previous studies on the physiology, growth, and metabolism of milkfish have demonstrated that its acclimation more stressful to FW than to SW (Ferraris et al., 1988; Hu et al., 2017; Jana et al., 2006; Kang et al., 2017; Lin et al., 2003). This study explored the protective mechanisms of HSPs in marine teleosts facing a hypotonic shock by using the euryhaline milkfish as the study model. Furthermore, the teleost gills are important osmoregulatory organs directly exposed to the external environments (Edwards and Marshall, 2013; Evans et al., 2005; Hiroi and McCormick, 2012; Hwang and Lee, 2007; Hwang et al., 2011). Therefore, the present study illustrated the significant roles of HSPs in the gills of the milkfish under hypotonic stress. To make sure that all the change was induced from external environments, dietary influence was avoid by fasted milkfish for 24 h before sampling or the transfer experiments.

Two major families of milkfish HSPs (CcHSP70 and CcHSP90) were found from the milkfish transcriptome database (Hu et al., 2015). In this study, the expression of Ccchsc70, Ccchsp70, and Ccchsp90β showed no salinity dependence in the gills of both SW- and FW-acclimated milkfish, while the expression of Ccchsp90α was significantly lower in the FW group than in the SW group. Similar findings have been reported in salmon gills, where in the hsp90 mRNA levels were higher in SW than in FW-acclimated salmon, however, the higher expressions of hsp90 in salmon were induced within 24 h after the hypertonic stress (Palmisano et al., 2000; Pan et al., 2000). The SW- and FW-acclimated milkfish were reared for at least four weeks to get physiological homeostasis of the fish. Therefore, the higher expression of Ccchsp90α might not be caused by the acute osmotic stress. The hsp90 can also influence the function of glucocorticoid receptors. After SW-acclimation, the concentration of plasma cortisol in milkfish decreased compared to the FW group (Hu et al., 2019), which implied that the higher expression of Ccchsp90α might be related to lower cortisol levels in plasma of SW milkfish. On the other hand, relative protein abundance of CcHSP70 and CcHSP90 showed no difference between SW- and FW-acclimated milkfish. According to the datasheet of the manufacturer, the HSP70 antibody used in the present study could detect both inducible and constitutive members. In contrast to the present findings in milkfish, the upregulation of HSPs with changing environmental salinities has been reported in other euryhaline teleosts (Choi and An, 2008; Deane et al., 2002; Deane and Woo, 2004; Tang and Lee, 2013a, 2013b; Tang et al., 2014). The differences in the effects of salinity on the expression of gill HSPs between milkfish and other teleosts were probably due to different periods of acclimation between different studies. In this study, milkfish were reared in SW or FW for at least 4 weeks before sampling. However, in previous studies, the periods of acclimation of experimental fish to SW/BW and FW were less than 7 days. Hence, in the present study, the time-course experiments were performed within 7 days (168 h) in order to figure out the relationship between the expression of CcHSPs and acute hypotonic challenge in milkfish.

The time-course experiments of acute salinity exposure in milkfish revealed dynamic changes in the mRNA expression of the Ccchsp genes. The HSPs might have a protective role, and their induction is transitory during or after stress conditions. The mRNA expression of both Ccchsp70 and Ccchsp90α was induced and increased rapidly within 12 h after exposure to hypotonic stress and recovered within 24 h. Lin et al. (2006) revealed that the modulation of cell homeostasis in milkfish was usually completed within approximately 24 h after transfer to FW, in agreement with the present results. However, the levels of Ccchsc70 and Ccchsp90β (the constitutive forms) were stable during the FW exposure. The hsc70 is often considered to be part of constitutive cell functions in non-stress condition such that an increased challenge may either decrease or has no effect on the expression of this gene (Yeh and Hsu, 2002; Yamashita et al., 2004; López-Maury et al., 2008). Meanwhile, hsp90β was thought to be associated with “long-term” cellular adaptation compared to hsp90α (Sreedhar et al., 2004). Expression of Ccchsp90β, similar to that of Ccchsc70, was also stable when milkfish was exposed to hypotonic stress. It seems that Ccchsp70 and Ccchsp90α were truly involved in hypotonic stress response by supporting mRNA to protein level in the gill in contrast to Ccchsc70 and Ccchsp90β.

Fig. 4. Time-course changes of the representative immunoblots and relative protein abundances of branchial HSP70 (A, C) and HSP90 (B, D) in milkfish directly transferred from seawater (SW) to fresh water (FW; study group) or to SW (control group). Values are means ± S.E.M. (N = 5 in the study group, and N = 3 in the control group). The asterisks (*) indicate significant differences between various time points and 0 h (P < .05, by student’s t-test). M, marker (kDa).
the CcHSP proteins were activated earlier than the expression of mRNA. Similarly, FXYD11 expression in gills of the Indian medaka was also reported in the time-course experiments after transfer to FW directly from SW (Chang et al., 2016a, 2016b). The current mRNA was suggested to translate immediately to meet an urgent need upon acutely hypotonic challenge. Therefore, relative protein abundance of HSP70 and HSP90 elevated without change in gene expression. However, the CcHsp70 and CcHsp90a were still needed when milkfish was exposed continuously thus the gene levels increased after the rise of proteins. Protein abundance of HSP70 and HSP90 elevated within 3 and 6 h, respectively, and then returned to the levels before stress at 24 h post-transfer. The results indicated that CcHSP70 and CcHSP90 responded to hypotonic stress in milkfish gills for the abrupt salinity stress.

To further clarify the potential roles of HSPs in hypotonic stress, the interaction between HSPs and NKA (the primary driving force for ion transporters in gill ionocytes for osmoregulation) was studied by performing co-IP experiments. Using the ATP-depleted LLC-PK1 cells as a cell injury model, Riordan et al. (2005) demonstrated that the binding of HSP70 to NKA were upregulated under renal injury. Ruete et al. (2008) also reported that the abundance of HSP70 increased to interact with NKA in rats recovering from a low-protein diet. In addition, HSP70 and HSP90 were suggested to play vital roles in refolding proteins and eliminating irregular proteins to maintain cellular homeostasis in fishes under hypoosmotic shock (Deane and Woo, 2011; Tang et al., 2014; Valkova and Kültz, 2006). Since the subunit α of NKA is responsible for its main function in osmoregulation of fish (Hwang and Lee, 2007; Tang et al., 2009), the present study assessed whether CcHSP70 and CcHSP90 contributed to the functional maintenance (or protection) of NKA through their binding with the NKA α subunit. These results indicated that, under stable environments, branchial NKA required little contribution of CcHSP70 and CcHSP90 to receive structural stabilization and protein substitution. However, during time-course experiments, branchial CcHSP70 and CcHSP90 indeed increased their interaction with branchial NKA intensely during a hypotonic challenge (from 3 to 24 h post-transfer) in parallel to the elevation in protein abundance. Overall, NKA provides driving force to other ion channels in ionocytes, maintaining the conformation of NKA represents maintaining the working of ion channels. A rapid increase in CcHSP70 and CcHSP90 combined with an increased interaction with NKA during hypotonic challenge seemed an efficient cellular response in order to overcome acute salinity stress in this euryhaline species. The results found in milkfish also explained an increased expression of HSPs in the gills of other teleosts (e.g., salmonids, sea breams, and tilapia) when exposed to acute hypoosmotic environments for 7 days.

The present study demonstrated differential expressions of branchial HSPs in milkfish upon exposure to a hypotonic challenge. The mRNA expression patterns of the Cchsp genes in the gills of SW- and FW-acclimated milkfish as well as time-course FW transferred milkfish varied with their inducible or constitutive function. The activation of CcHSP70 and CcHSP90 found in the time-course experiments indicated their roles in damage repair or construction maintenance upon exposure to sudden hypotonic stress (within a week), and the earlier elevation of protein abundance than mRNA expression might expound the urgent requirement of CcHSP70 and CcHSP90 for primary protection from acute hypoosmotic stress. The co-IP results provided the evidence that CcHSP70 and CcHSP90 in the gills of the milkfish exposed to hypoosmotic environments play an important role in osmo-protection. Thus, the HSP chaperone machinery should be considered as one of the essential components of protein quality control in aquatic teleosts facing acute changes in environmental salinities (Tang and Lee,
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Author contributions

K.U., H.J.C., W.K.Y., and T.H.L. conceived and designed the experiments. K.U., H.J.C., and L.C. performed the experiments. K.U., H.J.C., W.K.Y., and Y.C.W. and W.Y.W. analyzed the data. K.U. and H.J.C. wrote the manuscript. T.H.L. and C.L.N. organized the whole project and manuscript. All authors have read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that there is no conflict of interests regarding the publication of this paper. The authors alone are responsible for the content and writing of the paper.

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