Comparison of Thermal Stress Induced Heat Shock Factor 1 (HSF1) in Goldfish and Mouse Hepatocyte Cultures

So-Sun Kim[1], Jae-Hyeong So[1] and Jang-Su Park[1,2*]

[1]Department of Chemistry, Pusan National University, Busan 609-735, Korea
[2]Graduate School of Social Enterprise, Pusan National University, Busan 609-735, Korea

Heat shock proteins (HSPs) are induced in response to various physiological or environmental stressors. However, the transcriptional activation of HSPs is regulated by a family of heat shock factors (HSFs). Fish models provide an ideal system for examining the biochemical and molecular mechanisms of adaptation to various temperatures and water environments. In this study, we examined the pattern differentials of heat shock factor 1 (HSF1) and expression of heat shock protein 70 (HSP70) in response to thermal stress in goldfish and mouse hepatocyte cultures by immune-blot analysis. Goldfish HSF1 (gfHSF1) changed from a monomer to a trimer at 33°C and showed slightly at 37°C, whereas mouse HSF1 (mHSF1) did so at 42°C. This experiment showed similar results to a previous study, indicating that gfHSF1 and mHSF1 play different temperature in the stress response. We also examined the activation conditions of the purified recombinant proteins in human HSF1 (hmHSF1) and gfHSF1 using CD spectroscopy and immune-blot analysis. The purified recombinant HSF1s were treated from 25°C to 42°C. Structural changes were observed in hmHSF1 and gfHSF1 according to the heat-treatment conditions. These results revealed that both mammal HSF1 (human and mouse HSF1) and fish HSF1 exhibited temperature-dependent changes; however, their optimal activation temperatures differed.

Key words : Goldfish, heat shock factor1, heat shock protein70, hepatocyte, trimerization

Introduction

Heat shock proteins (HSPs) are induced immediately when cells respond to stresses, such as high or low temperatures, heavy metals, oxidative stress, toxins, and bacterial infections [20]. The expression of heat shock proteins (HSPs) is regulated at both the transcriptional and post-transcriptional levels. The transcriptional regulation of the HSP genes is mediated by the heat shock transcription factor (HSF), which is present as an inactive form in unstressed cells and is activated by stress stimuli [21]. This is a cellular defense against the deleterious effects of physiological and environmental stresses that are mediated by the heat shock transcription factors (HSFs) through a mechanism called the heat shock response. In vertebrates, four members of the HSF family have been identified. Of these, HSF1 and HSF2 are expressed ubiquitously and conserved [22, 23, 25, 28, 29]. HSF1 and HSF2 are expressed as inert monomers and dimers, respectively, in unstressed cells. HSF1 is activated by classical stresses, such as heat shock and heavy metals, and responds to elevated temperatures *in vitro* [2, 3]. The activation of HSF1 in response to cellular stress is related to its trimerization, phosphorylation and binding to DNA in the promoter regions containing the heat shock elements (HSEs), which are present mainly in the heat shock genes [17, 24, 33]. HSF1 is also involved in development and plays critical roles in longevity and cancer [6, 10, 19, 35]. Previous studies have indicated that mammalian HSF1 trimer formation depends on intermolecular hydrophobic noncovalent interactions in its trimerization domain [7, 26, 37]. EGS [ethylene glycol bis(succinimidyl succinate)], a unique sulfhydryl (SH) group cross-linker, was added during the majority of HSF1 trimerization experiments, which recommends that intermolecular disulfide bonds [SS bond(s)] participate in trimerization [5, 30].

The pollution of fresh water ecosystems by heavy metals is one of the main environmental issues [11]. Fish models provide an ideal system for investigating the biochemical and molecular mechanisms of adaptation to various temper-

*Corresponding author

Tel : +82-51-510-2294, Fax : +82-51-516-7421

E-mail : jaspark@pusan.ac.kr

atures and contaminated water environments, including the regulation of thermally sensitive genes [8]. In Korea, goldfish (*Carassius auratus*) are distributed widely in both polluted and unpolluted water. This species has been used as a model to evaluate environmental contamination [13]. This study was designed to show the trimerization of HSF1s (mHSF and gfHSF1) exposed to thermal stress. The HSF1s were investigated to analyze their protein synthesis pattern in response to thermal stress from hepatocyte cultures. Also, we showed the heat-induced expression of HSP70s from hepatocyte cultures, because HSP70 expression is regulated by HSFs, which are the best characterized stress-responsive genes with enhanced expression [1, 34]. In addition, the structural changes of the purified HSF1s (hmHSF1 and gfHSF1) were similar to the temperature dependent protein synthesis patterns observed in hepatocyte cultures.

Materials and Methods

Animals and materials

Goldfish (*Carassius auratus*), approximately 10-15 cm in length and 20 g in weight, were purchased from a local market and maintained in dechlorinated freshwater tanks. The fish were killed by a sharp blow to the head followed by severance of the spinal cord, and were dissected on ice. The animal protocol used in this study was reviewed and approved by the Pusan National University-Institutional Animal Care and use Committee (PNU-IACUC) according to their ethical procedures and scientific care (approval number: ED-PNU2015-0267). The mouse hepatocyte cells (AML12) were purchased from ATCC and grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, 90%; fetal bovine serum, 10%; pH 7.4, at 37°C in T-75 tissue culture flasks (Sarstedt).

Isolation of liver from goldfish and primary culture of hepatocytes

The isolation and primary culture of the hepatocytes were performed using the methods reported elsewhere [4]. The fish were killed by an anesthetic overdose with MS-222 (Sigma), and their liver tissue was isolated carefully and perfused with a hepatocyte buffer (HB, 136.9 mM NaCl, 5.4 mM KCl, 0.81 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.33 mM Na_2HPO_4, 5.0 mM $NaHCO_3$, pH 7.6) for 10 min at room temperature.

The liver tissue was digested with HB buffer containing collagenase (0.3 mg/ml) (Sigma, St. Louis, MO). The softened tissue was minced and sieved through a nylon mesh (50 μm). The resulting cell suspension was washed three times with HB containing 1.5 mM $CaCl_2$. After isolation, the cells were seeded in a Microtest™ tissue culture plate and cultured in Leibovitz-15 medium (L-15, Sigma, St. Louis, MO) at a density of 1×10^6 cell/well. The media was changed every 24 hr and kept at 25°C.

Exposure of hepatocytes and Western blot analysis

Approximately 48 hr after initiating the culture, the AML 12 cells were exposed to a temperature of 42°C and 25°C for 24 hr and, then, the hepatocytes of the goldfish were exposed to a temperature of 33°C and 37°C for 24 hr. After exposure, the harvested cells were extracted using RIPA buffer (Sigma) for western blot analysis. The Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell electroblotted SDS-PAGE samples, including the 42°C and 25°C treated cell pellet, 33°C and 37°C treated cell pellet and control cell pellets in transfer buffer (15.6 mM Tris-HCl, 120 mM glycine, pH 8.3) to the nitrocellulose membrane, were examined. After being blocked with 3% bovine serum albumin (BSA) in Tris-Buffered saline (TBS), the polyclonal antibodies of anti-goldfish HSP70, HSF1 and β-actin, which were prepared in this laboratory [4, 12], a rat monoclonal antibody HSF1 Ab-1, anti-HSP70 and β-actin were purchased from Thermo scientific and used for specific binding. After treating primary antibodies, secondary antibodies (anti-mouse IgG antibody conjugated with alkaline phosphatase) were diluted at 1:5000. The bands were developed using the phosphatase substrate solutions.

Characterization by circular dichroism (CD) and in vitro cross-linking experiments

The purified gfHSF1 and hmHSF1 were obtained in previous studies by our laboratory [12, 14] and diluted with Tris-HCl buffer (pH 7.8) to a final concentration of 0.45 mg/ml. The CD spectra were used to examine the changes in the conformation of the proteins. The CD scans were performed on a Jasco J-715 spectropolarimeter in the far-UV range at 25°C, 33°C and 37°C in the case of gfHSF1 and at 25°C, 37°C and 42°C in the case of hmHSF1. A cell with a 0.1 cm optical path was used to obtain the spectra at a scan speed of 25 nm/min. The spectra were averaged from five individual scans and the results are presented as the

mean molar ellipticity. The trimerization activities of the HSF1s were measured in cross-linking experiments. The purified HSF1 proteins were incubated with 1 mM ethylene glycol bis[succinimidyl succinate] (EGS). The gfHSF1 was left untreated (25°C) or heat-activated for 30 min at different temperatures (33°C and 37°C). The hmHSF1 was treated at 25°C, 37 °C or 42°C, resolved by electrophoresis and analyzed by immunoblotting using the polyclonal antibodies of anti-goldfish HSF1 and a rat monoclonal antibody HSF1 Ab-1.

Statistical analysis

All values are expressed as mean ± SE of 3 replicates in one representative experiment. Statistical analysis was performed using the SPSS (version11.0) program.

Results

This study showed that gfHSF1 and mHSF1 exhibit responses in response to heat stress. In the hepatocyte cultures of goldfish and mouse, the expression of inducible HSF1 were measured during the heat stress. As shown in Fig. 1, gfHSF1 and mHSF1 protein were both detected in monomeric bands at 25°C by Western blot analysis. Although gfHSF1 was monomeric at the control temperature (25°C), it formed a trimer upon heat shock. Trimers of gfHSF1 was observed at 33°C stronger than at 37°C(Fig. 1A). In contrast,

mHSF1 formed a trimer at 42°C, it was observed a monomeric at 25°C and 37°C (Fig. 1B). The β-actin expression was monitored in all hepatocyte cultures as control (Fig. 1C and Fig. 1D). To observe the induction of the HSP70 protein in hepatocyte culture, an inducible HSP70 of goldfish was expressed recombinantly in *E. coli* and purified for the production of polyclonal antibodies. The levels of HSP70 in the cultured hepatocytes were detected by western blot using the polyclonal antibody of goldfish and the monoclonal antibody of mouse. These results refer to the previous studies of this laboratory [13]. The HSP70 expression at 25°C was used as a control to normalize the expression of other temperatures. The levels of HSP70 induced expression in goldfish hepatocyte culture were significantly increased at 33°C than at 25°C, it was slightly increased at 37°C (Fig. 2A and Fig. 2C). In case of mouse hepatocyte culture, it was significantly increased at 42°C as compared to other temperatures (Fig. 2B and Fig. 2D). This study confirmed that fish and mouse have different activated temperature by thermal stress. Circular dichroism (CD) can screen the changes in the conformation of biopolymers and has previously been used to analyze the effects of heat stress on hmHSF1 and gfHSF1 [12]. In humans, HSF1 undergoes heat-inducible monomer-to-trimer conversion and binds to its cognate regulatory site, the heat shock element (HSE) [27]. The purified recombinant HSF1s were incubated at temperatures ranging from 25°C to 42°C for 30min. As a result, structural changes
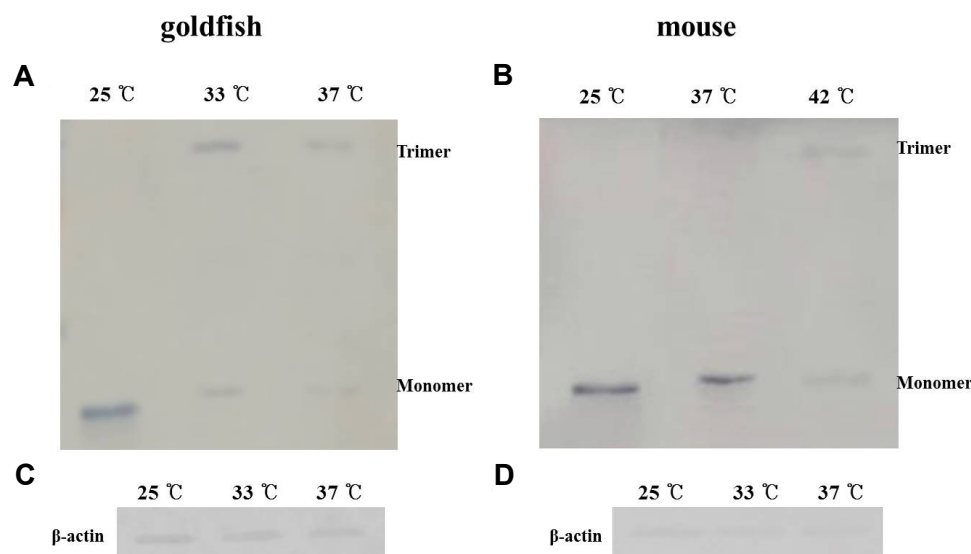

Fig. 1. Western blotting gfHSF1 (A) and β-actin (C) protein in the hepatocytes of goldfish and mHSF1 (C) and β-actin (D) in the mouse hepatocyte cells (AML12). Polyclonal-antibody of anti-goldfish HSF1 and β-actin were produced and monoclonal antibody of mHSF1 (HSF1 Ab-1) and mouse β-actin (AC-15) were purchased from Thermo Scientific. All antibodies were performed to detect the HSF1 and β-actin in hepatocyte cultures.

goldfish

A   25 ℃   33 ℃   37 ℃

HSP70

mouse

B   25 ℃   37 ℃   42 ℃

HSP70

C

Relative HSP70 increment (fold of control)

D

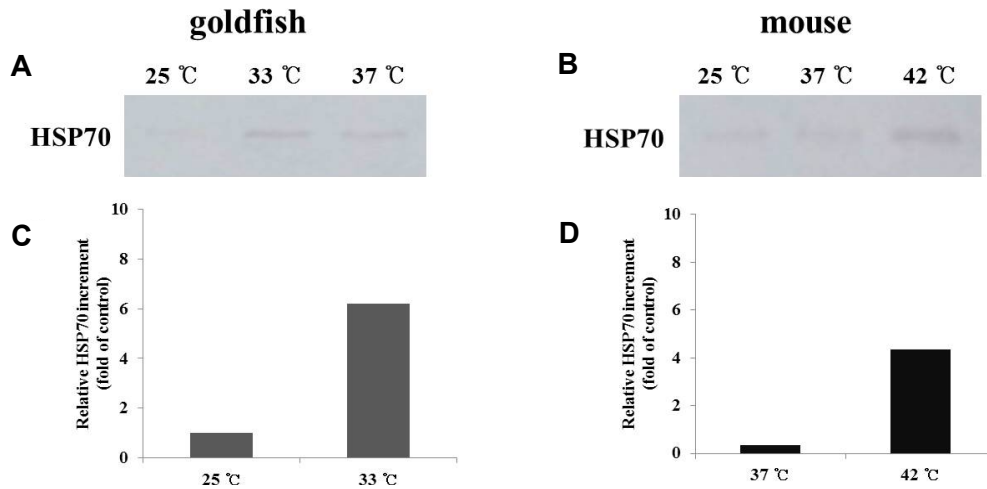Relative HSP70 increment (fold of control)

Fig. 2. Expression of HSP70 in the culture of hepatocytes (A-D). HSP70 synthesis in primary culture of hepatocytes exposed to different temperatures of goldfish (A and C) and mouse (B and D). Western blotting HSP70 protein in the hepatocytes of goldfish (A) and the mouse hepatocyte cells (B). The relative level of gfHSP70 and mHSP70 were showed at C and D. The values are reported as the mean ± standard error of three experiments. The increase in HSP70 protein during heat exposure compared to the control at 25°C.

in hmHSF1 were observed under heat conditions. The CD analysis of hmHSF1 at 42°C revealed an increase in the intensity near 208 nm compared to the control hmHSF1 at 37°C, indicating a conformation change in the secondary structures (Fig. 3A). On the other hand, at lower temperatures (similar patterns in the CD spectra were observed at 25°C) the CD analysis of HSF1 showed a decrease in intensity, which was caused by thermal denaturation (Fig. 3A).

human HSF1

A

(a) 25℃
(b) 37℃
(c) 42℃

goldfish HSF1

B

(a) 25℃
(b) 33℃
(c) 37℃

C   25℃   37℃   42℃

Trimer
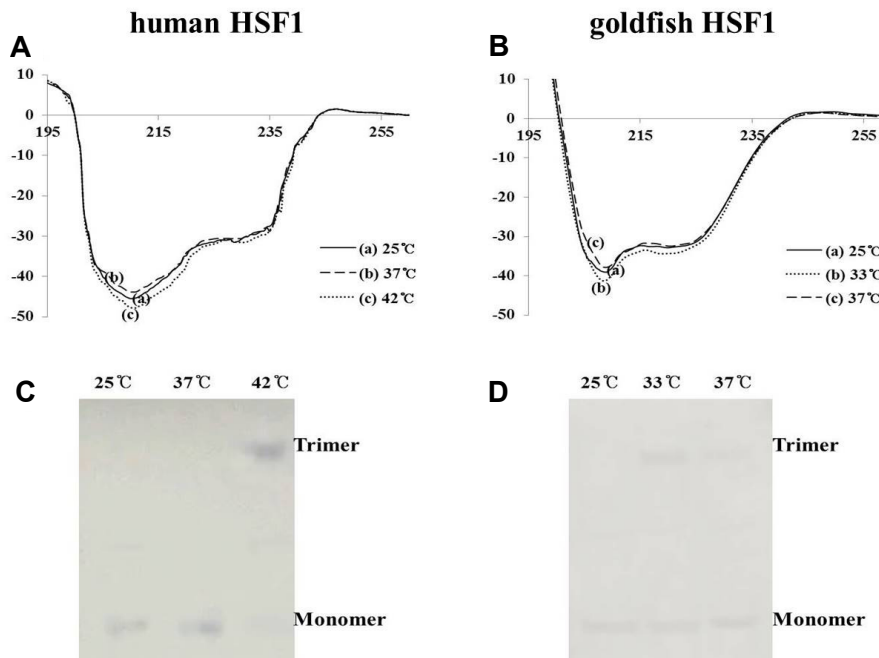
Monomer

D   25℃   33℃   37℃

Trimer

Monomer

Fig. 3. Characterizations of recombinant hmHSF1 and gfHSF1 (A and B). A, Circular dichroism analysis of heat stressed hmHSF1. Bacterial recombinant hmHSF1 were treated three point temperature, 25°C, 37°C and 42°C. The CD spectra were recorded in the far UV region (200-270 nm). The spectra were presented as the mean residue ellipticity, which is expressed as deg·cm²/dmol. Each spectrum is the average of three separate scans. B, Circular dichroism analysis of heat stressed gfHSF1. HmHSF1 and gfHSF1 confer heat inducible trimerization *in vitro* (C and D). Purified hmHSF1 and gfHSF1 was incubated at each other temperature for 30 min after EGS cross-linking and detection by Western blotting using their specific antibodies.

In contrast, the CD analysis of gfHSF1 showed an increase in intensity at 33°C compared to the control held at 25°C and a decrease at 37°C (Fig. 3B). The purified gfHSF1 and hmHSF1 proteins from *E. coli* were analyzed *in vitro* by immune blotting after EGS cross-linking to identify their oligomerization states at each temperature (hmHSF1 was examined at 25°C, 37°C and 42°C, whereas gfHSF1 was examined at 25°C, 33°C and 37°C). As shown in Fig. 3C, hmHSF1 was mostly monomeric at the control temperature (37°C) and room temperature. On the other hand, at 42°C, the formation of band at higher molecular weights was observed, which are consistent with the predicted sizes of the trimer. In contrast, gfHSF1 was detected as a trimer at temperatures lower than the corresponding temperatures for hmHSF1 (Fig. 3D).

Discussion

The temperature range to which a fish species can adapt is dependent on its adaptive cellular functions and stress responses. In a previous study, the adaptable temperatures ranged from 20°C to 32°C for the experimental laboratory fish, platyfish (*Xiphophorus maculatus*) and zebrafish (*Danio rerio*) and the cultured cells of these species can also be maintained in a similar temperature range [36]. The cells responded to unexpected adverse environmental changes by processes referred to as thermal or heavy metal responses, which were accompanied by a rapid increase in the synthesis of stress proteins [9]. This study aimed to understand the changes and roles of HSF1 in hepatocytes. Upon stress, HSF1 is the key transcriptional activator of chaperones, cochaperones and ubiquitin, and coordinates the expression of many transcriptional and translational regulators, signaling molecules and mitotic determinants [18, 31, 32]. The biochemical characterization of HSF1 and the mechanism of HSF1-mediated transcription have been extensively studied and are well understood [16, 27]. The results of the present study revealed the different optimal temperature activation of two HSF1s (mHSF1 and gfHSF1) under heat stress conditions in cultured hepatocytes. GfHSF1 was activated at 33°C, whereas mHSF1 was activated at 42°C. The levels of HSP70 induced expression in hepatocyte cultures showed similar pattern like trimers of HSF1. Here, we suggest that fish and mouse differ in an activation temperature by thermal stress. In previous studies, CD spectroscopy indicated that the unfolded structure of the carboxyl-terminal domain of human HSF1 changes to a more compact structure under stress [15].

In the present study, the CD spectra showed the activation of the purified hmHSF1 and gfHSF1 in a temperature dependent manner. HmHSF1 showed the optimal conformational change at 42°C, whereas for gfHSF1 the change was observed at lower temperatures. In these experiments, immune-blotting analysis revealed similar results to CD spectroscopy in that the HSF1s changed from a monomer to trimer upon exposure to heat stress. This difference in the conformational change between humans and fish has not previously been reported. The *in vivo* results were also the same as the in-vitro ones. These results suggest that fish and mammals (mouse and human) differ in optimal temperature in response to heat shock responses. This may be due to their structure or the differences in the amino acid sequences in each domain; mammal HSF1 and fish HSF1 did not correspond to important sequences forming trimers. It would seem important to study the different structural changes of fish HSF1and mammal HSF1. These functional differences will need to be examined in a future study to determine the reasons for them.

In conclusion, the heat shock factor1s (gfHSF1 and mHSF1) from hepatocyte cultures showed similar patterns in vitro, in that there was a difference in their activation temperatures. This study confirmed that their optimal temperatures forming trimer were different by immunoblot analysis and circular dichroism analysis.

Acknowledgment

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초록 : 붕어와 마우스의 간세포 배양에서 열 스트레스에 의해 유도되는 heat shock factor1 (HSF1)의 비교

김소선[1] · 소재형[1] · 박장수[1,2]*

([1]부산대학교 화학과, [2]사회적기업 겸임교수)

Heat shock proteins (HSPs)은 다양한 생리학적인 또는 환경적 스트레스에 응답하여 유도된다. 그러나 HSPs의 전사 활성은 heat shock factors (HSFs)에 의해 조절 된다. 현재 연구에서는 붕어와 마우스의 간세포 배양에서 열 스트레스에 의한 heat shock factor 1 (HSF1)의 패턴 차이와 heat shock protein 70 (HSP70)의 발현을 면역분석법을 이용하여 조사하였다. 붕어의 간세포는 33℃에서 trimer를 이루지만 마우스의 간세포는 42℃에서 trimer를 이루었다. 이 연구는 붕어와 마우스의 HSF1은 열 스트레스로부터 다른 온도에서 반응을 한다는 것을 보여준다. 또한 재조합 단백질을 이용하여 붕어와 인간의 HSF1의 온도에 따른 활성 조건을 CD spectroscopy와 면역분석을 이용하여 조사하였다. 이러한 결과들은 인간과 마우스 HSF1과 붕어의 HSF1은 온도에 의한 활성 변화를 보이지만 그들의 최적 활성 온도는 다르다는 것을 알 수 있다.