Different Responses in Brain Regions upon Heat Shock in Adult Zebrafish (*Danio rerio*)

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ABSTRACT : HSP70 has widely been induced in *in vivo* hyperthermia conditions in various organisms to study gene regulation and recently neuroprotectve roles of the induced gene expression under varying conditions. We investigated different responses among various tissues in zebrafish under heat shock to evaluate whether spatial and temporal expression pattern of zebrafish (z) hsp70 in transcriptional and translational level under heat shock stress in different brain regions. Heat shock groups were given for 1 h at 37℃ after recovery by transferring the treated animals back to 28℃ for 1, 2 and 24 h for recovery, respectively. Control (CTRL) group was kept at 28℃. At the end of treatments, five animals were collected and used for isolation of total RNAs and peptides from the corresponding tissues. Expression of zhsp70 mRNA showed different patterns in recovery periods in the tissues including the brain, eye, intestines, muscles, heart and testis by RT-PCR. Unlike the RT-PCR analysis, Northern blot analysis demonstrated nearly 30-fold increase in zhsp70 at 1 h heat shock, suggesting that RT-PCR may not be appropriate in unmasking regulation of the time-dependent zhsp70 expression. In the experiment involving different brain regions, the cerebellum showed gradual activation at 1 h to R1h and decreases in R2h and R24h, while the medulla oblongata and optic tectum showed gradual increase at R1h and decrease at R24h, indicating that different brain tissues respond specifically to heat shock in inducing zhsp70 and recovering from the heat shock status. Western blot analysis also demonstrated that the intracellular levels of zHSP70 in three different brain regions including the cerebellum, medulla oblongata and optic tectum are differently induced and recovered to normal state. These results clearly demonstrate that different regions of the body and the brain tissues are responding differently to heat shock in the aspects of its level of expression and speed of recovery.

Key words : zhsp70, Heat shock, Induction, Recovery, Different tissues.

INTRODUCTION

Heat shock among many other sources of stress to organisms triggers a cellular program called the heat shock response, i.e. elevated expression of specific classes of proteins known as heat shock proteins (HSPs). HSPs are associated with many cellular processes including protein synthesis, folding and translocation as well as assembly of larger protein complexes, all of which can be impaired upon under the stress (Lindquist & Craig, 1988). HSP70 has widely been induced in *in vivo* hyperthermia conditions in the brain tissues including the oligodendrocytes of white and gray matter, astrocytes and microglia (Li et al., 1992; McCabe & Simon, 1993). The expression of hyperthermic hsp70 mRNA appears in the rabbit brain where hsp70 mRNA was expressed in oligodendrocytes and microglia, but not in GFAP-positive astrocytes of the forebrain white matter (Foster & Brown, 1997). However, even a strong expression of hsp70 mRNA following heat stress could be dampened at the translational level (Krueger et al., 1999). The roles of the induced hsp70 have been reported by demonstrating that neuronal injury is markedly reduced after ischemic injury in rat brain (Tsuchiya et al., 2003; Yenari et al., 2005), and that hsp70 protects from the injury caused by strokes through anti-inflammatory responses (Zheng et al., 2008).

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The aim of the study is to evaluate whether spatial and temporal expression pattern of zebrafish (z) hsp70 in transcriptional and post-tranlational level under heat shock stress in different brain regions, we tried to northern blot and western blot. And, to determine response of heat shock in zebrafish tissues, zhsp70 mRNA levels analyzed using semi-quantitative analysis, RT-PCR.

MATERIALS AND METHODS

1. Heat Shock

Zebrafish, *Danio rerio*, were purchased from a local aquarium store and allowed to acclimatize for 1 month prior to exposure to stress. They were kept in a light- and temperature-controlled facility and maintained at optimal breeding conditions (Westerfield, 2000). To subject to heat shock stress, each group of adult fish were placed in 10 ℓ of aquarium that was pre-warmed at 37℃ in a water bath. Heat shock groups were given for 1 h at 37℃ after recovery by placing the treated animals back to 28℃ for 1, 2 and 24 h for recovery, respectively. Control (CTRL) group was kept at 28℃. At each sampling, five fish per treatment were removed from their tanks and all tissues were immediately frozen at liquid nitrogen gas. Samples were collected and analyzed from three independent experiments.

2. Quantitative RT-PCR Analysis

Total RNA was purified using standard procedures (TRIzol Reagent, Invitrogen, Carlsbad, CA, USA) from various tissues at adult zebrafish. Synthesis of cDNA was performed with 2 ㎍ of total RNA isolated from various tissues (brain, eye, intestines, heart, liver, muscles, ovary and testis) at 42℃ for 45 min with oligo dT primers (Invitrogen, Carlsbad, CA, USA) and AMV reverse transcriptase (Promega, Madison, WI, USA). The forward and the reverse primers used for RT-PCR analysis include: 5'-atcctgaccattgaagtcgg-3' and 5'-tgttcagttctctgccgttg-3' for zebrafish hsp70 (457 bp); 5'-ttgctgatccacatctgctg-3' and 5'-gacaggatgcagaaggagat-3' for zebrafish β-actin (180 bp). The reaction mixture was first heated to 95℃ for 5 min, and 30 cycles of PCR were performed with denaturation at 95℃ for 30 sec, annealing at 55℃ for 30 sec and elongation at 72℃ for 45 sec. All semi-quantitative RT-PCR analysis has performed by the drop-PCR. β-Actin was performed from 10 cycle and 30 cycles for zhsp70 were done in the same way. After PCR, the reaction mixtures were kept at 72℃ for 15 min. Finally, the contents of PCR mixtures were electrophoresed on a 1.2% agarose gel and ethidium bromide-stained DNA band intensities were quantitated by densitometric analysis with an image documentation system (Amersham, USA).

3. Western Blot Analysis

A 30 ㎍ sample of whole cell extract was separated on a 10% SDS-PAGE gel and subsequently, the separated proteins were transferred to nitrocellulose membranes using an electrotransfer kit (Bio-Rad, CA, USA). The membranes were blocked for 2h in 5% nonfat dry milk and 10 ㎎/㎖ BSA in 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5 (TBST) with 0.1% Tween 20. Immunoreaction was performed using three monoclonal mouse anti-HSP70 antibodies, 3A3 (1:50,000, Affinity Bioreagent, Golden, CO, USA) with TBST. The membranes were washed five times for 10 min each with TBST. After washing with TBST, the blots were incubated for 1 h with affinity- purified goat anti-mouse (A9316, Sigma) conjugated with alkaline phosphatase diluted 1:5000 with TBST, washed five times for 10 min each with TBST. Immunoreactivity was visualized using nitroblue tetrazolium chloride (NBT, at 340 ㎍/㎖) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 175 ㎍/㎖). The immunoreactivities of HSP70 were quantified using an loading control peptide with an anti-β-actin antibody (1:10,000 dilution, A5541, Sigma).

RESULTS

To determine response of heat shock in zebrafish tissues, zhsp70 mRNA levels analyzed using semi-quantitative

RT- PCR. After heat shock, heat shock responses showed sharp increases among the different tissues examined. Although there were different levels of zhsp70 expression induced after heat shock, the brain, eye, and intestines showed highly increased zhsp70 mRNA level, when compared with the rest of tissues. The muscle, heart, liver and testis also showed increased level of zhsp70 expression, although the response of heat shock was rather intermediate in the ovary (Fig. 1). Expression of zhsp70 mRNA showed different patterns in recovery periods. The brain, eye and intestines showed slight decrease in R1h. In the meantime the muscles, heart and testis showed prolonged expression of zhsp70 mRNA upto R2h. Especially, the expression almost disappeared in the intestines at R2h. Liver showed gradual increase up to R1h, and then rapidly decreased at R2h. In case of ovary, heat shock response of zhsp70 mRNA was comparatively low, as compared with the rest of tissues, and rapidly decreased at R1h. Thus, depending on the tissue the intensity of heat shock response and recovery to normal state were varies.

Since we focused on the induction of zhsp70 mRNA in the brain tissue, we evaluated whether spatial and temporal expression pattern of zhsp70 at transcriptional level without RT-PCR to verify the mRNA amount newly made upon heat shock and during recovery to normal temperature. Fig. 2 summarizes the marked induction of zhsp70 transcripts after heat shock. About 99-fold of increase in the zhsp70 expression level was noticed in whole brain tissues (Fig. 2B). Among the induced amount of mRNAs, only one third (36.4-fold) remained after 1 h recovery. Although the amounts of mRNA rapidly decreased at 2 and 24 h recovery, they were still 8- and 2.6-folds of control expression level, respectively.

To define any different heat shock response among the brain tissues, adjacent regions including the cerebellum, the medulla oblongata and the optic tectum of the brain were isolated, and separately analyzed for zhsp70 by RT-PCR (Fig. 3A). The gradual decrease of zhsp70 expression was found similarly in all brain regions after the explosive induction of 1h heat shock. However. the levels of zhsp70 expression decreased about 3- and 2-folds at 1 and 2h recovery at normal temperature, respectively (Fig. 3B).

To demonstrate zHSP70 expression at translational level, we analyzed zHSP70 peptide expressions in different regions of the brain including the cerebellum, the medulla oblongata and the optic tectum by Western blot. Unlike zhsp70 mRNA expression, all the brain tissues showed ubiquitous expressions of zHSP70 in the control lanes. During 1h of heat shock, the increases in the level of zHSP70 expression were found, but the delayed increase of zHSP70 expression was noticed in the recovery of 1h at normal temperature. This delayed zHSP70 expression continued up to 24 h of recovery in the cerebellum. The explosive zHSP70 expression was induced in whole brain after 1h heat shock (Fig. 4A). However, the levels of zHSP70 expression decreased very slowly unlike the levels of mRNA.

We analyzed the intracellular levels of zHSP70 under heat shock in three different brain regions. Cerebellum was gradually activated from 1h to R1h and decreased in R2h and R24h. medulla oblongata and optic tectum were gradually increased to R1h and gradually decreased to R24h. Although zHSP70 was different from degree, zHSP70 existed with basically level in control of all regions. Despite that RT-PCR directly showed the absence of heat shock inducible zhsp70 mRNA transcript in control when a large amount of zHSP70 was spontaneously synthesized, there existed a possibility. zHSP70 was detected in all tissues at 72 kDa. β-Actin protein was used as a loading control (43 kDa). From these results, we found that in brain, it showed similar responses to zHSP70 in brain regions like transcriptional levels. Maroni et al. (2003) reported that hyperthermia caused HSF activation and the induction of hsp70 mRNA and protein to a greater extent in the cerebellum than in the hippocampus. Northern blot analysis indicated that the increase in zhsp70 mRNA levels in the cerebellum was more prompt and greater than

in the hippocampus. However, the increase in zhsp70 mRNA led to a slight increase in zHSP70 protein levels in the cerebellum and the hippocampus, as demonstrated by our Western blot analysis, too.

DISCUSSION

Hyperthermia refers to a higher than normal body temperature with the induction of a characteristic cellular heat shock response and the induction of the HSP interrupting and altering the physiological homeostatic mechanisms of the cell and body (Walsh et al., 1997). Studies on early embryos demonstrated that post-blastula stage embryos maintained at 28.5℃ (control temperature) first exhibit inducible hsp47, hsp70 and hsp90 mRNA accumulation following one hour heat shock at 34℃ with maximum induction occurring at 37℃ (Krone & Sass, 1994; Pearson et al., 1996; Sass et al., 1996). In the adult fish, heat shock (25℃) of 10℃ acclimation in rainbow trout led to increases in hsp70 mRNA in blood, brain, heart, liver, red and white muscle with level in blood being amongst the highest. Hsp30 mRNA is also increased with heat shock in all tissues with the exception of blood (Currie et al., 2000).

Our study indicates a tissues-specific pattern of zhsp70 mRNA expression in the zebrafish tissues during both heat shock and recovery periods. And, the modulation of zhsp70 mRNA against heat shock was dramatically activated after heat shock. The brain, eye, intestines, liver and testis exhibited highest increase. And the other tissues also showed induced expression of zhsp70 mRNA high, although the level is not much as those of the above-mentioned tissues. There is also different degree of recovery responses when compared the intensities of RT-PCR results at R1h and R2h, suggesting different responses among the tissues.

Similar increases in zHSP70 expression have been shown in non-mammalian species, including the rainbow trout (Iwama et al., 1998). Lele et al. (1999) have examined differences in the spatial and temporal regulation of stress-induced hsp47 and hsp70 gene expression following exposure of zebrafish embryos to heat shock. Using Northern blot analysis, they also found that levels of hsp47 and hsp70 mRNA were dramatically elevated during heat

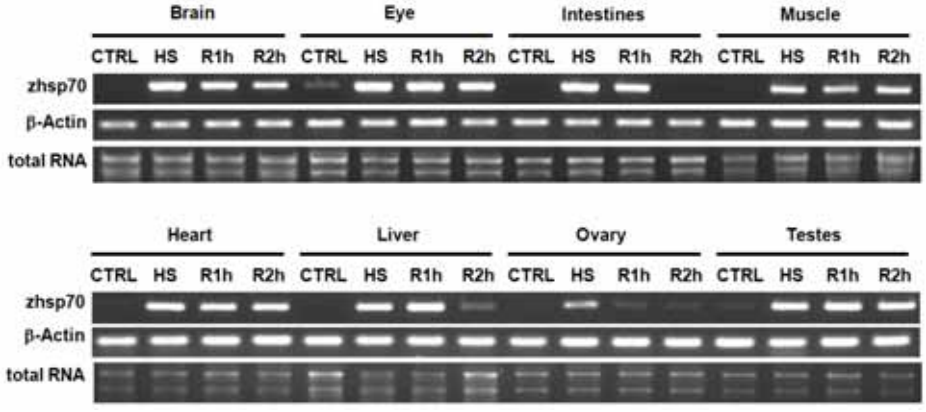

Fig. 1. Time-course zhsp70 mRNA expressions in various tissues of zebrafish revealed by RT-PCR during and after heat shock. Among the tissues examined there were specific differences in their heat shock responses. For example, the intestines and the liver recovered more quickly than the rest did during recovery periods. The induction of zhsp70 was weaker in the ovary than the testes, and the recovery in the testes was far slower than the ovary. CTRL, no heat shock; HS, 1h heat shock, and R1h and R2h, recovery for 1h and 2 h after 1h of heat shock treatment, respectively. Five adult male and female zebrafish were treated for heat shock for 1 h. At each time point, corresponding tissues were collected from 5 zebrafish, analyzed for mRNA expression by RT-PCR after total RNA isolation. A representative pattern of RT-PCR result is presented from three replicate experiments.

shock in 2-day-old embryos (Lele et al., 1999). However, as our result demonstrated, Northern blot result differentiates the expression levels of induced zhsp70 and the expression levels of recovery at even R1h in the brain tissues (Fig. 2) from those of RT-PCR results (brain tissues in Fig. 1). Therefore, the interpretation of the RT-PCR result should take careful consideration due to the exponential amplification of extremely little gene expressed.

In the experiments involving different regions of the brain, there is a region-specific recovery in particular at R24h, although the difference is much less than those shown in Fig. 1. As reported for hsp70 mRNA, hsp70 protein reached higher levels in the cerebellum than in the hippocampus (Maroni et al., 2003). In the present study, zHSP70 expression was relatively high in the brain and different brain regions under control as well as after 1h heat shock. These results correlate well with studies examining stress protein expression in various species where tissue- and stress-specific responses have been demonstrated (Airaksinen et al., 1998; Menzerra et al., 1997).

Exposing tissues to elevated temperatures has been

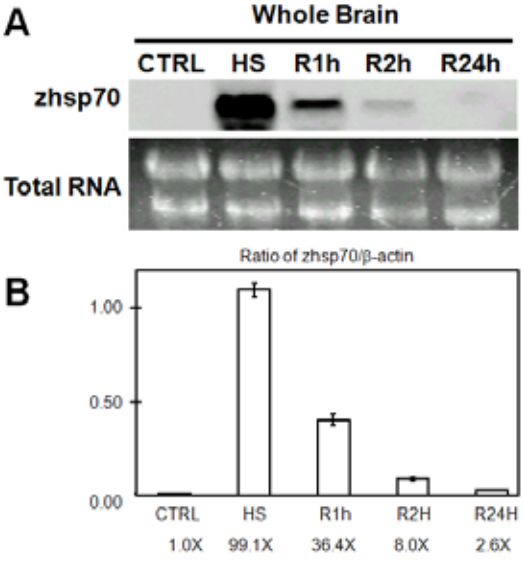

Fig. 2. Northern blot analysis of zhsp70 mRNA expressions in whole brain tissues. Twenty mg of total RNAs were loaded in each lane. The RNAs were transferred to a nitrocellulose membrane, followed by hybridization with a specific probe for zhsp70. The image was obtained by exposing an X-ray film using anti-Dig antibody and subsequently alkaline phosphatase reaction. The explosive zhsp70 expression was induce in whole brain after 1h heat shock (A). However. the levels of zhsp70 expression decreased about 3- and 10-fold at 1 and 2 h recovery at normal temperature, respectively. Total RNA shows the amounts of loading sample. The semi-quantitations revealed dramatic induction and decreases during recovery from the heat shock (B). CTRL, no heat shock; HS, 1h heat shock, and R1h, R3h and R24h, recovery for 1, 2 and 24 h after 1 h of heat shock treatment, respectively. A representative expression pattern of zhsp70 mRNA in whole brain obtained from three replicate experiments.

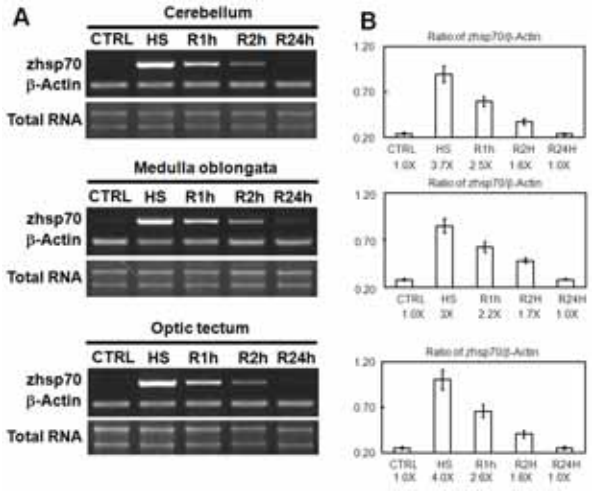

Fig. 3. RT-PCR analysis of zhsp70 mRNA expressions in different regions of the brain. The gradual decrease of zhsp70 expression was found in whole brain after the explosive induction of 1h heat shock (A). However, the levels of zhsp70 expression decreased about 3- and 2-folds at 1 and 2 h recovery at normal temperature, respectively. Total RNA shows the amounts of loading sample. The semi-quantitations revealed dramatic induction and decreases during recovery from the heat shock (B). CTRL, no heat shock; HS, 1 h heat shock, and R1h and R2h, recovery for 1h and 2 h after 1 h of heat shock treatment, respectively. Each brain region was dissected out for isolation of total RNAs, followed by RT-PCR analysis for mRNA expression at different time points to reveal different expression patterns of zhsp70 mRNA. A representative pattern of RT-PCR result is presented from three replicate experiments.

shown to induce HSPs in almost all organisms examined to date (Morimoto et al., 1997). In this study, it was shown that zebrafish are no exception, as an ambient temperature increase from 28℃ to 37℃ induced HSP70 expression in various tissues of the zebrafish examined. Airaksinen et al. (1998) also demonstrated that a temperature increase from 18℃ to 26℃ induced the synthesis of 67 kDa and 69 kDa proteins (members of the Hsp70 family of stress proteins) in cultured *Oncorhynchus mykiss* hepatocytes, gill epithelial cells and gonadal fibroblasts. In mammalian species, heat shock also results in induction of Hsp70 proteins (Bechtold et al., 2000).

From the results obtained in this study, the level of zhsp70mRNA increases markedly after the heat shock in different tissues. Adult brain regions respond differently in their recovery of zhsp70 back to normal state. This was clearly demonstrated in the analysis of zHSP70 expression in different regions of the brain tissues in its level of expression and speed of recovery. This may reflect the position and size of the regional tissues in the brain. Further investigation of distributions of the mRNA and peptide should provide more understanding of vulnerability and neuroprotection from hyperthermic stress.

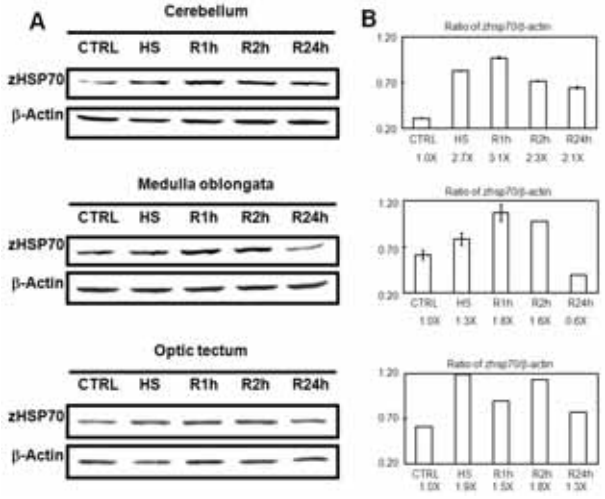

Fig. 4. Western blot analysis of zHSP70 peptide expressions in different regions of the brain including the cerebellum, the medulla oblongata and the optic tectum. Each brain region was dissected out for extraction of cellular proteins, followed by SDS-PAGE and Western blotting at different time points to reveal different expression patterns of zHSP70 peptide expression. All the brain tissues showed ubiquitous expressions of zHSP70 in the control lanes. During 1h of heat shock, the increased expression levels of zHSP70 are found, but the delayed increase of zHSP70 expression is also noticed in the recovery of 1 h at normal temperature. This delayed zHSP70 expression continued up to 24 h of recovery in the cerebellum. The elevated zhsp70 expression was induced in whole brain after 1 h heat shock (A). The semi-quantitations revealed dramatic induction and decreases during recovery from the heat shock (B). CTRL, no heat shock; HS, 1h heat shock, and R1h and R2h, recovery for 1 h and 2 h after 1 h of heat shock treatment, respectively.

ACKNOWLEDGEMENTS

This research was performed by a special grant provided by Korea University.

REFERENCES

Airaksinen S, Rabergh CM, Sistonen L, and Nikinmaa M (1998) Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. J Exp Biol 201:2543-2551.

Bechtold DA, Rush SJ, Brown IR (2000) Localization of the heat-shock protein HSP70 to the synapse following hyperthermic stress in the brain. J Neurochem 74: 641-646.

Currie S, Moyes CD, Tufts BL (2000) The effects of heat shock and acclimation temperature on hsp70 and hsp30 mRNA expression in rainbow trout: *in vivo* and *in vitro* comparisons. J Fish Biol 56:398-408.

Foster JA, Brown IR (1997) Differential induction of heat shock mRNA in oligodendrocytes, microglia, and astrocytes following hyperthermia. Mol Brain Res 45:207-218.

Iwama GK, Thomas PT, Forsyth RB, Vijayan MH (1998) Heat shock protein expression in fish. Rev Fish Biol Fisher 8:35-56.

Krone PH, Sass JB (1994) Hsp 90α and Hsp 90β genes are present in the zebrafish and are differentially regulated in developing embryos. Biochem Biophys Res Comm 204:746-752.

Krueger AM, Armstrong JN, Plumier J, Robertson HA, Currie RW (1999) Cell specific expression of Hsp70 in neurons and glia of the rat hippocampus after hyperthermia and kainic acid-induced seizure activity. Brain Res Mol Brain Res 71:265-278.

Lele Z, Engel S, Krone PH (1999) Hsp47 and hsp70 gene expression is differentially regulated in a stress- and tissue-specific manner in zebrafish embryos. Dev Genet 21:123-133.

Li Y, Chopp M, Yoshida Y, Levine SR (1992) Distribution of 72-kDa heat-shock protein in rat brain after hyperthermia. Acta Neuropathol 84:94-99.

Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Genet 22:631-677.

Maroni P, Bendinelli P, Tiberio L, Rovetta F, Piccoletti R, Schiaffonati L (2003) *In vivo* heat-shock response in the brain: signalling pathway and transcription factor activation. Brain Res Mol Brain Res 119:90-99.

McCabe T, Simon RP (1993) Hyperthermia induces 72 kDa heat shock protein expression in rat brain in non-neuronal cells. Neurosci Lett 159:163-165.

Menzerra P, Rush SJ, Brown IR (1997) Tissue-specific differences in heat shock protein hsc70 and hsp70 in the control and hyperthermic rabbit. J Cell Physiol 170:130-137.

Morimoto RI, Kline MP, Bimston DN, Cotto JJ (1997) The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. Essays Biochem 32:17-29.

Pearson DS, Kulyk WM, Kelly GM, Krone PH (1996) Cloning and characterization of a cDNA encoding the collagen-binding stress protein hsp47 in zebrafish. DNA Cell Biol 15:263-272.

Sass JB, Weinberg ES, Krone PH (1996) Specific localization of zebrafish hsp90α mRNA tomyoD-expressing cells suggests a role for hsp90α during normal muscle development. Mech Dev 54:195-204.

Tsuchiya D, Hong S, Matsumori Y, Kayama T, Swanson RA, Dillman WH, Liu J, Panter SS, Weinstein PR (2003). Overexpression of rat heat shock protein 70 reduces neuronal injury after transient focal ischemia, transient global ischemia, or kainic acid-induced seizures. Neurosurgery 53:1179-1187.

Walsh D, Li Z, Wu Y, Nagata K (1997) Heat shock and the role of the HSPs during neural plate induction in early mammalian CNS and brain development. Cell Mol Life Sci 53:198-211.

Westerfield M (2000) The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*). (4th ed.), University of Oregon Press, Eugene.

Yenari MA, Liu J, Zheng Z, Vexler ZS, Lee JE, Giffard RG (2005) Antiapoptotic and anti-inflammatory mechanisms of heat-shock protein protection. Ann N Y Acad Sci 1053:74-83.

Zheng Z, Kim JY, Ma H, Lee JE, Yenari MA (2008). Anti-inflammatory effects of the 70 kDa heat shock protein in experimental stroke. J Cereb Blood Flow Metab 28:53-63.

(received 16 August 2009, received in revised form 19 September 2009, accepted 19 September 2009)