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Relationship between Heat Shock Protein 70 Synthesis and Development of Thermotolerance in the Olive Flounder, *Paralichthys olivaceus*

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Relationship between hsp 70 expression and development of thermotolerance in the olive flounder was investigated by exposing the fish to 23 or 26℃ for 1 h. After selected intervals, they were re-exposed to 31℃ for a duration of 15 min. Theremotolerance rapidly developed with increasing interval and peaked at 3 h interval. Subsequently, it gradually decayed and disappeared by 24 h interval. The flounder displayed remarkably high levels of hsp 70 mRNA and protein, as compared to control flounder. Hence, the olive flounder acquires thermotolerance, which is positively correlated with the synthesis of hsp 70.

Key words : Olive flounder, *Paralichthys olivaceus*, Heat shock protein 70, Thermotolerance

Introduction

Thermotolerance is a wide spread phenomenon among organisms and an important adoptation to surive changing environmental conditions. The capacity of an organism to survive extreme temperatures is one of the most investigated phenomena. This capacity is developed by the organisms following their prior but brief exposure to sublethal temperatures. This kind of exposure is known to induce resistance against high temperature conditions, that would otherwise be lethal (Li and Hahn, 1980; Nover, 1991; Parsell and Lindquist, 1994). The degree of resistance is indeed very wide; for example, mammalian cells expressing thermotolerance can exhibit survival levels several hundred times higher than normal cells, when both are subjected to lethal temperatures (Mizzen and Welch, 1988). This phenomenon appears to be highly conserved and universal from bacteria to mammalian cells (Schlesinger, 1990). During the conditioning heat shock, synthesis of a small set of highly conserved proteins known as heat shock proteins (hsps) is dramatically increased. Although the function of hsps continues to be elucidated, a well-established role for some of them, including the hsp70 family, involves their ability to act as molecular chaperones (Ellis, and Vies, 1991). In that capacity, they protect other proteins from unfolding, or refold denatured proteins, or target them for degradation, and it is clear that these processes are involved in the induction of thermotolerance (Gething and Sambrook, 1992; Morimoto et al., 1994).

The commercially important olive flounders survive from 10℃ during winter to about 28℃ in summer along the entire coast of Korea. Since 1990s, mass mortality of the flounders has become a recurring obstacle for growers. Several ecological factors appear to coincide with summer mortality of the olive flounder population; these include stress owing to altered water temperature, salinity and dissolved oxygen, and pathogens. Hsps appear to be involved in protecting organisms from the negative effects of heat and other stress at the cellular level, but their exact function is not fully understood at organismal level; it is still not clear whether there is a direct

cause-effect relationship between the synthesis of hsps and development of thermotolerance. As part of a broader study on mass mortality of the olive flounder, the acquisition of thermotolerance in the flounders was studied by heat-shocking them at a sublethal temperature, which was followed by an exposure to lethal temperature. Our results suggest that this acquired thermotolerance was associated with the expression of hsp 70.

Materials and Methods

Maintenance of olive flounders

Olive flounders, obtained from Koje Hatchery of National Fisheries Research and Development Institute, were kept in an aerated 2-ton running seawater aquaria and maintained at ambient temperature (12 ±1℃, monitored daily) under a natural photoperiod for at least 15 days until used in thermotolerance experiments. They were fed daily with a moist pellet and starved for 24 h prior to the heat shocking and thermotolerance experiments described below. Size of the flounder juveniles was 10-13 cm.

Heat shock and acquired thermotolerance

Lethal temperature caused 100 % mortality within 2 days of return to ambient temperature, while sublethal heat shock resulted in 100% survival within 2 days after return to ambient temperature. On the basis of results obtained from preliminary experiments, the following exposure temperature and duration of 23 or 26℃ for 1 h and 31℃ for 15 min representing the sublethal and lethal shock, respectively were selected. A two-step heat shock protocol was adopted for the heat-shocking and subsequent return to the ambient temperature. For this, the selected flounders were introduced into a 10 l inner plastic container, which was gradually heated to the desired temperature; the entire inner plastic container was immersed in a 50 l outer plastic container, set at the desired temperature. To examine acquired thermotolerance, the flounders (maintained at ambient temperature of 12±1℃) were exposed to a sublethal heat shock, as described above and then returned to ambient temperature for recovery. The lengths of the recovery period varied: 0, 1, 3, 6, 9, 12, or 24 h post-sublethal heat shock. At the end of each recovery period, the flounders were heat-shocked at 31℃ for 15 min and then returned to the ambient temperature and monitored daily. Olive flounders exposed to lethal temperature without prior exposure to sublethal temperature were used as control. Survival calculated as percentage remaining alive at ambient temperature, as described above, 2 days after each treatment. All experiments were repeated three times using 20 olive flounders per each treatment.

RNA isolation and Northern blot analysis

To determine whether a sublethal heat shock was associated with induction of hsp70, liver samples were prepared for Northern blot analysis. Because of their size, amenability to manipulation, and high resolution of liver RNA by Northern blot analysis, liver was chosen. After sublethal heat shock at 23 or 26℃ for 1 h, the flounders were maintained at ambient temperature (12±1℃) for the interval indicated in each experiment for recovery. At each recovery interval, the flounders were sacrificed and their livers were removed. Total RNA was isolated from liver samples using Trizol RNA isolation reagent (GIBCO/BRL) according to the manufacturer's instructions (Chomczynski and Sacchi, 1987). RNA samples (25 μg) were denatured in formamide and formaldehyde at 65℃ for 5 min and electrophoresed in an agarose gel containing formaldehyde (Sambrook et al., 1989). RNA was then transferred onto a nylon membranes (Bio-Rad) in 10 ×SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and fixed to the membrane using the UV cross linker. cDNA probe was labeled using [α-^{32}P] dCTP with random priming kit (Amersham) and hybridized to the membranes in hybridization buffer (50% formamide, 0.25 M $NaHPO_4$, 7% SDS and 1 mM EDTA) at 42℃ for 14 h. Membranes were washed in 2×SSC-0.1% SDS at room temperature (RT) and then twice for 15 min each time in 0.1×SSC-0.1% SDS at 65℃. The

mebrames were exposed to Kodak XR5 film at -80℃. The cDNA probe was nucleotide 1183 to 1542 representing a highly conserved part of olive flounder *hsp70*-related cDNA (Kim et al., 1999). This part binds both the heat-inducible *hsp70* and constitutive *hsc70*. The quantification of hsp70 transcripts was carried out using a densitometric scanner (Bio- Rad).

SDS-PAGE and Western blot analysis

At each recovery interval described above, the liver tissues were homogenized with a Teflon pellet pestle in a buffer (5 mM $MgSO_4$, 5 mM NaH_2PO_4 40 mM HEPES, 70 mM potassium gluconate, 1 mM PMSF, and 150 mM sorbitol, pH 7.5). SDS-PAGE was carried out on a 12.5 % polyacrylamide gel, as described by Laemmli (1970). Western blotting was performed, as described by Towbin et al. (1979). Primary antibody, namely the mouse monoclonal anti-hsp/hsc70 antibody (N27, a gift from Dr. W. J. Welch, University of Califonia, San Francisco, CA), which recognizes both inducible hsp70 and cognate hsc70 was used. The secondary antibody used was the alkaline phosphatase-conjugated goat anti-mouse IgG.

Results

Heat shock and acquired thermotolerance in olive flounder

Preliminary studies to determine the LT50 showed that the heat shock of temperatures below 28℃ for 1 h (on animals taken directly from ambient temperatures of about 12℃) did not cause mortality within 2 days after the heat-shocked olive flounders were returned to 12℃ (data not shown). Thus, 23 or 26℃ for 1 h were selected for the sublethal heat shock. The lowest lethal temperature regimen required to kill all the flounders, which were previously not exposed to heat shock, varied slightly from 31-32℃ for 15 min between different batches of fishes. Figure 1 shows the survival of control and heat-shocked olive flounder after exposure to a lethal temperature. All olive flounders, taken directly from 12℃ were killed by lethal temperature. In contrast, a large proportion of olive flounders, previously heat-shocked, then returned to 12℃, survived, when they were were exposed to a lethal temperature. Thermotolerance developed immediately after heat shock ("0" in Fig. 1), and peaked after a period of 6 and 3 h in the flounders, that were previously exposed to 23 and 26℃, respectively. Subsequently, the tolerance began to progressively decay, and disappeared by 12 and 24 h, after the termination of heat shock in the flounders that were previously shocked at 23 and 26℃, respectively. Apparently, thermotolerance is dependent upon the sublethal temperature, at which the fish was shocked.

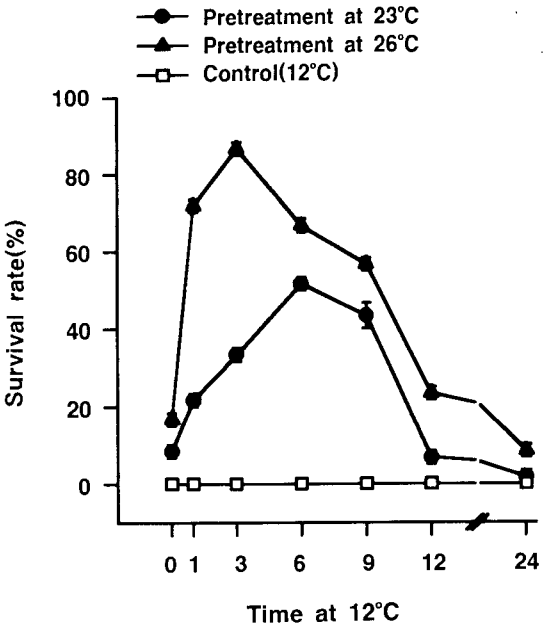

Fig. 1. Time course of the acquisition of thermotolerance. Olive flounders were exposed to a sublethal temperature of 23 or 26℃ for 1 h and then incubated for 0, 1, 3, 6, 9, 12, or 24 h at ambient temperature (12± 1℃) before being exposed to lethal temperature of 3 1℃ for 15 min. Survival was evaluated after 2 days at 12±1℃. Control refers to olive flounders exposed to lethal temperature without prior heat shock. Values are means ± S.E.M. for 3 different experiments, each using 20 olive flounders per treatment.

Expression of hsp 70 transcripts in heat-shocked olive flounder

To determine whether the heat shock at a sublethal

temperatures was associated with elevated expression of hsp 70, Northern blot analysis was made using a highly conserved part of the flounder hsp *70*-related cDNA. Analysis of RNA samples, obtained from the heat-shocked olive flounders, showed a rapid accumulation of hsp *70* transcripts, reaching a maximum 6 h after the 23℃ heat shock (Fig. 2A). Hsp *70* transcripts were also accumulated rapidly in the flounders, that were heat-shocked at 26℃, but maximum levels of hsp *70* transcripts were observed at 3 h recovery (Fig. 2B). Hsp *70* transcripts were detected neither in livers obtained from control olive flounders nor after 24 h of recovery following the heat shock at 23℃. Densitometric analysis of the autoradiograms showed approximately a 3-fold increase in hsp *70* transcripts at 3 h recovery after heat shock at 26℃, as compared to 3 h recovery after heat shock at 23℃. In the flounders that were heat shocked at 26℃, there are as much as 40-fold increase of hsp *70* transcripts levels during the peak of thermotolerance (e.g. at 3 h recovery).

But in those heat shocked at 23℃, levels of hsp *70* transcripts induced are only by 15-fold during the peak of thermotolerance (e.g. at 6 h recovery) compared to control levels. From the Northern blot analysis, signals corresponding to hsp *70* transcripts were not however observed throughout the recovery after the heat shock at 23 or 26℃ (data not shown). Therefore, hsp *70* mRNA observed in acquisition of thermotolerance of the flounder indicates the inducible hsp *70*.

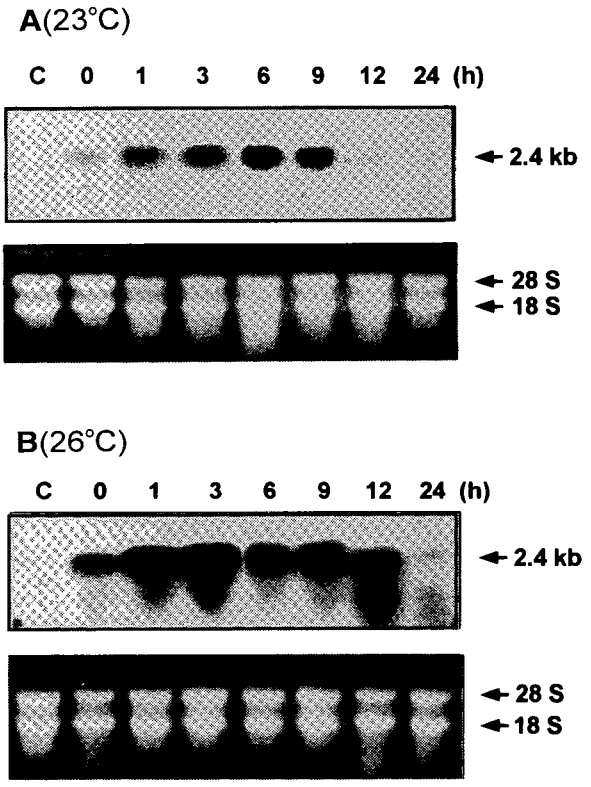

Fig. 2. Expression of hsp *70* mRNA in the acquisition of thermotolerance. Olive flounders were exposed to a sublethal temperature of 23 (panel A) or 26℃ (panel B) for 1 h and then incubated for 0, 1, 3, 6, 9, 12, or 24 h at ambient temperature (12±1℃). At each of the recovery time, total liver RNA (20 μg) was isolated, eletrophoresed in an agarose gel, transferred to nylon membrane, and hybridized with a highly conserved part of olive flounder hsp *70*-related cDNA. Arrow indicates a 2.4 kb corresponding to hsp *70* transcripts. Ribosomal RNA (28S and 18S) as a control for equivalent loading and RNA integrity are shown at the bottom of each panel.

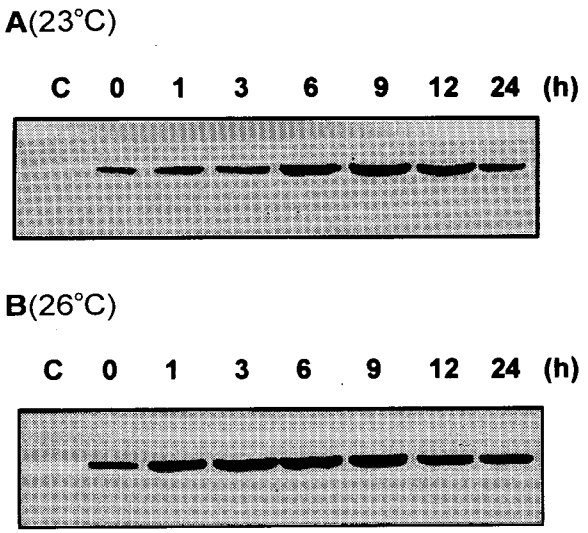

Fig. 3. Changes of hsp 70 levels in the acquisition of thermotolerance. Olive flounders were exposed to a sublethal temperature of 23 (panel A) or 26℃ (panel B) for 1 h and then incubated for 0, 1, 3, 6, 9, 12, or 24 h at ambient temperature (12±1℃). At each of the recovery time, the polypetides of liver tissues were isolated, electrophoresed using SDS/polyacryamide gel (12.5 %), transferred onto nitrocellulose paper, and probed with anti-hsp 70 antibody (N27).

Accumulation of hsp *70 in olive flounder after heat shock*

Olive flounders were heat-shocked at 23 or 26℃ for 1 h and then were allowed to recover to ambient temperature (12℃). At different intervals during the recovery, proteins of liver samples were obtained. Equal amounts of proteins were separated by SDS-PAGE, transferred onto nitrocellulose filters, and probed with monoclonal antibody N27. Hsp 70 levels were maximized after 9 h of recovery from heat shock at 23℃ (Fig. 3A). The maximal levels of hsp 70 was observed after 3-6 h of recovery from heat shock at 26℃ and the level persisted for 24 h after recovery (Fig. 3B). Control olive flounders did not show any detectable levels of hsp 70.

Discussion

Thermotolerance is defined as a transient resistance to one or other environmental factor, at lethal level, which can be influenced by prior exposure to the same factor but at sublethal level (Laszlo, 1988). A level of sublethal temperature and exposure duration to that selected sublethal temperature are apparently important in inducing thermotolerance (Trent et al., 1990). One hundred percent olive flounders, heat shocked at sublethal temperature of 23 or 26℃ for 1 h, survived for 2 days after returned to ambient temperature; remarkably, these flounders tolerated not only immediate transfer to a temperature of 11-14℃ above the holding (physiologic) environment, but also acquired thermotolerance, that remains for at least 12 h (Fig. 1); a similar acquisition of thermotolerance has been described in a number of aquatic animals like rainbow trout (Mosser et al., 1987), tilapia (Chen et al., 1988), and oyster (Clegg et al., 1998) and other animals (Morimoto et al., 1990; Nover, 1991; Weber, 1992). Briefly, the time course of acquiring thermotolerance in the olive flounder was comparable to that observed by Hahn (1982).

In mammalian cells, levels of hsp70 were shown to correlated fairly well with the development and decay of thermotolerance as well as with the heat sensitivity (Laszlo and Li, 1985; Li and Mak, 1988). This correlation was also true for the olive flounder. Maximum levels of hsp *70* mRNA and hsp 70 (protein) were observed at 3h recovery after the heat shock at 26℃. The hsp *70* mRNA observed in development of thermotolerance of the flounder indicates the inducible hsp *70* mRNA. To precisely investigate the expression of *hsc70* transcripts in the development of thermotolerance, RT-PCR experiments using specific primers for olive flounder *hsc70* cDNA is now in process.

Although the immediate role of the heat shock-induced hsp 70 in the flounder is unknown, previous studies have suggested a correlation between the induction of thermotolerance and heat shock protein expression in a variety of cells and intact organisms (Weber, 1992; Morimoto et al., 1994; Sanders er al., 1994; Roberts et al., 1997). The biochemical nature of heat-induced, higher resistance to elevated temperature is not completely understood; several lines of evidence have suggested involvement of heat shock proteins (Lindquist and Craig, 1988). Results from this study suggest that exposure to sublethal but elevated temperatures enhanced thermotolerance and that there is a correlation between the acquired thermotolerance and hsp 70 synthesis. As demonstrated in many other organisms (Nover, 1991; Wynn et al., 1994), the appearance of hsp 70 in olive flounders may be intimately associated with resistance to other physical and ecological stresses in the environment (Lindquist, 1986). Whether acquired thermotolerance can result in increased survival during summer mortality is currently under investigation. This may be a mechanism adapted by olive flounders to increase survival under natural conditions. Thus, hsp70 may be a potential biomarker for stress in olive flounders.

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