Modulation of ASK1 expression during overexpression of Trx and HSP70 in stressed fish liver mitochondria

Ekambaram Padmini · Bose Vijaya Geetha

Received: 18 November 2008 / Revised: 13 January 2009 / Accepted: 16 January 2009 / Published online: 17 February 2009
© Cell Stress Society International 2009

Abstract Mitochondrial heat shock protein 70 (mtHSP70) is found to play a primary role in cellular defense against physiological stress like exposure to environmental contaminants and helpful in the maintenance of cellular homeostasis by promoting the cell survival. In the present investigation, the environmental-stress-induced increase in mtHSP70 levels along with the quantification of apoptosis signal regulating kinase 1 (ASK1) and thioredoxin (Trx) were measured in the liver mitochondria of grey mullets (*Mugil cephalus*) collected from the polluted Ennore estuary and the unpolluted Kovalam estuary for a period of 2 years. The results showed elevated lipid peroxide (LPO) and decreased total antioxidant capacity along with the decrease in mitochondrial viability percentage. Mitochondrial HSP70, ASK1, and Trx levels were increased under this stress condition. A 42% increase in LPO levels and 18% decrease in mitochondrial survivality were observed in the polluted-site fish liver mitochondria when compared to the results of unpolluted estuary. We also report that, under observed oxidative stress condition in Ennore fish samples, the ASK1 levels are only moderately elevated (13% increase). This may be due to mitochondrial-HSP70-induced adaptive tolerance signaling for the activation of Trx (22% increase) which suppresses the ASK1 expression thereby promoting the cell survival that leads to the maintenance of the cellular homeostasis.

Keywords Estuarine contamination · Mitochondrial HSP70 · Thioredoxin · Mitochondrial viability · Cell survival

Abbreviations

HSP70	heat shock protein 70
ASK1	apoptosis signal regulating kinase 1
Trx	thioredoxin
LPO	lipid peroxide
MDA	malondialdehyde
TAC	total antioxidant capacity

Introduction

Estuarine environment is recognized as a complex ecosystem which is under constant disturbance to a greater extent by the industrial effluents which will in turn affect the ecosystem particularly the fish (Padmini et al. 2008c). One such contaminated estuary that is facing a great threat due to metal contamination caused by the surrounding industries is Ennore estuary located in North Chennai, Tamil Nadu, India (Padmini and Vijaya Geetha 2007a). Kovalam estuary located in South Chennai, Tamil Nadu, India was selected as the unpolluted study site, as the comparison between the polluted and unpolluted estuary gives a direct and effective evidence for the extent of contamination (Padmini and Kavitha 2003).

Fish are widely used as sentinels of contamination and can be used as bioindicators of heavy metal pollution as they are exposed to bewildering array of anthropogenic chemicals. Heavy metals accumulated in the tissues of fish may catalyze reactions that generate reactive oxygen species (ROS) which may lead to oxidative stress (Farombi et al. 2007; Ryan and Hightower 1994). Mitochondria are

E. Padmini (✉) · B. Vijaya Geetha
Research Department of Biochemistry,
Bharathi Women's College,
Chennai 600108 Tamil Nadu, India
e-mail: dstpadmini@rediffmail.com

B. Vijaya Geetha
e-mail: viji.bwc@gmail.com

important dynamic organelles for cell survival and function, as the biogenesis of mitochondria is involved in the control of cell metabolism and signal transduction (Nisoli and Carruba 2006). They are the primary sites of ROS formation and play a major role in the mediation of cellular damage (Thannickal and Fanburg 2000). In a normal cell, there is always a balance between prooxidant and antioxidant pathways. Upon stress stimuli, an imbalance of the redox milieu develops that leads to the accumulation of ROS which in turn affects the survival of cell (Gutterman 2005). The increased amount of ROS in mitochondria is likely to decrease the cellular viability by promoting the denaturation of proteins (Sakurai and Cederbaum 1998).

As stress is unavoidable in the polluted environment, a cellular level stress response has been observed in nearly all organisms whose characteristic feature is the induction of heat shock proteins (HSPs) that encompass several families of cytoprotective proteins (Basu et al. 2001). Stress proteins are known to provide information on the biological impact of toxic chemicals to organisms and to predict adverse consequences of that exposure (Sanders and Martin 1993). HSP70 is found to have a protective role against oxidative stress and also found to be upregulated to assist in the refolding of proteins that are damaged during stress condition (Guo et al. 2007). HSP70 has been proposed as a significant biochemical marker of environmental stress caused during toxicant exposure (Tiano et al. 2004; Simon et al. 1995).

Mitochondria contain the set of HSP70 that are essential for the import of proteins from the cytoplasm into the mitochondria and folding of the proteins which are found to be essential for cell viability (Bolliger et al. 1994). MtHSP70 is essential for driving preproteins across the mitochondrial membrane into the matrix for maintaining the mitochondrial membrane potential that constitutes the motor unit of mitochondrial protein import machinery (Schatz and Dobberstein 1996). Recently, we have reported that the increase in the level of mitochondrial heat shock protein 70 (mtHSP70) is associated with the prevention of NO-dependent increase in cellular free iron thereby maintaining the Fe-S clusters which is responsible for the intactness and integrity of mitochondrial respiratory chain complexes and hence reduced free radical damage (Padmini et al. 2009). Thioredoxin (Trx) is a widely distributed intracellular redox protein which constitutes an endogenous antioxidant system in addition to glutathione and superoxide dismutase systems by suppressing free radical formation. It regulates intracellular processes, including transcriptional activity, proliferation, and mitochondrial-dependent apoptosis by forming an inactive complex with apoptosis signal regulating kinase 1 (Chen et al. 2002; Saitoh et al. 1998). The increased ROS production under stressed condition would dissociate the Trx-ASK1 complex thereby increasing mitogen activated protein kinase

(MAPK) pathway activity (Hsieh and Papaconstantinou 2006). Previously, it was reported that the knockout or knockdown of the Trx may be detrimental to cell survival (Chiueh et al. 2005). Also, Pouis and Montfort (2001) suggested that oxidative stress mediated ROS activate ASK1 in part by oxidizing Trx which releases ASK1 from the complex. The released ASK1 acts as a critical mediator in ROS-induced cell damage that leads to cell death.

Our previous studies demonstrated that the fish inhabiting the Ennore estuary are able to survive in the heavy metal pollution induced stressed situation (Padmini et al. 2008b). It is important to understand the various damages that occur in these fish liver mitochondria during stress conditions and the mechanism that are involved in providing resistance to such damages which allow the fish to survive. As the development of fish tolerance towards estuarine contamination is in association with mtHSP70, we examined the levels of mitochondrial HSP70 along with Trx and ASK1 to understand the alterations in their expressions under natural stress conditions and the results were compared with the unpolluted Kovalam estuary.

Materials and methods

Chemicals

Malondialdehyde (MDA), thiobarbituric acid, 2,2'-azinobis, 3-ethylbenzothiaziline-6-sulfonate (ABTS), trolox, β -aminocaproic acid, phenylmethanesulfonyl fluoride, *N*-ethylmaleimide and dithionitrobenzoic acid were purchased from Sigma-Aldrich, USA. *O*-phthalaldehyde, hydrogen peroxide and ethylenediaminetetraacetic acid were from Sisco Research Laboratories, Mumbai, India. HSP70 enzyme-linked immunosorbent assay (ELISA) kit (EKS 700B), mouse monoclonal HSP70 antibody (SPA-810), rabbit polyclonal ASK1 antibody (AAP-480), mouse monoclonal thioredoxin antibody (MSA-150), and rabbit polyclonal β -actin antibody (CSA-400) were obtained from Stressgen Bioreagents, Victoria, Columbia, Canada.

Animals and study site

Mugil cephalus (grey mullets), a natural inhabitant of the estuaries, identified by the use of the Food and Agriculture Organization's species identification sheets was chosen as the experimental animal for the study (Fischer and Bianchi 1984).

Two estuaries were chosen as the experimental sites for the study: (1) Kovalam estuary (12° 49' N and 80° 5' E), an unpolluted site situated about 35 km south of Chennai is far away from the influence of industrial pollution and (2) Ennore estuary (13° 14' N and 80° 20' E), situated about 15 km north of Chennai is a highly polluted estuary due to

the discharge of effluents from industries like oil refineries, fertilizer company, thermal power stations, etc. surrounding this site. Contamination of the Ennore estuary by heavy metals like Fe, Pb, Cd, Mg, Zn, etc. to a significant extent was already confirmed by our previous studies (Padmini and Vijaya Geetha 2007b).

Fish sampling

Grey mullets (*M. cephalus*) with average length of 30 cm were collected freshly twice in a month for 2 years (April 2006–March 2008) from both the estuaries to study the effect of pollution on oxidative stress status of the fish. Sampling fish was collected with baited minnow traps in a shallow estuary and were placed immediately into insulated containers filled with aerated estuarine water at ambient temperature. Fish were sacrificed by severing their spinal cord and the liver was removed immediately for the isolation of mitochondria.

Isolation of fish liver mitochondria

The procedure of Johnson and Lardy (1967) with slight modifications was employed to isolate mitochondria. About 1 g of tissue was weighed and washed twice with ice-cold buffer and 5 ml of Kohler's homogenizing buffer. The homogenate was centrifuged in the refrigerated centrifuge at $500\times g$ for 10 min. The supernatant was recentrifuged at $8,000\times g$ for 30 min. The resulting pellet containing the mitochondrial fraction was resuspended and recentrifuged under the same experimental conditions to obtain the crude fraction of mitochondria. We purified the crude mitochondrial fraction by resuspending such fraction in three packed-cell volumes of mitochondrial suspension buffer (10 mM Tris HCl pH 6.7, 0.15 mM $MgCl_2$, 0.25 M sucrose, 1 mM phenylmethanesulfonyl fluoride, and 1 mM dithiothreitol) and centrifuging at $9,500\times g$ for 15 min for repelleting the pure mitochondria. It was then suspended in 0.25-M sucrose solution (pH 7.4) and homogenized for 1 min, which was then used for further studies. The presence of mitochondria in the pellet fraction was confirmed by the assay of succinate dehydrogenase enzyme (marker enzyme). Proteins were estimated by the method of Bradford (1976) with the use of bovine serum albumin as the standard.

Measurement of lipid peroxidation and total antioxidant capacity levels

Lipid peroxidation was measured by high-performance liquid chromatography according to Lykkesfeldt (2001) method as described previously (Padmini et al. 2008a) which involves measurement of the MDA adduct extracted

from mitochondrial samples using thiobarbituric acid reagent. The results were expressed as nanomoles of MDA formed per milligram of mitochondrial protein.

Total antioxidant capacity (TAC) was determined according to Erel (2004) method using ABTS reagent as described previously (Padmini et al. 2008a). The degree of free radical quenching in individual samples is indicative of its antioxidant potential. The TAC of the samples were quantified by comparing with the traditional standard trolox and the assay results were expressed in terms of millimole of trolox equivalent per liter.

3-(4,5-Dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide assay

The survivality of liver mitochondria was determined by the 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium (MTT) assay (Mosmann 1983). The mitochondrial suspension obtained by centrifugation was resuspended in phosphate-buffered saline (PBS) buffer and serially diluted to a concentration of 200 μg of mitochondrial protein per milliliter of mitochondrial suspension using the same buffer system. One-hundred microliters of the dilutions were plated out into the wells of a microtiter plate in duplicates. In the control wells, PBS alone was incubated to provide the blanks for absorbance reading. Ten microliters of MTT reagent was added to each well including controls. After incubation for 1 h, the visualized purple precipitate (formazan product) was solubilized with 1 ml acidic isopropanol. The plates were covered and left in the dark for 4 h at room temperature. Absorbance was measured at 560 nm in a spectrophotometer. The relative survivality was calculated by dividing the optical density of sample by the optical density of control well having the PBS buffer solution with MTT reagent and multiplying by 100.

Coimmunoblot analysis of HSP70, ASK1, and Trx

The fish liver mitochondrial protein aliquots containing 50- μg proteins were ran on two 10% sodium dodecyl sulfate-polyacrylamide gels that were placed together in the dual-gel electrophoretic system according to the method of Laemmli (1970). Both the gels were then blotted on to polyvinylidene fluoride (PVDF) membranes (BioTrace PVDF 0.4 μm , Pall Corporation, Germany) according to the method of Towbin et al. (1979) after which the blotted membranes were blocked with 5% (w/v) nonfat dry milk in 0.1 M phosphate-buffered saline–Tween20 buffer (PBS–Tween buffer) pH 7.4 containing 5 mM sodium azide for 2 h at 25°C. Both the blotted membranes were cut into two equal parts. One part of the blotted membrane was thoroughly washed and then incubated with mouse monoclonal antibody

against HSP70 alkaline-phosphatase-conjugated (Stressgen SPA810) for 3 h at 25°C. The 3-bromo-4-chloro-indolylphosphate–nitroblue tetrazolium (BCIP–NBT) substrate (Sigma, St. Louis, MO, USA) system was used to detect the alkaline phosphatase conjugate as described by the manufacturer for the quantification of specific immunoreactive proteins. Another part of the blot was incubated with rabbit polyclonal antibody against ASK1 diluted 1:3,000 (AAP-480) for 3 h at 25°C. The membrane was then incubated with appropriately diluted alkaline-phosphatase-labeled goat antimouse IgG secondary antibody prepared in PBS–Tween buffer. The BCIP–NBT substrate system was used to detect the bands as described above. The remaining blot sheet was thoroughly washed and then incubated with mouse monoclonal antibody against Trx diluted 1:3,000 (MSA-150) for 3 h at 25°C. The membrane was then incubated with appropriately diluted alkaline-phosphatase-labeled goat antimouse IgG secondary antibody prepared in PBS–Tween buffer. In the remaining blot sheet, the immunoblotting was performed for β -actin by incubating it with rabbit polyclonal β -actin antibody diluted 1:3,000 (CSA-400) for 3 h at 25°C followed by secondary antibody treatment and color development using BCIP–NBT substrate system. The band intensities were scanned with the HP Scan Imager and quantified using the Total Lab Software, GELS, USA. The results were confirmed by individually performing the blotting studies of HSP70, ASK1, and Trx. The results obtained for HSP70 were verified with the results obtained by the quantification of mtHSP70 by ELISA using HSP70 ELISA kit (EKS 700B).

Statistical analysis

Data were analyzed using commercially available statistical software package (SPSS for windows 7.5 version). Wilcoxon Mann Whitney test was performed to find out the significance of variations between the polluted-site and unpolluted-site fish samples and Spearman correlation test was conducted for correlation analysis. Results were presented as mean $\pm$ SD. A p value <0.05 was considered to be statistically significant.

Results

Oxidative stress in response to environmental pollutants

The level of MDA, a measure of lipid peroxidation, was increased significantly in Ennore estuary fish when compared to liver mitochondria of fish collected from Kovalam estuary (42%; $*p<0.05$). The significant increase in LPO levels indicates a substantial increase in oxidative stress in the fish samples collected from the polluted site. In the fish liver mitochondria from Ennore estuary, the level of TAC was severely affected which is indicated by a significant decrease in their values to an extent of 24% when compared to the fish collected from unpolluted site ($*p<0.05$ Table 1). The results indicate elevated oxidative stress condition and defective antioxidant status in liver mitochondria of fish that inhabits the highly contaminated site.

Environmental stress decreases the viability of mitochondria

The viability of the fish liver mitochondria was assayed to determine the role of environmental pollutant induced stress on the cytotoxicity of liver cells. The mitochondrial viability percentage displayed a moderate variation between the two study sites (insignificant, Fig. 1). In Ennore estuary, the mitochondrial survivality is about 72% and dropped to an extent of 18% when compared with the Kovalam estuary (84%). The result demonstrated in the study suggests that there exists a factor in the mitochondrial system which would promote the survivality of mitochondria under such high oxidative stress conditions.

Environmental stress induced elevation of mtHSP70 levels

To evaluate the antistress mechanism developed by *M. cephalus* in response to stress situation, the levels of mtHSP70 were determined in liver mitochondria of fish from Kovalam and Ennore estuaries. The expression of mtHSP70 was markedly increased in response to environmental pollutant induced oxidative stress. In Ennore estuary samples,

Table 1 Effect of pollution induced stress on the levels of lipid peroxide (LPO), total antioxidant capacity (TAC) and mitochondrial HSP70 (mtHSP70) levels in fish liver mitochondria collected from polluted and unpolluted sites

Parameters	Fish from unpolluted site	Fish from polluted site
Lipid peroxides, nanomoles of MDA per milligram of mitochondrial protein	1.67 $\pm$ 0.116	2.37 $\pm$ 0.14*
Total antioxidant capacity, milliequivalents per liter	0.87 $\pm$ 0.058	0.70 $\pm$ 0.097*
mtHSP70, nanograms of HSP70 per milligram of mitochondrial protein	4.46 $\pm$ 0.26	6.27 $\pm$ 0.21*

Data were mean $\pm$ SD of 16–20 fish per estuary for LPO and TAC estimations and data were mean $\pm$ SD of eight to ten fish per estuary for HSP70 estimation

* $p<0.05$, statistically significant versus unpolluted site fish

Fig. 1 Effect of pollution induced stress on the mitochondrial survival percentage of the isolated fish liver mitochondria collected from polluted and unpolluted sites. Data were mean $\pm$ SD of 16–20 fish per estuary. *NS* statistically nonsignificant versus unpolluted-site fish

the level of mtHSP70 was augmented by 31% when compared to Kovalam estuary samples ($*p<0.05$ Table 1). The defective antioxidant status which is not sufficient to cope up with increased oxidative stress would evoke the cellular response that leads to elevated mtHSP70 levels in Ennore estuary fish sample. Hence, increase in HSP70 observed in response to stress situation may exert its protective effect against stress-induced damage and ultimately aid survival.

Pollution stress induced HSP70 overexpression mitigates stress induced ASK1 expression by elevating Trx expression

The expression of ASK1 (Fig. 2) under contaminated stressed condition has increased to a small extent in fish samples collected from Ennore estuary when compared with the unpolluted Kovalam estuary fish sample which was insignificant. But the Trx expression in Ennore estuary fish liver mitochondria was significantly increased when compared to the results obtained from Kovalam estuary samples (Fig. 3 $*p<0.05$). In order to analyze the factors behind the cytoprotective nature of HSP70, the expression levels of mtHSP70 were assessed and correlated with the expression levels of ASK1 and Trx. The insignificant alteration in ASK1 level along with comparatively significant increase in Trx levels may have a positive role to play with mtHSP70 in promoting the cell survival under polluted conditions.

Correlation studies between the oxidative stress parameters, mitochondrial viability percentage, HSP70, ASK1, and Trx levels

The Spearman rank correlation test was done for the variable LPO in comparison with the TAC, mitochondrial viability percentage, mtHSP70, Trx and ASK1 values. It was also carried out for the variable mtHSP70 in comparison with TAC, mitochondrial viability percentage, Trx,

Fig. 2 Effect of pollution induced stress on the expression of ASK1 in fish liver mitochondria collected from both polluted and unpolluted sites. *C*—unpolluted site fish; *T*—polluted site fish. *Bar graph* representing the quantity of ASK1 in the fish liver mitochondria of eight to ten fish experiments that are inhabiting Ennore estuary compared with the values obtained from the experiments using Kovalam fish. Data were mean $\pm$ SD of eight to ten fish per estuary. *NS* statistically nonsignificant versus unpolluted-site fish

and ASK1 values (Table 2). A significant positive correlation between the level of LPO–mtHSP70, LPO–Trx, and mtHSP70–Trx and a significant negative correlation between the level of LPO–TAC and mtHSP70–TAC were observed in polluted-site fish liver mitochondria ($*p<0.05$ Table 2). In the analysis, the correlation between the levels of LPO–mitochondrial viability percentage, mtHSP70–

Fig. 3 Effect of pollution induced stress on the expression of Trx in fish liver mitochondria. *C*—unpolluted site fish; *T*—polluted site fish. *Bar graph* representing the quantity of Trx in the fish liver mitochondria of eight to ten fish experiments that are inhabiting Ennore estuary compared with the values obtained from the experiments using Kovalam fish. Data were mean $\pm$ SD of eight to ten fish per estuary. $*p<0.05$, statistically significant versus unpolluted-site fish

Table 2 Spearman rank correlation coefficient between the LPO, TAC, mitochondrial survivality percentage and mtHSP70 levels ($n=20$)

Variable1	Variable 2	r_s	p
LPO	TAC	-0.387	* p
	Mitochondrial viability %	-0.020	^{NS} p
	mtHSP70	0.385	* p
	Trx	0.395	* p
	ASK1	-0.063	^{NS} p
mtHSP70	TAC	-0.387	* p
	Mitochondrial viability %	0.134	^{NS} p
	Trx	0.450	* p
	ASK1	0.113	^{NS} p

NS nonsignificant

* $p<0.05$

mitochondrial viability percentage, LPO–ASK1 and mtHSP70–ASK1 was found to be insignificant.

Discussion

Domestic sewage, combustion emission, mining operations, metallurgical activities, and industrial effluents are among the sources of anthropogenic metal inputs and heavy metals such as Pb, Cd, Cu, and Zn are released as a result of these processes (Chinni and Yallapragda 2000). Estuarine and coastal environments in particular are affected by a wide range of pollutants due to increased anthropogenic activity from a burgeoning population in the coastal zones. The Ennore estuarine region, the study site of the present investigation, is known to be highly polluted due to increasing industrial activities (Padmini and Sudha 2004). In the last few decades, attempts were made regarding the evaluation of metal pollution, measurement of metal-pollution-induced oxidative stress, and the expression of HSP70 in *M. cephalus* surviving in this polluted estuary (Padmini et al. 2008b; Padmini and Usharani 2008).

In Ennore estuary fish liver mitochondrial samples, the comparison of LPO values with the result of the TAC values showed that the TAC level decreases proportionately with increased levels of LPO reflecting the defective antioxidant status when compared with Kovalam estuary samples (Table 1 * $p<0.05$). The negative correlation that is shown to exist between the LPO and TAC levels may be due to the presence of heavy metals in the Ennore estuary. Under such oxidative stress conditions, the toxic effects of the pollutants may overwhelm the antioxidant defenses (Bebianno et al. 2004). The results suggest that the elevated generation of reactive oxygen species in the mitochondria causes prooxidative state that damages mitochondria and

leads to a loss in cell viability in the fish collected from Ennore estuary.

In these samples, about 42% increase in LPO levels and 24% decrease in TAC levels were observed (Table 2). Under such high oxidative stress conditions, there was only 18% decrease in mitochondrial survivality percentage (insignificant; Fig. 1) which confirms the presence of factors that decrease the mitochondrial survivality percentage to a smaller extent and promotes the survival of fish.

Mitochondria are the main energy-transducing organelles of eukaryotic cells and play a crucial role in the cellular metabolism (Lill and Kispaal 2000). Hence, the maintenance of mitochondrial activity by preserving protein content and function is essential under stressed conditions. From the quantification of mtHSP70 expression levels (Fig. 2), it is found that there is about 31% increase in mtHSP70 levels in the polluted estuary inhabiting fish liver mitochondria when compared to the unpolluted estuary fish sample. In the polluted estuary inhabiting fish, the oxidative stress is in excess to be compensated by the fish antioxidant machinery and the overexpression of mtHSP70 is meant to repair the excessive oxidative damage of proteins caused by the pollutants.

HSPs have a role to play in long-term adaptation and increased stress tolerance (Morimoto and Santoro 1998). The mitochondria also depend on the import transport of numerous proteins from the cytosol which relies on mitochondrial chaperones including HSP70, as well as specific import machinery including the translocases of the inner membrane and outer membrane (Neupert 1997; Wickner and Schekman 2005). Zhao et al. (2007) showed that HSP70 contributes to thermotolerance, as the elevated HSP70 levels decrease cell sensitivity to heat stress and protect the cell against toxicity induced by tumor necrosis factor, ultraviolet ray, oxidative stress, and some chemicals. Most of the studies show that the level of HSP70 induction is correlated positively with the degree of cell tolerance against temperature and oxidative stress but the potential mechanism responsible for the protective effects of HSP70 on cell survivality remains unexplored.

In the Ennore estuary sample, the positive correlation that exists between LPO and mtHSP70 values proved that under high oxidative stress conditions, the cellular stress response in terms of HSP70 would get elevated in order to counteract the stress situation. In addition, the comparison between the mtHSP70 expression levels and the TAC levels shows a significant negative correlation which proves that the elevated expression of mtHSP70 (31% increase when compared to Kovalam estuary) is meant to cope up with the imbalance in antioxidant machinery and to promote the survival of mitochondria under contaminated stress condition (Table 2). But the results obtained from Kovalam-estuary-inhabiting fish shows comparatively less mtHSP70

levels indicating that these species did not experience significant pollution induced stress.

In the polluted site fish liver mitochondria, the insignificant reduction in the mitochondrial viability may be due to the cytoprotective strategy exhibited by mtHSP70 (Table 2). As HSP70 is thought to protect cells against cellular stress, the adaptive role of elevated levels of HSP70 in Ennore estuary fish liver mitochondria was correlated with ASK1 and Trx expression levels.

Trx can exert its protective function either directly as an antioxidant by reducing the activity of oxidized Trx peroxidase to scavenge ROS such as H₂O₂ or indirectly by binding to signaling components or modulating their function (Nordberg and Arner 2001). ASK1 is one of the transcription factors involved in signal transduction that are sensitive to regulation by reactive species. Trx-ASK1 complex preexists in the normal unstressed cells and appears to be the target of many extracellular stimuli such as ROS that activates ASK1 by disrupting the Trx-ASK1 complex (Liu et al. 2000). The complexity of Trx with the N terminus of ASK1 determines the ROS-mediated regulation of p38 MAPK and JNK pathways (Saitoh et al. 1998). Further, Trx is found to be identified as direct physiological inhibitor of ASK1 and plays an important role in the maintenance of cellular redox balance (Adler et al. 1999; Salinas and Wong 1999).

The individual comparison of LPO levels and the mtHSP70 levels against the Trx levels in the fish collected from Ennore estuary shows a significant positive correlation. Hence, the simultaneous elevation in Trx levels along with LPO and mtHSP70 would inhibit the activity of ASK1. The above fact was also confirmed by the insignificant correlation that exists between LPO-ASK1 and mtHSP70-ASK1 (Table 2). The inhibitory effect of increased levels of mtHSP70 may modulate the level of ASK1 in the polluted-site samples. Consistent with this, it has been reported that the biological function of ASK1 is negatively regulated by ASK1-interacting proteins including thioredoxin, protein serine/threonine phosphatase 5, glutathione *S*-transferase, 14-3-3 protein, Akt, and HSP70 (Morita et al. 2001; Cho et al. 2001).

When compared to Kovalam estuary fish, the minimal increase in ASK1 levels (about 13%) in Ennore estuary fish liver mitochondria may be a protective mechanism provided by elevated levels of mtHSP70 (31%) and Trx (22%). Leppa et al. 1997 reported that the kinetics of the transcriptional induction of Trx was similar to that of HSP70. It was demonstrated that in the central nervous and the cardiovascular system, the preconditioning adaptation by means of HSP overexpression may induce the expression of Trx as a compensatory mechanism for cell survival (Chiueh et al. 2005). It was also reported that the Trx enables mitochondria to communicate with the cytosol in a

wide range of basic cellular reactions (energetics, metabolism, and protein synthesis) in response to change in environment (adaptation to stress; Balmer et al. 2004).

The exposure of cells to pollution could generate oxidative stress which may transmit signals that separate the Trx from stress-activated signal molecules thereby promoting the activation of ASK1. The chaperones of the HSP70 family may inhibit the activity of ASK1 by having physical association thereby inhibiting the homo-oligomerization of the kinase and act as an endogenous inhibitor of ASK1 (Park et al. 2002). As HSP70 may inhibit the activity of ASK1, the expression of ASK1 may be downregulated and hence there is only 13% increase in its expression levels. The evidence presented by the above study indicates that the overexpression of HSP70 along with the elevated expression of Trx could mitigate ASK1 expression to prevent stress-induced cellular injury thereby promoting cell survival in Ennore estuary fish.

In Ennore estuary fish liver mitochondria, the 42% increase in oxidative stress along with significantly decreased TAC levels (24% decrease) induces about 31% increase in mtHSP70. The increased mtHSP70 levels associated with 22% increase in Trx expression levels may be the reason for minimal increase in ASK1 levels (only by 13% increase) which would lead to minimal reduction of mitochondrial survivability percentage.

Conclusion

The present study clearly demonstrates an interesting correlation between HSP70 and Trx levels along with ASK1 expression which may enable the fish cells to resist stress and promote survival during pollution. The elevated levels of mtHSP70 may play a key role in environmental adaptation process in the gray mullets that are inhabiting Ennore estuary and protect the cell from oxidative stress in natural field conditions. The study of the role of Trx and ASK1 in promoting the cytoprotective function of mtHSP70 might be helpful in understanding the adaptive mechanism followed by the gray mullet inhabiting the stressed condition that promotes the cell survival against oxidative stress conditions. The inhibitory effect of increased level of HSP70 along with Trx on the expression of ASK1 appears to be a major contributor to the acquired tolerance against pollution stress and the adaptation of fish in contaminated Ennore estuary.

Acknowledgement This study is a part of the DST Project Ref. No. DST: SP/SO/AS-10/2003 funded by the Ministry of Science and Technology, Government of India. The Council of Scientific and Industrial Research is acknowledged with thanks for providing Senior Research Fellowship to B. Vijaya Geetha.

References

- Adler V, Yin Z, Fuchs SY, Benezra M, Rosario L, Tew KD, Pincus MR, Sardana M, Henderson CJ, Wolf CR, Davis RJ, Ronai Z (1999) Regulation of JNK signaling by GSTp. *EMBO J* 18:1321–1334 doi:10.1093/emboj/18.5.1321
- Balmer Y, Vensel WH, Tanaka CK, Hurkman WJ, Gelhaye E, Rouhier N, Jacquot JP, Manieri W, Schtermann P, Droux M, Buchanan BB (2004) Thioredoxin links to the regulation of fundamental processes of plant mitochondria. *Proc Natl Acad Sci USA* 101:2642–2647 doi:10.1073/pnas.0308583101
- Basu N, Nakano T, Grau EG, Iwama GK (2001) The effects of cortisol on heat shock protein 70 levels in two fish species. *Gen Comp Endocrinol* 124:97–105 doi:10.1006/gcen.2001.7688
- Bebiano MJ, Geret F, Hoarau P, Serafim MA, Coelho MR, Gnassia-Barelli M, Romeo M (2004) Biomarkers in *Ruditapes decussatus*: a potential bioindicator species. *Biomarkers* 9:305–330 doi:10.1080/13547500400017820
- Bolliger C, Deloche O, Glick BS, Georgopoulos C, Jenö P, Kronidou N, Horst M, Morishima N, Schatz GA (1994) The mitochondrial homolog of bacterial GrpE interacts with mitochondria Hsp70 is essential for viability. *EMBO J* 13(8):1998–2006
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254 doi:10.1016/0003-2697(76)90527-3
- Chen Y, Cai J, Murphy TJ, Jones DP (2002) Overexpressed human mitochondrial thioredoxin confers resistance to oxidant induced apoptosis in human osteosarcoma cells. *J Biol Chem* 277:33242–33248 doi:10.1074/jbc.M202026200
- Chinni S, Yallapragda R (2000) Toxicity of copper, cadmium, zinc and lead to *Penaeus indicus* postlarvae: effects of individual metals. *J Environ Biol* 21:255–258
- Chiueh CC, Andoh T, Chock PB (2005) Induction of thioredoxin and mitochondrial survival proteins mediates preconditioning induced cardioprotection and neuroprotection. *Ann N Y Acad Sci* 1042:403–418 doi:10.1196/annals.1338.034
- Cho SG, Lee YH, Park HS, Ryoo K, Kang KW, Park L (2001) Glutathione S-transferase Mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. *J Biol Chem* 276:12749–12755 doi:10.1074/jbc.M005561200
- Erel O (2004) A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem* 37:277–285 doi:10.1016/j.clinbiochem.2003.11.015
- Farombi EO, Adelowo OA, Ajimoko YR (2007) Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African catfish (*Clarias gariepinus*) from Nigeria Ogun River. *Int J Environ Res Public Health* 4(2):158–165 doi:10.3390/ijerph2007040011
- Fischer W, Bianchi G (1984) FAO identification sheets for fishery purposes. Western Indian Ocean. FAO, Rome
- Guo S, Wharton W, Moseley P, Shi H (2007) Heat shock protein 70 regulates cellular redox status by modulating glutathione related enzyme activities. *Cell Stress Chaperones* 12(3):245–254 doi:10.1379/CSC-265.1
- Gutterman DD (2005) Mitochondria and reactive oxygen species: an evolution in function. *Circ Res* 97:302–304 doi:10.1161/01.RES.0000179773.18195.12
- Hsieh CC, Papaconstantinou J (2006) ThioredoxinASK1 complex levels regulate ROS-mediated p38 MAPK pathway activity in livers of aged and long-lived snell dwarf mice. *FASEB J* 20:259–268 doi:10.1096/fj.05-4376com
- Johnson D, Lardy H (1967) Isolation of mitochondria. *Methods Enzymol* 10:94–96 doi:10.1016/0076-6879(67)10018-9
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680 doi:10.1038/227680a0
- Leppä S, Pirkkala L, Chow SC, Eriksson JE, Sistonen L (1997) Thioredoxin is transcriptionally induced upon activation of heat shock factor 2*. *J Biol Chem* 272:30400–30404 doi:10.1074/jbc.272.48.30400
- Lill R, Kispal G (2000) Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem Sci* 25:352–356 doi:10.1016/S0968-0004(00)01589-9
- Liu H, Nishitoh H, Ichijo H, Kyriakis JM (2000) Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. *Mol Cell Biol* 20:2198–2208 doi:10.1128/MCB.20.6.2198-2208.2000
- Lykkesfeldt J (2001) Determination of malondialdehyde as dithiobarbituric acid adduct in biological samples by HPLC with fluorescence detection: comparison with ultraviolet visible spectrophotometry. *Clin Chem* 47(9):1725–1727
- Morimoto RI, Santoro MG (1998) Stress inducible responses to heat shock proteins: new pharmacological targets for cytoprotection. *Nature Biotechnol* 16:833–838 doi:10.1038/nbt0998-833
- Morita K, Saitoh M, Tobiume K, Matsuura H, Enomoto S, Nishitoh H, Ichijo H (2001) Negative feedback regulation of ASK1 by protein phosphatase 5 (PP5) in response to oxidative stress. *EMBO J* 20:6028–6036 doi:10.1093/emboj/20.21.6028
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63 doi:10.1016/0022-1759(83)90303-4
- Neupert W (1997) Protein import into mitochondria. *Annu Rev Biochem* 66:863–917 doi:10.1146/annurev.biochem.66.1.863
- Nisoli E, Carruba MC (2006) Nitric oxide and mitochondrial biogenesis. *J Cell Sci* 119:2855–2862 doi:10.1242/jcs.03062
- Nordberg J, Arner ES (2001) Reactive oxygen species, antioxidants and the mammalian Thioredoxin system. *Free Radic Biol Med* 11:1287–1312 doi:10.1016/S0891-5849(01)00724-9
- Padmini E, Kavitha M (2003) Seasonal pollution assessment through comparative hydrobiological studies in Ennore and Kovalam estuaries. *Ind Hydrobiol* 6:139–144
- Padmini E, Sudha D (2004) Environmental impact on gill mitochondrial function in *Mugil cephalus*. *Aquacult* 5(1):89–92
- Padmini E, Usharani M (2008) Impact of seasonal variation on HSP70 expression quantitated in stressed fish hepatocytes. *Comp Biochem Physiol Part B* 151:278–285 doi:10.1016/j.cbpb.2008.07.011
- Padmini E, Vijaya Geetha B (2007a) Seasonal influences on hydrographic parameters and pollution status of Ennore estuary. *J Environ Hydrol* 15:paper 15
- Padmini E, Vijaya Geetha B (2007b) A comparative seasonal pollution assessment study on estuary with respect to metal accumulation in *Mugil cephalus*. *Oceanol Hydrobiol Stud* 35:91–103 doi:10.2478/v10009-007-0031-4
- Padmini E, Usha Rani M, Vijaya Geetha B (2008a) Differential HSP90α expression in fish hepatocytes from polluted estuary during summer. *Fisheries Sci* 74(5):1118–1126 doi:10.1111/j.1444-2906.2008.01631.x
- Padmini E, Usha Rani M, Vijaya Geetha B (2008b) Studies on antioxidant status in *Mugil cephalus* in response to heavy metal pollution at Ennore estuary. *Environ Monit Assess.* doi:10.1007/s10661-008-0430-3
- Padmini E, Vijaya Geetha B, Usha Rani M (2008c) Liver oxidative stress modulation shows seasonal impact in *Mugil cephalus*. *Braz J Med Biol Res* 41:951–955
- Padmini E, Vijaya Geetha B, Usha Rani M (2009) Pollution induced oxidative stress and heat shock protein 70 overexpression in fish liver mitochondria. *Sci Tot Environ* 407:1307–1317

- Park HS, Cho SG, Kim CK, Hwang HS, Noh KT, Kim MS, Huh SH, Kim MJ, Ryoo K, Kim EK, Kang WJ, Lee JS, Seo JS, Ko YG, Kim S, Choi EJ (2002) Heat shock protein Hsp72 is a negative regulator of apoptosis signal-regulating kinase 1. *Mol Cell Biol* 22:7721–7730 doi:[10.1128/MCB.22.22](https://doi.org/10.1128/MCB.22.22)
- Pouis G, Montfort WR (2001) Properties and biological activities of thioredoxins. *Annu Rev Pharmacol Toxicol* 41:261–295 doi:[10.1146/annurev.pharmtox.41.1.443](https://doi.org/10.1146/annurev.pharmtox.41.1.443)
- Ryan JA, Hightower LE (1994) Evaluation of heavy-metal ion toxicity in fish cells using a combined stress protein and cytotoxicity assay. *Environ Toxicol Chem* 13:1231–1240 doi:[10.1897/1552-8618\(1994\)13\[1231:EOHITI\]2.0.CO;2](https://doi.org/10.1897/1552-8618(1994)13[1231:EOHITI]2.0.CO;2)
- Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, Kawabata M, Miyazono K, Ichijo H (1998) Mammalian thioredoxin is a direct inhibitor of apoptosis signal regulating kinase (ASK1). *EMBO J* 17:2596–2606 doi:[10.1093/emboj/17.9.2596](https://doi.org/10.1093/emboj/17.9.2596)
- Sakurai K, Cederbaum AI (1998) Oxidative stress and cytotoxicity induced by ferric-nitrosyltriacetate in Hepg2 cells that express Cytochrome P450 2E1. *Mol Pharmacol* 54(6):1024–1035
- Salinas AE, Wong MG (1999) Glutathione S-transferases—a review. *Curr Med Chem* 6:279–309
- Sanders BM, Martin LS (1993) Stress proteins as biomarkers of contaminant exposure in archived environmental samples. *Sci Tot Environ* 139–140:459–470 doi:[10.1016/0048-9697\(93\)90043-6](https://doi.org/10.1016/0048-9697(93)90043-6)
- Schatz G, Dobberstein B (1996) Common principles of protein translocation across membrane. *Science* 271:1519–1526 doi:[10.1126/science.271.5255.1519](https://doi.org/10.1126/science.271.5255.1519)
- Simon MM, Reikerstorfer A, Schwatz A, Luger TA, Jaattela M, Schwatz T (1995) Heat shock protein 70 overexpression affects the response to ultraviolet light in murine fibroblasts. Evidence for increased cell viability and suppression of cytokine release. *J Clin Invest* 95:926–933 doi:[10.1172/JCI117800](https://doi.org/10.1172/JCI117800)
- Thannickal VJ, Fanburg BL (2000) Reactive oxygen species in cell signaling. *Am J Physiol* 279:L1005–L1028
- Tiano L, Davies I, Craft J, Falcioni G (2004) Analysis of stress induced gene expression in trout red blood cells following tributyltin chloride exposure. *Fish Physiol Biochem* 30:231–240 doi:[10.1007/s10695-005-8244-5](https://doi.org/10.1007/s10695-005-8244-5)
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–4354 doi:[10.1073/pnas.76.9.4350](https://doi.org/10.1073/pnas.76.9.4350)
- Wickner W, Schekman R (2005) Protein translocation across biological membranes. *Science* 310:1452–1456 doi:[10.1126/science.1113752](https://doi.org/10.1126/science.1113752)
- Zhao Y, Wang W, Qian L (2007) Hsp70 may protect cardiomyocytes from stress-induced injury by inhibiting Fas-mediated apoptosis. *Cell Stress Chaperones* 12:83–95 doi:[10.1379/CSC-231R.1](https://doi.org/10.1379/CSC-231R.1)