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Stress-Protein Response in Tributyltin-Exposed Clams

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The heat-shock proteins (HSPs) or stress proteins are a family of diverse proteins containing both constitutive and stress-induced members (Hightower, 1991). Under normal conditions, these proteins are involved in the transport, folding and assembly of newly synthesised proteins; under adverse environmental situations, their synthesis increases and they act to repair and protect cellular proteins and to minimise protein aggregation (Cheng et al. 1989; Chiang et al. 1989; Martin et al. 1992; Kim et al. 1993). The most abundant and best studied groups are the 70 kDa (HSP70) stress protein family and the 60 kDa (HSP60) family. Both of them are known to be induced in response to a wide variety of physical and chemical stressors, including UV light, salinity, temperature, anoxia, but also oxidizing agents, heavy metals, and organic xenobiotics (Sanders, 1990). Thus, over the past decade, an increasing number of studies have explored the possibility of using HSPs as biomarkers of adverse effects at the cellular level and some authors have proposed that the induction and subsequent accumulation of HSPs may be useful in environmental monitoring (Sanders, 1990; Sanders and Martin, 1993). The use of HSPs as biomarkers has been assessed in laboratory and field experiments, and their induction by metals and other environmental contaminants or physical parameters has been demonstrated in molluscs (Hightower, 1993; Roberts et al. 1997).

Tributyltin (TBT) is used as a biocide in a variety of consumer and industrial products and it enters the aquatic environment mainly via leaching from antifouling paints. Molluscs are among the most sensitive species to TBT exposure. A variety of toxic effects have been described, viz. shell malformations in oysters, reduced scope for growth, population decline, etc. (Alzieu, 1991; Widdows and Page, 1993). Potential reproductive impairment occurs at trace concentrations of few nanograms of TBT per litre which are responsible of causing imposex in neogastropods (Bryan et al. 1986). Thus, there is a great need of looking for early warning stress signals in TBT exposed molluscs.

This study was designed to assess the usefulness of two major stress proteins, HSP-70 and HSP-60, as a diagnostic tool to screen cell injury caused by tributyltin (TBT) exposure in *clams -Ruditapes decussata-*. To this end, clams were exposed to different doses of TBT (91, 454 and 2268 ng/l as Sn) for seven

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days, and the relationship between accumulation of TBT and levels of stress proteins determined. In a second experiment, clams collected from an unpolluted environment were transplanted to a TBT-polluted marina for a period up to 5 weeks and both bioaccumulation of organotin compounds and HSP response monitored over time.

MATERIALS AND METHODS

Clams *(Ruditapes decussata)* were collected in February 1996 from the bivalve farms located in the Ebro Delta (NE Spain), a non-polluted environment in terms of organotin compounds (Morcillo et al. 1997). For the laboratory exposure experiment, four groups of approximately 90 specimens each were placed in four glass aquaria filled with 45 1 of filtered seawater fitted with constant air bubbling. Water temperature was maintained at 14-15°C. TBT was dissolved in acetone and the necessary amount of the stock solutions added to the aquaria in order to get 91, 454 and 2268 ng/l of TBT as Sn. Acetone at a concentration of 4 µl/l was added to the control tank. Water was changed daily and fresh TBT added. Animals were fed every 48 h with a commercial available plankton preparation. For the transplant experiment, clams were randomly selected and divided into four groups of 60-75 specimens each. Those used as control were immediately taken to the laboratory and the other three groups were placed in net bags and transplanted to El Masnou, a recreational marina that has consistently showed high organotin pollution (Tolosa et al. 1992; Morcillo et al. 1997). Organisms were kept buried in the sediment and sampled 1, 3 and 5 weeks after transplant.

For stress protein analysis, gills were immediately dissected out, frozen in liquid nitrogen and stored at -80°C. Gills were homogenised in 6 volumes (w/v) of calcium-magnesium free saline (CMFS) buffer pH 7.3 (20 mM HEPES, 500 mM NaCl, 12.5 mM KCl) containing 1% Nonidet P-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg/ml trypsin inhibitor. The homogenate was centrifuged at 19,000 g for 90 min at 4°C and the remaining supernatant frozen at -80°C until analysis. Proteins were determined by the method of Lowry et al. (195 1).The homogenate was thawed and samples of equivalent total protein content (2.5 mg/ml) were boiled for 5 min in SDS-PAGE sample buffer (Laemmli, 1970) and loaded for their separation on 10% polyacrylamide gels topped by 3% polyacrylamide stacking gels. After electrophoresis, separated proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham) using a buffer containing 150 mM glycine in 20 mM Tris and 20% methanol (v/v). Transfer was carried out for 30 min at room temperature using a Trans-Blot semi-dry cell (Bio-Rad). The nitrocellulose membranes were then blocked for 30 min in Tris buffered saline pH 8.0 (TBS) which contained 0.2% Tween 20, 0.5% gelatine and 0.1% sodium azide. The membranes were probed using two monoclonal antibodies directed against bovine brain HSP-70 (Sigma H-5147) and human HSP-60 (Sigma H-3524). Blots were incubated overnight at room temperature and rinsed three times with TBS containing 0.2% Tween 20 and 0.5% gelatin. The membrane was incubated for one hour in a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma Chemical Co.). Excess of secondary antibody was removed by a further three washes in 0.2% Tween 20 in TBS and the sites of binding of the

antibody visualised by incubation with the substrates *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate. When the optimal signal-background ratio had been achieved, rinsing the membrane in water stopped the development reaction. A clam -*Ruditapes decussata*- protein standard (heat-shocked organisms) was included on every blot, thus results from different blots could be compared. Western blots were quantitated by scanning with a Laser Densitometer (Epson GT-8000) and the absorbance of the samples expressed as percents of the standard.

Organotin analyses of exposed and transplanted clams is reported in Morcillo et al. (1998) and Morcillo and Porte (2000) respectively.

One-way ANOVA analysis were performed using Sigmastat program. A Student-Newman-Keuls method was used and when $p<0.05$, considered significant.

RESULTS AND DISCUSSION

In the laboratory experiment, immunoblotting of gill tissue extracts with the HSP-60 monoclonal antibody showed specific cross-reactivity with one single 60 kDa peptide, much more evident in TBT-exposed organisms than in controls. Semiquantitative western blot analysis (Table 1) indicated low levels of HSP-60 in control organisms and a dose-dependent HSP-60 increase, up to 3.8-fold, in TBT-exposed clams. Nevertheless, differences were not statistically significant due to high interindividual variability. Western blots incubated with the anti-HSP70 antibody detected two bands at approximately 78 and 72 kDa in the heat-shocked clams used as control, and only the lower molecular weight band (approx. 72 kDa) in blots from control and TBT-exposed clams. It was observed a relative increase, up to 2-fold of this band, but this difference was not statistically significant either (Table 1). Bioconcentration of TBT and its metabolites dibutyltin (DBT) and monobutyltin (MBT) was monitored. MBT and DBT represented less than 10% of total butyltins, thus only the concentration of TBT -the most toxic compound- is reported in Table 1. TBT was bioaccumulated by clams in a dose-dependent manner and tissue residues increased up to 120-fold in the high-dose exposed group (Morcillo et al. 1998).

In the field transplant study, similarly to the laboratory observations, only a band was observed after incubation with HSP-60. Semiquantitative image analysis of this band revealed a time-dependent increase on HSP-60 levels and the highest content observed the fifth week of transplant (Table 2). In contrast, HSP-70 protein was rarely detected, levels were very low and not clear trend was evidenced along the transplant experiment (Table 2). The bioaccumulation of TBT was also monitored, it increased from 5 ng/g wet weight at time zero to 290 ng/g wet weight 3 weeks after transplant (Morcillo and Porte, 2000).
Evidence on HSP-60 induction has been observed in TBT-exposed clams, both in laboratory and field conditions, and a relationship between TBT body burden and HSP-60 levels was observed (Fig. 1). However, levels of HSP-70 in gills did not reflect TBT exposure, and the 2-fold HSP-70 increase recorded in the laboratory was not confirmed in the transplant experiment. Contradictory results have been

reported in terms of HSP-70 induction, viz. HSP-70 levels increased in juvenile mussels feed with TBT adsorbed to algae (Steinert and Pickwell, 1993), but no response was observed in mussels *(Mytilus edulis)* and oysters *(Crassostrea virginica)* exposed to 0.7 µg/l TBT (Pickwell and Steinert, 1988).

Table 1. Whole tissue residues of TBT and relative levels of HSP-60 and HSP-70 in gills of *Ruditapes decussata* exposed to different concentrations of TBT in water for a period of seven days.

TBT	TBT[a]	HSP-60[b]	HSP-70[b]
Control	6.8 ± 0.5	16.5 ± 11.4	4.3 ± 2.3
Low (90 ng/l as Sn)	52.8 ± 1.6	37.6 ± 20.2	10.0 ± 5.4
Medium (454 ng/l as Sn)	246.8 ± 3.5	47.2 ± 21.7	8.8 ± 3.7
High (2268 ng/l as Sn)	831.5 ± 77.5	63.0 ± 28.9	9.8 ± 3.8

[a] values are mean ± SEM (n=3) in ng/g w.w. as Sn. Data from Morcillo et al. (1998).

[b] values are mean ± SEM (n=6) in arbitrary units/µg protein

Table 2. Whole tissue residue of organotin compounds and PAHs, and relative levels of HSP-60 and HSP-70 in gills of *Ruditupes decussatu* transplanted to a TBT-polluted marina for a period up to 5 weeks.

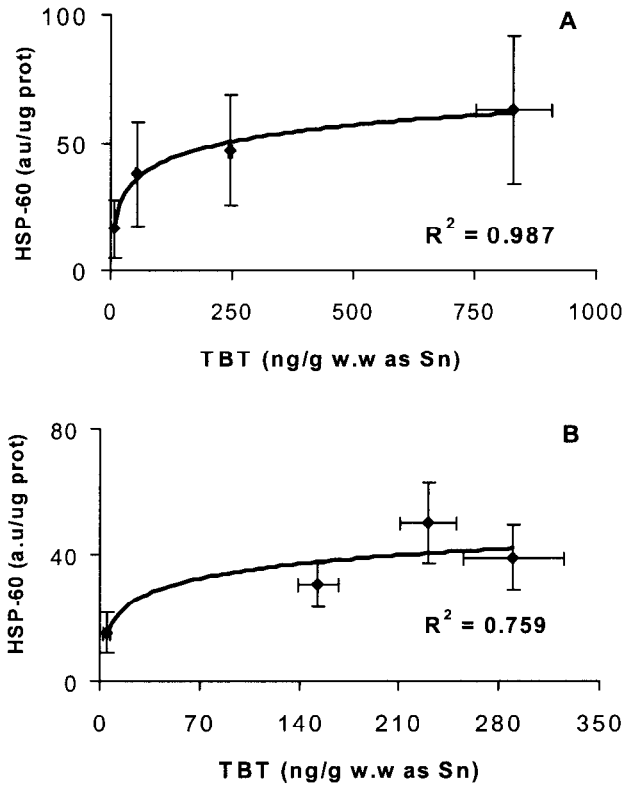
	TBT[a]	HSP-60[b]	HSP-70[b]
Control	4.9 ± 2.6	20.1 ± 6.3	2.6 ± 1.7
1 week	153.1 ± 14.1	30.3 ± 7.2	1.9 ± 1.8
3 weeks	289.8 ± 35.2	39.1 ± 10.4	0.3 ± 0.3
5 weeks	230.5 ± 19.8	50.1 ± 12.7	0.6 ± 0.6

[a] values are mean ± SEM (n=3) in ng/g w.w. as Sn. Data from Morcillo and Porte (2000).

[b] values are mean ± SEM (n=6) in arbitrary units/µg protein

From our exposure experiment data, a concentration of TBT of 53 ng/g w.w. is enough to induce a 2-fold increase in HSP-60, however, in a field situation, higher TBT body burdens (230-290 ng/g w.w.) are needed to get a similar response. In the transplant experiment, clams are faced with a real environment, where TBT is probably the major threat, but where other bioavailable chemicals (PAHs, persistent organic pollutants, metals, etc.) and physical variables can affect the expression of the stress proteins (Werner et al. 1998). Lundebye et al. (1997) reported that stress proteins (HSP-60 and HSP-70) did not reflect the degree of TBT contamination of mussels sampled around the island of Fyn (Denmark). Only HSP-60 gave a certain response, and mussels from a polluted area -with TBT body burdens of approximately 260 ng/g as Sn- showed a 1.7-fold increase in HSP-60. Clayton et al. (2000) reported an increase in stress response (HSP-60 and HSP-70) of 2.5-3 times in *Dreissena polymorpha* exposed to environmentally-relevant doses of TBT. These results are in agreement with our

findings and they point out the small response of mussel and clam stress proteins to TBT exposure. In addition, the relationship between TBT uptake and the stress response is not linear (Fig. 1), and it better fits a logarithmic function as indicated



by Clatyon et al. (2000).

Figure 1. Regression between whole tissue residues of TBT and gill HSP-60 levels in clams *Ruditapes decussata:* (A) exposed to different concentrations of TBT in the laboratory, and (B) transplanted to a TBT-polluted marina.

It is important to relate stress proteins to higher organisation stress indices, such as growth inhibition or effects on reproduction, to support their potential as biomarkers. Sanders et al. (1991) related HSP-60 with the physiological parameter Scope For Growth (SFG), and observed that the stress response was manifested at levels one order of magnitude lower than SFG. Also Steinert and Pickwell, (1993) indicated a linear relationship between HSP-70 induction and reduction of the clearance rate across the gills in TBT exposed mussels. However, other studies have failed to detect any relationship between stress proteins and mussel condition indexes (Lundebye et al. 1997). Levels above 2 µg/g dry weight of TBT (approximately 400 ng/g w.w.) are reported to cause adverse effects in SFG in adult mussels (Page and Widdows, 1991; Widdows and Page, 1993). In our work, only clams from the laboratory experiment, exposed to the highest TBT dose, exceeded this concentration. TBT residues determined in this work are in the

range of those detected in bivalves from harbours and marinas from the NW Mediterranean (Tolosa et al. 1992; Morcillo et al. 1997), and changes on testosterone/estradiol titres and cytochrome P-450 aromatase activity have been reported (Morcillo et al. 1998; 1999). The pronounced individual variability and the existence of a plateau in the stress response at the higher TBT doses limit the applicability of HSPs in field studies, where the existence of a wide range of chemicals and environmental stressors, with synergistic or antagonistic effects among them, will probably affect the synthesis of stress proteins and interfere with their response.

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