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TRIBUTYLTIN CHLORIDE-INDUCED EFFECTS ON PROTEIN TYROSINE PHOSPHORYLATION AND ON EXTRACELLULAR-SIGNAL-REGULATED KINASE (ERK) PHOSPHORYLATION IN ASCIDIAN *PHALLUSIA MAMMILATA*

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Key words: Tributyltin-induced effect; Tyrosine kinase signalling; MAPK; ERK (p44/42); Ascidian embryos.

Abstract: Ascidians represent an intriguing candidate experimental system for studying the effects of environmental stress. We studied TBT effects and probable related pathways were investigated on ascidian embryos by using Western immunoblotting. Among the various signal transduction pathways involved in response to environmental stress, both tyrosine kinase signalling and MAPKs have been played a significant role. To better understand molecular mechanisms after exposure to TBT we studied the two signal transduction pathways above mentioned. Attempting to unravel the molecular effects of TBT-induced on ascidians embryogenesis, TBT treatments carried out in *Phallusia mammillata* embryos at gastrula stage. We found different levels of tyrosine protein phosphorylation in response to the incubation with TBT in µM range and a remarkable ERK phosphorylation inhibition dose-dependent with 10, 50 and 100 µM of TBT solution. Based on these data, the use of tyrosine phosphorylation leves and MAPK signal transduction could be considered as biomarkers in the response of marine organisms to pollutant.

Introduction

Tributyltin (TBT) is a biocide that enters the aquatic environment mainly from its employment in antifouling paints to prevent the growth of organisms such as barnacles on the hull of ships. Extensive use in antifouling paints led to the widespread distribution of TBT and its breakdown products in the global marine, sediment and biota (Elgethun et al., 2000; Connelly et al., 2001; De Brito et al., 2002; Lee et al., 2005). High levels of TBT in the waters are shown to be very toxic to aquatic life, by inhibiting embryogenesis and larval development, in a variety of marine organisms. The most notable effect of TBT is 'imposex' in some marine invertebrates, in which females exposed to TBT develop male characteristics, notably a penis and vas deferens (Bryan et al., 1986; Miller et al., 1999; Ten Hallers-Tjabbes et al., 2003; Santos et al., 2004).

Ascidians are a good model for the study of embryonal development and they are also sensitive bio-indicators of habitat degradation. The effects of tributylin (IV) chloride (TBT chloride) solutions on ascidian embryos of *Ciona intestinalis* at different stages of development have been described in some works. These studies showed morphological and ultrastructural modifications of the embryos and larvae after incubation in TBT chloride. In particular, the results obtained showed modifications of cytomembranes and mitochondria and anomalous blastomere arrangement during gastrulation (Mansueto et al., 1993; Gianguzza et al., 1996; Dolcemascolo et al., 2005).

Among the various signal transduction pathways involved in response to environmental stress, MAPKs have been shown to play a significant role (Schaffer and Weber, 1999; Widmann et al., 1999; Kyriakis and Avruch, 2001); MAPK cascades are important amplifying modules that can transduce stress signals into cellular responses (Poonam et al., 2002; Ranganna et al., 2002). In ascidian embryos, a Fibroblast Growth Factor (FGF-like) signal has been proposed to be involved in induction of notochord and mesoderm formation (Nakatani and Nishida, 1994; Kim and Nishida, 1999). The mitogen-activated protein kinases (MAPKs) are serine/threonine kinases that transduce signals from the plasma-membrane to the cell nucleus (Garrington and Johnson, 1999; Cobb and Goldsmith, 2000). They have key roles to play in animal responses to a wide variety of environmental stresses. Kim and Nishida (2001) suggested that a MEK-MAPK signalling cascade is widely involved in embryonic induction in ascidians. Tyrosine kinase signalling is the major mechanism for receptor signal transduction that mediate cell growth, differentiation, host defence and metabolic regulation (Fischer, 1999). Different stressors are known to stimulate tyrosine kinase activities and this could explain a wide spectrum of effects pollutants produce on different organisms.

We have previously shown that exposure to TBT in embryos of *Ciona intestinalis* induced changes in the total pattern of phosphotyrosine and in the phosphoytlation levels of ERK 1/2 (Damiani et al., 2009). In the present research, to confirming the effects of the TBT treatments on cell signalling, a study of mitogen-activated protein (MAP) kinases p44/42 of protein tyrosine phosphorylation levels in *Phallusia mammilata* embryos were assessed by Western immunoblotting.

MATERIALS AND METHODS

Animals

Adult specimens of *Phallusia mammillata* were collected from the Gulf of Palermo. They were taken away and transferred into aquaria at water temperature of

18°C to the laboratory of the Biology and Genetic section of Department of Biopathology and Medical and Forensic Biotechnologies of Palermo University.

Tributyltin chloride solutions

Concentrated stock solution of Tributyltin (IV) chloride (Schering Bergkamen, Germany) was obtained by dissolving of compound at 10 mM concentration in dimethylsulfoxide (DMSO). Working solutions were obtained by further diluition of the stock in Millipore-filtered seawater (MFSW). These solutions were diluited at the final concentration of 0,5, 1, 10, 50 and 100 µM.

Experimental procedure

In each experiment female and male gametes were removed from gonoducts and transferred into agar-coated Syracuse dishes containing filtered sea water at 22 °C for cross-fertilization. Ten minutes after fertilization, sperm excess was removed and sea water was renewed. Principally we focused our attention on gastrula stage, in particular observation have been carried out on the following groups:

Control (Ctrl): *P. mammillata* gastrulae in 0.1% DMSO;

P. mammillata gastrulae treated for 60 min with 0.5µM, 1µM, 10 µM, 50 µM, 100 µM TBT chloride solution

The final DMSO concentration chosen to make up the experimental solution was 0.1%; this represents a no-toxic concentration which is less than the one reported by Bellas et al. (2005); in fact the controls treated with this concentration of DMSO did not show significant differences with controls in FSW.

Electrophoresis and Western blotting

The levels of tyrosine phosphorylation and phosphorylated ERK in whole cell extracts from ascidian embryos were determined using specific antibodies.

Different lots of ascidian embryos were incubated with TBT solutions for 60 min. Then the sample were centrifugated and lysed in buffer (300mM NaCl, 50 mM Tris-HCl pH 7.6, 0.1% Triton, 1% protease inhibitor cocktail (Sigma-Aldrich), 4 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 100mM sodium fluoride) on ice for 120 min. The lysates were centrifuged at high speed (14.000 r.p.m.) for 15 min and an aliquot of the supernatant was assayed to determine protein concentration by the Bradford method. Equal amounts of proteins (30 µg) were separated by 12% SDS- polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA) in 0.1M 3-(cyclo-hexylamino)-1-propanesulfonic acid (CAPS; Sigma-Aldrich),

pH 11; 10% Methanol, at 170 mA for 45 min. Membranes were stained with Ponceau S, incubated in block solution (3% Albumin from Bovine Serum, 10% Fetal Bovine Serum in Phosphate buffer), and probed overnight with anti-phosphotyrosine antibody (PY20; Santa Cruz Biotechnology, CA). After 5 washes with washing solution (1x Phosphate buffer, 0.1% Tween-20) the membranes were incubated with alkaline phosphatase-conjugated secondary antibody (Promega Corporation Madison, USA). Then the membranes were washed with alkaline phosphatase buffer (0.1 M Tris HCl, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5) and proteins were detected with 5-Bromo-4chloro-3-indolyl Phosphate/Nitroblue Tetrazolium Liquid Substrate System (BCIP/NBT liquid substrate system Sigma, Saint Louis, Missouri, USA). For p44/42 MAP kinase (ERK 1/2) and phospho-p44/42 MAP kinase (p-ERK 1/2) protein level evaluation, the samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane and then the membranes were probed overnight with specific antibodies against ERK 1/2 (Cell Signalling Technology, Beverly, MA) and p-ERK 1/2 (Cell Signalling Technology, Beverly, MA), respectively. Protein corresponding to ERK 1/2 and p-ERK 1/2 were indentified by using the detection protocol mentionated earlier.

Data analysis

Data from desitometric analyses of Western blots are means ± SD of three independent experiments. Statistical evaluation of the data was performed with the Student's t test and $p< 0,05$ was assumed to be statistically significant.

RESULTS

Effect of TBT chloride on tyrosine phosphorylation proteins pathway

As a marker for signal transduction, we initially looked at tyrosine phosphorilation. Western blot analyses with a monoclonal antibody that specifically recognize phosphorylated tyrosine residues allowed to detect different levels of phosphorylation in various proteins obtained by treating different embryos of *P. mammillata* with TBT.

Embryos of *Phallusia mammillata* at gastrula stage, treated with 0,5 µM, 1 µM, 10 µM 50 µM and 100 µM TBT solution for 60 min showed an increase, at 10 µM TBT, in phosphorylation level of proteins with molecular size nearby 48 KDa and 55 KDa. The bands nearby 36 KDa showed a slight increase at 0,5 µM and 1 µM and then decrease until the control levels at 10 µM (Fig. 1A).

As shown in Fig. 1B, the highest increase in tyrosine phosphorylation was found at 10 µM TBT (0.18-fold above the control; $p< 0.001$); exposure of embryos to 50

and 100 µM TBT resulted in a remarkable decrement in tyrosine phosphorylation levels of total pattern (0.6- and 0.7- fold beneath the control respectively; $p< 0.01$).

Because variations of densitometric analysis were evaluated as a mean of the total pattern of tyrosine protein phosphorylation, the decrease of a single band gives an unimportant contribution respect to the density increase of the other proteins.

Effect of TBT chloride on ERK 1/2 and phospho- ERK levels

We chose to focus on MAPK signalling cascades for two reasons. First, generally an activation of an ERK signalling pathway has a role in mediating cell division, migration and survival. Second, it has previously been reported that MAPK pathways are well conserved in ascidians genome (Satou et al., 2003; Hotta et al., 2003).

To determine whether ERK 1/2 signalling pathway was activated in response to TBT treatments, whole embryos extracts were prepared and immunoblotted with antibodies specifically recognizing the phosphorylated and active forms of ERK 1/2 (p42 and p44). TBT treatments were carried out in *P. mammillata* embryos at gastrula stage exposing embryos to 1 µM, 10 µM, 50 µM and 100 µM TBT solution. As shown in Figure 2A (top panel), with 10, 50 and 100 µM a remarkable ERK phosphorylation inhibition dose-dependent was evident, reaching an average decrease of 0.75-fold beneath the control at 100 µM TBT ($p < 0.001$) (Figure 2B). There were no changes about endogenous levels of total ERK induced by TBT treatment (Figure 2A, bottom panel).

As we previously described for *C. intestinalis* (Damiani et al., 2009), we confirmed that also for *P. mammillata* high concentrations of TBT inhibit ERK signalling as shown by above mentioned results.

DISCUSSION

Ascidians are able to survival in a wide range of marine pollutions and some species are most abundant in highly transformed and polluted environments such as ports and industrial areas. For this reason, numerous studies have outlined the importance of this group as pollution bio-indicators (Papadopouolu et al., 1972; Papadopouolu and Kanias, 1977; Bell et al., 1982; Galletly et al., 2007).

In the present study we observed the response of proteins tyrosine phosphorylation and ERK 1/2 signalling pathway to TBT exposure. Different stressors such as heavy metals, pro-oxidants and pollutants are known to stimulate tyrosine kinase signalling (Rahman et al., 1993; Nakashima et al., 1994; Katano et al., 1995; Burlando et al., 2006).

Among signalling mechanisms, tyrosine kinase-dependent pathways are triggered by citokines, growth factors and hormones and they are implicated in cell signalling, cell growth, differentiation and apoptosis (Fischer, 1999).

In the first place it has been shown that levels of tyrosine phosphorylation, evaluated by quantitative Western immunoblotting, can be used in the assessment of pollutant effects. To evaluate whether the effects of TBT might be associated with a change in tyrosine phosphorylation levels, embryos of *P. mammillata* at gastrula stage, were exposed for 60 min to µM range of TBT solution. The treatment with 10 µM TBT, induced a significant increase in phosphotyrosine levels ($p< 0.001$). At higher concentrations (50 µM and 100 µM TBT) a remarkable decrease in tyrosine phosphorylation levels beneath the control of total pattern was observed. We think that a decrease could be connected to apoptotic or necrotic events caused by high concentrations of TBT. It has been suggested that distinct upstream mechanisms exist leading to apoptosis and necrosis by different concentrations of an organotin compound (Gunasekar et al., 2001; Jurkiewicz et a., 2004).

Future investigations will be directed towards better comprehension of the role of tyrosine kinase signalling and mechanism of TBT-induced apoptosis in ascidian embryos. However, the results obtained suggest that protein tyrosine phosphorylation may represent a key element in the signal transduction of ascidian embyos when exposed to marine pollution and it could be also used as biomarker for detect the involvement of cell signalling in organisms exposed to pollutant or stressors.

The ERK signalling pathway responds mainly to growth factors and mitogens, stimulating transcriptional responses in the nucleus and its activation has a role in mediating cell division, migration and survival (Garrington and Johnson, 1999; Cobb and Goldsmith, 2000). In the present study we observed the response of proteins tyrosine phosphorylation and ERK 1/2 signalling pathway to TBT exposure.

About the role of ERK 1/2 signalling pathway, the results revealed that 10, 50 and 100 µM TBT induced a remarkable ERK phosphorylation inhibition dose-dependent, while the levels of total ERK were unvaried.

In conclusion, these results together with our previous data about the same experiments on *C. intestinalis* have shown that phosphorylation status of ERK could be considered a key event in the response of marine organism to xenobiotics.

Considering that the MAPK family may play an important role in coordinating gene responses to various stresses, we want to verify the possibility of phosphorylation and hence activation of stress-activated protein kinases, p38 MAPK and JNKs in ascidian embryos, at different stage of development after TBT treatment.

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Fig. 1

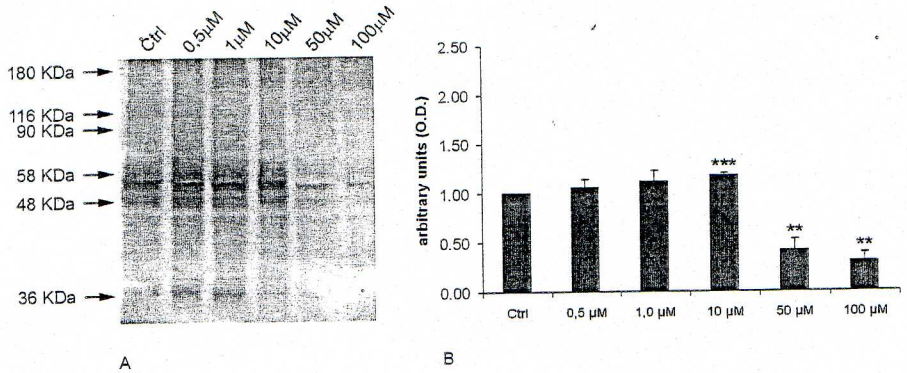

Fig. 2

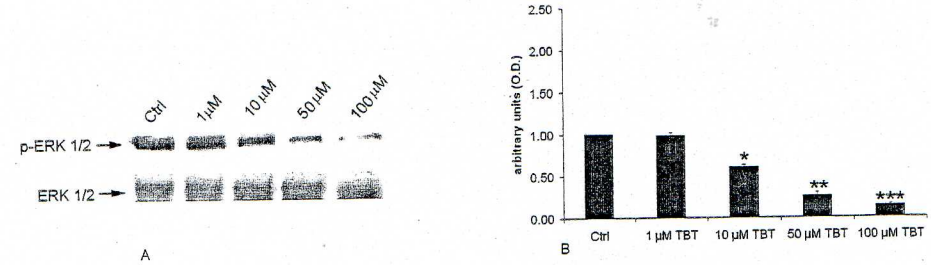

Figure 1. Effects of TBT chloride on protein tyrosine phosphorylation in *Phallusia mammillata* gastrulae. Protein extracts from ascidian embryos were subjected to 12% SDS-PAGE followed by Western blot using anti-phosphotyrosine antibody.
(A): representative Western blot showing variations in protein phosphorylation of *Phallusia mammillata* gastrulae after TBT treatment (0.5- 1- 10- 50- 100 ÌM). (B): densitometric analysis of phosphotyrosine bands; data plotted on bar charts represent the mean intensities (±S.D., n= 3) obtained from all the bands of each lane. Statistical evaluation of means were carried out using the Student's t test and statistically significant differences as compared to the control are indicated by asterisks. **$p< 0.01$, ***$p< 0.001$

Figure 2. Effects of TBT chloride on ERK 1/2 (p44/42) and phospho-ERK1/2 (phospho- p44/42) in *Phallusia mammillata* gastrulae. (A, top panel): representative experiment of phopho-ERK 1/2 levels obtained from embryos of control (Ctrl) and embryos treated with TBT (1-10-50- and 100 ÌM). (A, bottom panel): representative experiment of ERK 1/2 levels at the same concentration above-mentionated. (B): densitometric analysis of phospho-p44/42 bands. Results are means for three independent experiments (mean ±S.D.). Statistical evaluation of means were carried out using the Student's t test and statistically significant differences as compared to the control are indicated by asterisks.

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