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RESEARCH REPORT

Effects of tributyltin chloride in ascidian embryos: modulation of kinase-mediated signalling pathways

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Abstract

We studied the effects of various TBT concentrations by assaying the activity of ERK 1/2 (p44/42) and phospho-ERK1/2 (phospho-p44/42), proteins with a key role in ascidian development, and tyrosine kinase-dependent pathway. The effects of this xenobiotic and the role of some signalling mechanisms on ascidian embryos were examined by using Western immunoblotting. The tyrosine phosphorylation pattern in the ascidians *Ciona intestinalis* and *Phallusia mammillata* development was examined and different levels of protein phosphorylation were found as a response to TBT at µM range. To determine whether another key signalling pathway was activated, the effects of TBT on the phosphorylation state of a component of tyrosine kinase-mediated signal transduction MAPK, ERK 1/2 (p44/42) were evaluated. Embryos of *Ciona intestinalis* exposed to 0.1, 0.25 and 0.5 µM TBT showed a slight decrement in the level of phosphorylated ERK, while a remarkable decrement in level of phopshorylated ERK were observed at higher TBT concentrations (0.5 µM to 10 µM). These data indicated that exposures to TBT induced changes in the total pattern of phosphotyrosine and in the phosphorylation levels of ERK 1/2 but there were no changes on the overall level of total ERK in ascidian embryos.

Key words: tributyltin-induced effect; tyrosine kinase signalling; MAPK; ERK (p44/42); ascidian embryos

Introduction

Among the class of organotin compounds, tributyltin (TBT) is well known. Organotin compounds have many applications, which include use in PVC, as catalyst in chemical reactions, agricultural pesticides, glass coatings and food packaging materials (Forsyth *et al.*, 1993; Ohno *et al.*, 2002) and antifungal treatments for textile polymers (Allsopp *et al.*, 2000, 2001). In particular, TBT has been used in marine antifouling paints leading to the widespread distribution of TBT and its breakdown products in the marine sediments and biota (Elgethun *et al.*, 2000; Connelly *et al.*, 2001; De Brito *et al.*, 2002; Lee *et al.*, 2005). High levels of TBT dissolved in fresh and seawater impair reproduction, by inhibiting embryogenesis and larval development, in a variety of marine organisms (Coelho *et al.*, 2006; Beiras *et al.*, 2008). In invertebrates, symptoms of such a TBT exposure includes the development of male sexual characteristics as a penis and *vas deferens* by female (imposex) (Miller *et al.*, 1999; Ten Hallers-Tjabbes *et al.*, 2003; Santos *et al.*, 2004).

TBT effects have been studied in many marine organisms including ascidians, in which immunotoxic effects such as decreasing of phagocytic activity and phenoloxidase activity have been observed (Cooper *et al.*, 1995; Cima *et al.*, 1998; Cima and Ballarin, 2000; Tujula *et al.*, 2001; Arizza *et al.*, 1995; Cima *et al.*, 1995).

Ascidians are an useful model for developmental biology, and they are also sensitive bio-indicators of environment degradation.

Previous light and electron microscope studies on *Ciona intestinalis* embryos and larvae incubated in TBT chloride solutions showed morphological and fine structural changes in the embryos and larvae. In particular, changes in cytomembranes and mitochondria and anomalous blastomere arrangement during gastrulation were found (Mansueto *et al.*, 1993; Gianguzza *et al.*, 1996; Dolcemascolo *et al.*, 2005).

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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). A main pathway is a protein kinase transduction pathway, which include Ras, Raf, MEK and mitogen-activated protein kinase (MAPK). Kim and Nishida (2001) suggested that a MEK-MAPK signalling cascade is widely involved in embryonic induction in ascidians.

MAPKs are serine/threonine kinases that transduce signals from the plasma-membrane to the nucleus (Garrington and Johnson, 1999; Cobb and Goldsmith, 2000) and they play a critical role in controlling cell survival, proliferation, and differentiation (Chang and Karin, 2001). Three major subfamilies have been characterized, including the extracellular signal-regulated kinases (ERKs), the c-jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) and the p38-MAPKs (Kyriakis *et al.*, 1996; Seger *et al.*, 1995; Cohen, 1997). MAPKs have also a key role in responding to a wide variety of environmental stresses (Storey and Storey, 2001). Canesi *et al.* (2004a) have shown that in hemocytes of the mussel *Mitylus galloprovincialis*, 17β-estrediol (E2) induced shape changes, lysosomial membrane destabilization and release of hydrolytic enzymes by activation of the stress-activated p38 MAPK and Signal Transducers and Activators of Transcription (STAT).

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). 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). Therefore the possibility exists that both signal transduction pathways (tyrosine phosphorylation and MAPK) could be used to study signalling mechanisms in organisms under enviromental stress.

In the present paper, the effects of the TBT treatments on cell signalling, variation of protein tyrosine phosphorylation levels and ERK module in embryos of *Ciona intestinalis* and *Phallusia mammillata* embryos were examined by the immunoblotting method.

Materials and methods

Animals

Adult specimens of *Ciona intestinalis* and *Phallusia mammillata* were collected from the Gulf of Palermo and Termini Imerese harbour (Palermo). They were maintained in sea water and kept in aerated aquaria (200 liters). Ascidians (about 100 for every experiments set) were held at 18-20 °C for no more than 7 days before use and were daily fed with various food types including freeze-dried rotifers, green unicellular algae and artificial diet.

TBT chloride solutions

Concentrated stock solution of Tributyltin (IV) chloride (Schering Bergkamen, Germany) was obtained by dissolving the compound at 10 mM concentration in dimethylsulfoxide (DMSO). Working solutions were obtained by further diluitions of the stock in Millipore-filtered seawater (FSW). These solutions were diluited at 0.1, 0.5, 1, 10, 50 and 100 μM final concentrations.

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 min after fertilization, sperm excess was removed and sea water renewed. Gastrulae were separated to form the following groups:

(1) Control A (Ctrl): *C. intestinalis* or *P. mammillata* gastrulae in FSW
(2) Control B: *C. intestinalis* gastrulae in 0.1 % DMSO
(3) *C. intestinalis* gastrulae treated for 60 min with 0.1 μM, 0.5μM, 1μM, 10 μM TBT chloride solution
(4) *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 (300 mM 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 100 mM sodium fluoride) on ice for 120 min. The lysates were centrifuged at high speed (10.000xg) for 15 min and an aliquot of the supernatant was assayed to determine protein concentration by the Bradford method (Bradford, 1976). 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, USA) 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

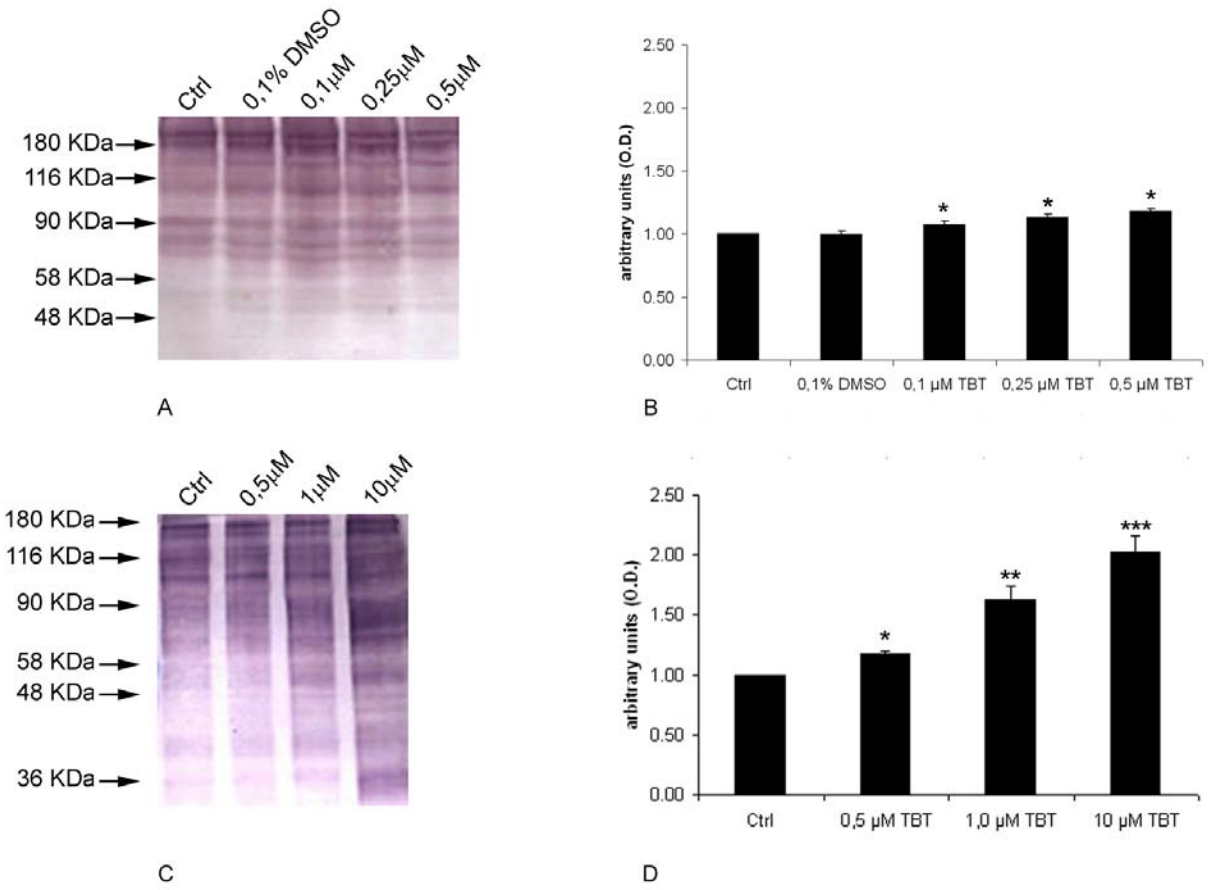

Fig. 1 Effects of TBT chloride on protein tyrosine phosphorylation in *C. intestinalis* gastrulae. Protein extracts from ascidian embryos were subjected to 12 % SDS-PAGE followed by Western blot using anti-phosphotyrosine antibody. (A): representative blot obtained from embryos controls and TBT treatment (0.1- 0.25- 0.5 µM); (C): representative blot obtained from embryos control and TBT treatment (0.5- 1- 10 µM). (B) and (D): densitometric analysis of phosphotyrosine bands; data plotted on bar charts represent the mean intensities (±SD, 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* < 0.05, ***p* < 0.01, ****p*< 0.001.

(PY20; Santa Cruz Biotechnology, CA, USA). 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, MS, 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, USA) and p-ERK 1/2 (Cell Signalling Technology), 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

Tyrosine phosphorylation as marker for signal transduction was examined. Western blot analyses with a monoclonal antibody that specifically recognizes phosphorylated tyrosine residues allowed to detect different levels of phosphorylation in various proteins obtained by treating different embryos of *C. intestinalis* and *P. mammillata* with TBT.

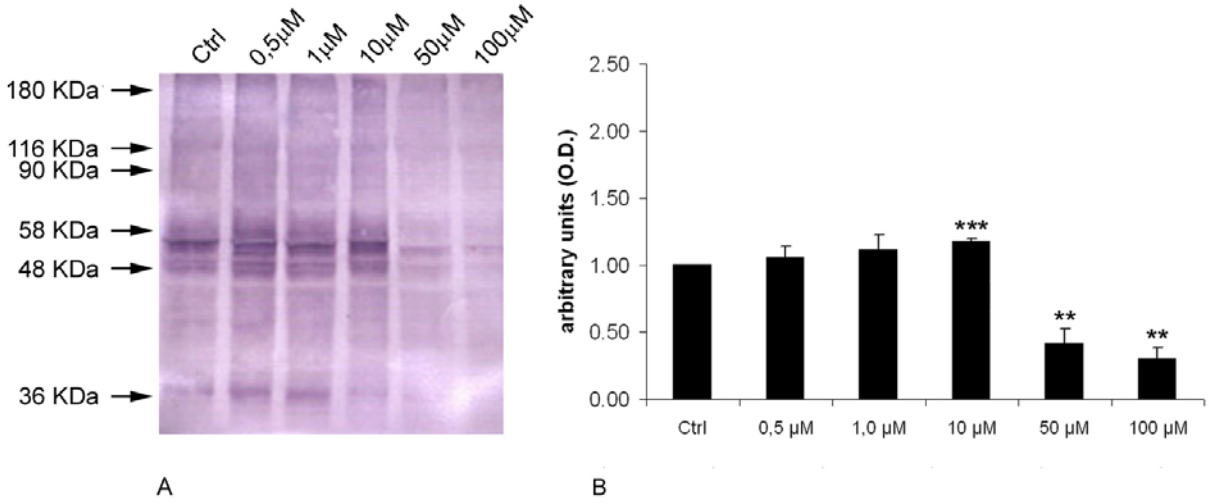

Fig. 2 Effects of TBT chloride on protein tyrosine phosphorylation in *P. mammillata* gastrulae. Protein extracts from ascidian embryos were subjected to 12 % SDS-PAGE followed by Western blot using anti-phosphotyrosine antibody. (A): representative Western blot showing variations in protein phosphorylation of *P. mammillata* gastrulae after TBT treatment (0.5- 1- 10- 50- 100 μM). (B): densitometric analysis of phosphotyrosine bands; data plotted on bar charts represent the mean intensities (±SD, 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.

Exposure of *C. intestinalis* embryos at gastrula stage to 0.1μM, 0.25μM or 0.5 μM TBT for 60 min induced only a slight increase of phosphorylated proteins around 116 KDa and 180 KDa. (Figs 1A, B). When embryos of *C. intestinalis* were treated with 0.5 μM, 1 μM and 10 μM TBT solution, they showed an increase of phosphotyrosine levels, reaching, at 10 μM TBT exposure, a significant variation of about 1-fold over the control ($p<0.001$) (Figs 1C, D). In particular, phosphorylated proteins of 36 KDa, 48 KDa, 58 KDa, 90 KDa and 180 KDa were mainly identified. Densitometry analysis values of the protein patterns were presented as mean density of the total bands in each SDS-PAGE lane.

The same experiments were carried out in *P. mammillata* gastrula stage by using a 0.5-100 μM TBT. At 10 μM TBT an increase of phosphorylation level of proteins with molecular size nearby 48 KDa and 55 KDa was found. The bands nearby 36 KDa, showed a slight increase at 0.5 μM e 1 μM and then decrease until the control levels at 10 μM (Fig. 2A). Because variations of the 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.

As shown in Fig. 2B, 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; $p < 0.01$).

Effect of TBT chloride on p44/42 and phospho p44/42 levels

The ERK module responds primarily to growth factors and mitogens and stimulates transcriptional responses in the nucleus. Generally, activation of an ERK signalling pathway has a role in mediating cell division, migration and survival.

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 (p44 and p42). At first a putative protein was identified in *Ciona* genomic database (BLAST genomic database and SWISS PROT, EMBL) showing about 90 % of sequence similarity with mammalian ERK1 and ERK2.

Exposure of *C. intestinalis* embryos to 0.1 μM, 0.25 μM, and 0.5 μM TBT induced only a slight decrease in the level of phosphorylated ERK (Fig. 3A, top panel); at 0.5 μM of TBT exposure the average decrement was 0,4-fold beneath the control ($p< 0.05$) (Fig. 3B), whereas the overall p44/42 levels remained did not change as shown by representative experiment in Fig. 3A (bottom panel). As shown in Fig. 4 A (top panel), when higher TBT concentration, from 0.5 μM to 10 μM, a remarkable dose-dependent ERK phosphorylation inhibition was evident, and an average decrease of 0.85-fold beneath the control at 10 μM TBT ($p < 0.001$) (Fig. 4B) was reached. There were no changes about endogenous levels of total ERK due to TBT treatment (Fig. 4A, bottom panel). High concentrations of TBT inhibit ERK signalling as shown by above mentioned results.

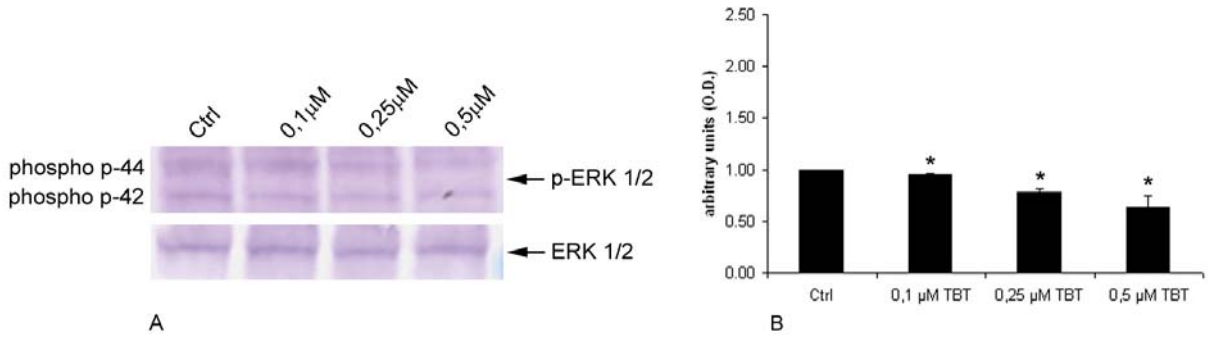

Fig. 3 Effects of TBT chloride on ERK 1/2 (p44/42) and phospho-ERK1/2 (phospho- p44/42) in *C. intestinalis* gastrulae. (A, top panel): representative experiment of phopho-ERK 1/2 levels obtained from embryos of control (Ctrl) and embryos treated with TBT (0.1- 0.25- 0.5 µM). (A, bottom panel): representative 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±SD). Statistical evaluation of means were carried out using the Student's *t* test and statistically significant differences ($p < 0.05$) as compared to the control are indicated by an asterisk.

Discussion

Ascidians represent an intriguing candidate experimental system for studying the effects of environmental stress. In fact, these organisms are able to survival in a wide range of marine pollutions and some species are abundant in highly transformed and altered environments such as harbors 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).

Among the various signal transduction pathways involved in response to environmental stress, both tyrosine kinase signalling and MAPKs appear to play a significant role (Burlando *et al.*, 2006; Schaffer and Weber, 1999; Widmann *et al.*, 1999; Kyriakis and Avruch, 2001). To further elucidate molecular mechanisms affected by TBT exposure we studied the two signal transduction pathways above mentioned. They have an important role in the response of marine organisms to pollutant, and are considered as biomarkers.

Different stressors are known to stimulate tyrosine kinase activities and this could explain a wide spectrum of effects produced by pollutants on different organisms.

At the beginning, we examined the effects of TBT exposure on tyrosine kinase signalling in ascidian embryos by Western immunoblotting. To evaluate whether the effects of the xenobiotic might be associated with a change in tyrosine phosphorylation levels, embryos of *C. intestinalis* at gastrula stage, were exposed for 60 min to µM range of TBT solution. The treatments with 1 µM and 10 µM TBT, showed an enhanced phosphorylation level for proteins ranging from 36 and about 180 KDa. The fold increase in phosphotyrosine levels, reached a significant variation of about 1-fold over the control ($p < 0.001$) at 10 µM TBT, compared to the control.

In *P. mammillata* embryos at gastrula stage, the exposure to 10 µM TBT, induced a significant increase in phosphotyrosine levels ($p< 0.001$). At higher TBT concentrations (50 µM and 100 µM) a remarkable decrease in the phosphorylated protein pattern was observed. The possibility exists that such a decrease can be linked to apoptotic or necrotic events caused by high TBT concentrations. Taking in account total pattern, the highest phosphorylation values were found after treatment with 10 µM TBT. It has been suggested that distinct upstream mechanisms exist leading to apoptosis and necrosis by different concentrations of an organotin compounds (Gunasekar *et al.*, 2001; Jurkiewicz *et al.*, 2004). Although further research are needed to elucidate the role of tyrosine kinase signalling and mechanism of TBT-induced apoptosis, the here reported results suggest that protein tyrosine phosphorylation may represent a key element in the signal transduction of ascidian embyos exposed to marine pollution. In this respect these embryos could be used for detecting the involvement of cell signalling in organisms exposed to pollutant or stressors.

MAPK cascades are important amplifying modules that can transduce stress signals into cellular responses (Kultz and Avila, 2001; Poonam *et al.*, 2002; Ranganna *et al.*, 2002). Studies on the characterization and function of MAPK modules have been carried out by using mammalian models and non mammalian experimental systems, for i.e. *Xenopus laevis*, *Drosophila melanoganster* and *Caenorhabditis elegans* (Widmann *et al.*, 1999), and recently mussel hemocytes or isolated digestive gland cells (Canesi *et al.*, 2001, 2002). In the present study the stimulation of MAPK signal transduction pathways by TBT treatment was examined in ascidian embryos. In particular, we observed the response of proteins tyrosine phosphorylation and ERK 1/2 signalling pathway to TBT exposure. The ERK signalling pathway responds mainly to growth factors and mitogens, stimulating

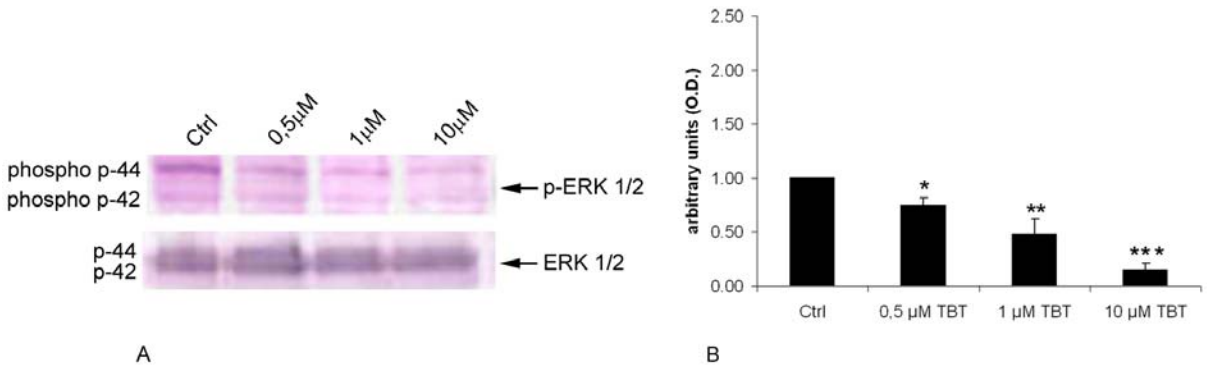

Fig. 4 Effects of TBT chloride on ERK 1/2 (p44/42) and phospho-ERK1/2 (phospho- p44/42) in *C. intestinalis* gastrulae. (A, top panel): representative experiment of phopho-ERK 1/2 levels obtained from embryos of control (Ctrl) and embryos treated with TBT (0.5- 1- and 10 μM). (A, bottom panel): representative 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 ±SD). 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* < 0.05, ***p* < 0.01, ****p* < 0.001.

transcriptional responses and its activation has a role in mediating cell division, migration and survival (Garrington and Johnson, 1999; Cobb and Goldsmith, 2000). As regards the role of ERK 1/2 signalling pathway, the results of our studies revealed that 0.1 μM, 0.25 μM and 0.5 μM TBT induced only a slight decrement in the level of phopshorylated ERK, while the levels of total ERK were unvaried. Higher TBT concentration up to 10 μM leads to a remarkable decrement in the level of phopshorylated ERK, but no effects on endogenous levels of total ERK were found. These results indicate that these TBT exposures caused an inhibition in the phosphorylation of ERK 1/2 while not changing their overall levels.

It could be suggested that TBT, a strong toxic agent, could interfere with normal mechanisms of signal transduction involved in ascidians embryo development. There are indications that the MAPK signalling cascade, mediated via the ERK family, plays a role in the onset of apoptosis during the embryogenesis (Kling *et al.*, 2002; Yao *et al.*, 2003) and that MAPK plays a pro-apoptotic role during the ascidian metamorphosis. In fact, Chambon *et al.* (2002) have demonstrated that the activation of the *C. intestinalis* MAPK/ERK (Ci-ERK) in tail cells precedes the wave of apoptosis, suggesting that the phosphorylated form of Ci-ERK transduces the death-activating signal in tail tissues during metamorphosis, whereas the inhibition of Ci-ERK blocks metamorphosis.

Moreover it has been proposed that mitogen-activated protein kinase (MAPK) signalling might be involved in the regulation of Hsp expression in blue mussels (Anestis *et al.*, 2007). Batel *et al.* (1993) have reported that in response to TBT, the sponge cells of *Geodia cydonium* not only undergoes apoptosis but also expresses the stress protein, Hsp70, to a higher degree. In preliminary experiments we also investigated the possible modulation of the heat shock protein-70 (Hsp70) by TBT treatment. Considering the TBT exposure cause Hsp70 induction in ascidian embryos (data not shown), it could be interesting to verify if there is a link between MAPK activation and Hsp 70 expression.

Acknowledgements

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References

Allsopp A, Santillo D, Johnston P. Hazardous chemicals in carpets. Greenpeace Research Laboratories, Technical Note 01/2001 (published under cover title "Poison Underfoot: Hazardous Chemicals in PVC Flooring and Hazardous Chemicals in Carpets, ISBN 90-73361-68-0), 2001.

Allsopp A, Santillo D, Johnston P. Hazardous chemicals in PVC flooring. Greenpeace Research Laboratories, Technical Note 14/00 (published under cover title "Poison Underfoot: Hazardous Chemicals in PVC Flooring and Hazardous Chemicals in Carpets, ISBN 90-73361-68-0), 2000.

Anestis A, Lazou A, Portner HO, Michaelidis B. Behavioral, metabolic, and molecular stress responses of marine bivalve *Mytilus galloprovincialis* during long-term acclimation at increasing ambient temperature. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293: 911-921, 2007.

Arizza V, Cammarata M, Tomasino MC, Parrinello N. Phenoloxidase characterization in vacuolar hemocytes from the solitary ascidian *Styela plicata*. J. Invertebr. Pathol. 66: 297-302, 1995.

Ballarin L, Cima F, Sabbadin A. Phenoloxidase and cytotoxicity in the compound ascidian *Botryllus schlosseri*. Dev. Comp. Immunol. 22: 479-92, 1998.

Batel R, Bihari N, Rinkevic B, Dapper J, Schacke H, Schroder HC, *et al.*. Modulation of organotin-induced apoptosis by the water pollutant methyl mercury in a human lymphoblastoid tumor cell

line and a marine sponge. Mar. Ecol. Prog. Ser. 93: 245-51, 1993.

Beiras R, Bellas J. Inhibition of embryo development of the *Mytilus galloprovincialis* marine mussel by organic pollutants; assessment of risk for its extensive culture in the Galician Rias. Acquaculture 277: 208-212, 2008.

Bell MV, Pirie VJ, Mcphail DB, Goodman BA, Falk-Petersen B. Contents of vanadium and sulphur in the blood cells of *Ascidia mentula* and *Ascidiella aspersa.* J. Mar. Biol. Ass. UK 62: 709-716, 1982.

Bellas J, Beiras R, Marino-Balsa JC, Fernandez N. Toxicity of organic compounds to marine invertebrate embryos and larvae: a comparison between the sea urchin embryogenesis bioassay and alternative test species. Ecotoxicology 14: 337-353, 2005.

Bradford M M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Burlando B, Berti E, Viarengo A. Effects of seawater pollutants on protein tyrosine phosphorilation in mussel tissue. Aquat. Toxicol. 78S: S79-S85, 2006.

Canesi L, Ciacci C, Betti M, Lorusso LC, Marchi B, Burattini S, Falcieri E, Gallo G. Rapid effect of 17β-estradiol on cell signaling and function of *Mytilus* hemocytes. Gen. Comp. Endocrinol. 136: 58-71, 2004a.

Canesi L, Betti M, Ciacci C, Scarpato A, Citterio B, Pruzzo C, *et al*. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAP kinases. Dev. Comp. Immunol. 26: 325-34, 2002.

Canesi L, Betti M, Ciacci C, Gallo G. insulin effect of zinc in *Mytilus* digestive gland cells: Modulation of tyrosine kinase-mediated cell signaling. Gen. Comp. Endocrinol. 122: 60-66, 2001.

Chambon JP, Soule J, Pomies P, Fort P, Sahuquet A, Alexandre D, *et al*. Tail regression in *Ciona intestinalis* (Prochordate) involves a Caspase-dependent apoptosis event associated with ERK activation. Development 129: 3105-14, 2002.

Chang L, and Karin M. Mammalian MAP kinase signalling cascades. Nature 410: 37-40, 2001.

Cima F, Ballarin L. Tributyltin induces cytoskeletal alterations in the colonial ascidian *Botryllus schlosseri* phagocytes via interaction with calmodulin. Aquat. Toxicol. 48: 419-429, 2000.

Cima F, Ballarin L, Bressa G, Burighel P. Cytoskeleton alterations by tributyltin (TBT) in tunicate phagocytes. Ecotoxicol. Environ. Saf. 40: 160-165, 1998.

Cima F, Ballarin L, Bressa G, Sabbadin A. Immunotoxicity of butyltins in tunicates. Appl. Organomet. Chem. 9: 567-72, 1995.

Cobb MH, Goldsmith EJ. Dimerization in MAP-kinase signalling. Trends Biochem. Sci. 25: 7-9, 2000.

Coelho MR, Langston WJ, Bebianno MJ. Effect of TBT on *Ruditapes decussatus* juveniles. Chemosphere 63: 1499-1505. 2006.

Cohen P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. Trends Cell Biol. 7: 353-361, 1997.

Connelly DP, Readman JW, Knap AH, Davies J. Contamination of the coastal waters of Bermuda by organotins and the triazine herbicide Irgarol 1051. Mar. Pollut. Bull. 42: 409-414, 2001.

Cooper EL, Arizza V, Cammarata M, Pellerito L, Parrinello N. Tributyltin affects phagocytic activity of *Ciona intestinalis* hemocytes. Comp. Biochem. Physiol. 112C: 285-9, 1995.

Cowan KJ, Storey KB. Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. J. Exper. Biol. 206: 1107-1115, 2003.

De Brito AP, Takahashi S, Ueno D, Iwata H, Tanabe S, Kubodera T. Organochlorine and butyltin residues in deep-sea organisms collected from the western North Pacific, off-Tohoku, Japan. Mar. Pollut. Bull. 45: 348-361, 2002.

Dolcemascolo G, Gianguzza P, Pellerito C, Pellerito L, Gianguzza M. Effects of trybutiltin (IV) chloride on neurulation of *Ciona intestinalis* (Tunicata, Ascidiacea): an ultrstructural study. Appl. Organomet. Chem. 19: 11-22, 2005.

Elgethun K, Neumann C, Blake P. Butyltins in shellfish, finfish, water and sediment from the Coos Bay estuary (Oregon, USA). Chemosphere 41: 953-964, 2000.

Fischer EH. Cell signalling by protein tyrosine phosphorylation. Adv. Enzyme Regul. 39: 359-369, 1999.

Forsyth DS, Dabeka R, Sun WF, Dalglish K. Speciation of organotins in poly(vinyl chloride) products. Food Addit. Contam. 10: 531-540, 1993.

Galletly BC, Blows MW, Marshall DJ. Genetic mechanisms of pollution resistance in a marine invertebrate. Ecol. Appl. 17: 2290-2297, 2007.

Garrido C, Gurbuxani S, Ravagnan L, Kroemer G. Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem. Biophys. Res. Commun. 286: 433-442, 2001.

Garrington TP , Johnson, G. Organization and regulation of mitogen-activated protein kinase signalling pathways. Curr. Opin. Cell Biol. 11: 211-218, 1999.

Gianguzza M, Dolcemascolo G, Mansueto C, Pellerito L. Effects of trybutiltin (IV) chloride exposure on larvae of *Ciona intestinalis* (Urochordata): an ultrstructural study. Appl. Organomet. Chem. 10: 405-413, 1996.

Gunasekar P, Li L, Prabhakaran K, Ebyl V, Borowitz JL, Isom GE. Mechanisms of the apoptotic and necrotic actions of trimethyltin in cerebellar granule cells. Toxicol. Sci. 64 : 83-89, 2001.

Jurkiewicz M, Averill-Bates DA, Marion M, Denizeau F. Involvment of mitochondrial and death receptor pathways in tributylin-induced apoptosis in rat epatocytes. Biochim. Biophys. Acta 1963: 15-27, 2004.

Katano Y, Pu MY, Akhand AA, Hamaguchi M, Koga Y, Isobe K, *et al*. Evidence of redox-linked signalling for producing a giant signal complex. J. Cell Biochem. 57: 432-439, 1995.

Kim GJ, Nishida H. Role of FGF and MEK signalling pathway in the ascidian embryo. Dev. Growth Differ. 43: 521-533, 2001.

Kim GJ, Nishida H. Suppression of muscle fate by cellular interaction is required for mesenchyme formation during ascidian embryogenesis. Dev. Biol. 214: 9-22, 1999.

Kyriakis JM, Avruch J. Protein kinase cascades activated by stress and inflammation. J. Biol. Chem. 271: 24313-24316, 1996

Kultz D, Avila K. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. Comp. Biochem. Physiol. 129B: 821-829, 2001.

Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol. Rev. 81: 807-869, 2001.

Lee CC, Wang T, Hsieh CY, Tien CJ. Organotin contamination in fishes with different living patterns and ist implications for human health risk in Taiwan. Environ. Pollut. 137: 198-208, 2005.

Mansueto C, Gianguzza M, Dolcemascolo G and Pellerito L. Effects of trybutiltin (IV) chloride exposure on early embryonic stages of *Ciona intestinalis*: *in vivo* and ultrstructural investigations. Appl. Organomet. Chem. 7: 391-399, 1993.

Miller KL, Fernandes TF, Read PA. The recovery of populations of dogwhelks suffering from imposex in the Firth of Forth 1987-1997/98. Environ. Pollut. 106: 183-192, 1999.

Nakashima I, Pu MY, Nishizaki A, Rosila I, Ma L, Katano Y, *et al*. redox mechanisms as alternative to ligand binding for receptor activation delivering disregulated cellular signals. J. Immunol. 152: 1064-1071, 1994.

Nakatani Y, Nishida H. induction of notochord during ascidian embryogenesis. Dev. Biol. 166: 289-299, 1994.

Ohno H, Suzuki M, Nakashima S, Aoyama T, Mitani K. Determination of organotin compounds in plastic products by GC/MS after ethyl derivatization with sodium tetraethylborate. Shokuhin Eiseigaku Zasshi 43: 208-214, 2002.

Papadopuolou C, Kanias G. Tunicate species as marine pollution indicators. Mar. Pollut. Bull 8: 229-331, 1977.

Papadopuolou C, Hadzistelios I, Grimanis AP. Trace element uptake by *Cynthia claudicans* (Savigny). Limnol. Oceanogr. 11: 651-663, 1972.

Poonam AK, Wuster RD, Lucchesi PA. Mitogen-activted protein kinase activation and oxidant signalling in astrocytoma cells. J. Neurol. oncol. 56: 109-117, 2002.

Rahman SM, Pu MY, Hamaguchi M, Iwamoto T, Isobe K, Nakashima I. Redox-linked ligand-independent cell surface triggering for extensive protein tyrosine phosphorylation. FEBS Lett. 317: 35-85, 1993.

Ranganna K, Yousefipour Z, Nasif R, Yatsu FM, Milton SG, Hayes BE. Acrolein activates mitogen-activated protein kinase signal transduction pathways in rat vascular smooth muscle cells. Mol. Cell. Biochem. 240: 83-98, 2002.

Robinson MJ, Cobb MH. Motogen-activated protein kinase pathways. Curr. Opin. Cell Biol. 9: 180-186, 1997.

Santos MM, Vieira N, Reis-Henriques MA, Santos AM, Gomez-Ariza JL, Giraldez I, *et al*. Imposex and butyltin contamination off the Oporto Coast (NW Portugal): a possible effect of the discharge of dredged material. Environ. Int. 30: 793-798, 2004.

Schaeffer HT, Weber MJ. Mitogen-activated protein kinases: specific messages from ubiquitous messengers. Mol. Cel.l Biol. 19: 2435 -2444, 1999.

Seger R, Krebs EG. The MAPK signaling cascade. FADEB J. 9: 726-735, 1995.

Storey KB and Storey JM. Signal transduction and gene expression in the regulation of natural freezing survival. Rev. Cell Mol. Responses to Stress. 2: 1-19, 2001.

Sugisawa N, Matsuoka M, Okuno T, Igisu H. Suppression of cadmium-induced JNK/p38 activation and Hsp70 family gene expression by LL-Z1640-2 in NIH3T3 cells. Toxicol. Appl. Pharmacol. 196: 206-214, 2004.

Takashi U, Masayuki K, Shinji T, Yasunobu O, Yasuyuki N. Possible involvment of p38 MAP kinase in Hsp70 expression induced by hipoxia in rat primary astrocytes. Brain Res. 823: 226-230, 1999.

Takayama S, Reed J, Homma S. Heat-shock proteins as regulators of apoptosis. Oncogene 22: 9041-9047, 2003.

Ten Hallers-Tjabbes CC, Wegener JW, Van Hattum BA, Kemp JF, ten Hallers E, Reitsemae TJ, *et al*. Imposex and organotin concentrations in *Buccinum undatum* and *Neptunea antiqua* from the North Sea: relationship to shipping density and hydrographical conditions. Mar. Environ. Res. 55: 203-233, 2003.

Tujula N, Radford J, Nair SV, Raftos DA. Effects of tributyltin and other metals on the phenoloxidase activating system of the tunicate, *Styela plicata.* Aquat. Toxicol. 55:191-201, 2001.

Zhang X, Beuron F, Freemont PS. Machinery of protein folding and unfolding. Curr. Opin. Struct. Biol. 12: 231-238, 2002.

Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three kinase module from yeast to human. Physiol. Rev. 79:143-180, 1999.