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Modulation of organotin-induced apoptosis by the water pollutant methyl mercury in a human lymphoblastoid tumor cell line and a marine sponge

Renato Batel[1], Nevenka Bihari[1], Baruch Rinkevich[2], Jutta Dapper[3], Heike Schäcke[3], Heinz C. Schröder[3], Werner E. G. Müller[3,*]

[1] Institute Ruder Boskovic, Center for Marine Research, 552210 Rovinj, Croatia

[2] Israel Oceanographic and Limnological Research, National Institute of Oceanography, Tel Shikmona, PO Box 8030, Haifa 31080, Israel

[3] Institut für Physiologische Chemie, Abteilung Angewandte Molekularbiologie, Universität Mainz, Duesbergweg 6, W-6500 Mainz, Germany

ABSTRACT: Apoptosis is a special form of cell death in which the cells actively participate in the process of dying. We found that the water pollutants tributyltin (> 1 µM) and methyl mercury (MeHg; at concentrations of > 3 µM) induce apoptosis in human T-lymphoblastoid CEM cells and in tissue of the marine sponge *Geodia cydonium*. At concentrations of > 5 µM, MeHg causes alkaline-labile sites in DNA of CEM cells. At the lower dose of 0.3 µM, MeHg abolishes the tributyltin-induced apoptosis in both CEM cells and sponge tissue. Incubation of sponge tissue with 3 µM of tributyltin induces not only apoptosis but also an increased expression of heat shock protein-70.

INTRODUCTION

Ions from heavy metals as well as their organic derivatives, such as tin (e.g. tributyltin; TBT) or mercury (methyl mercury; MeHg), are conservative pollutants because they are not broken down over a long time scale; they effectively become permanent additions to the aquatic environment (Hall & Pinkney 1985, Fitzgerald & Clarkson 1991, Mason 1991). They cause acute toxicity in animals and man (Attahiru et al. 1991, McLauchlan 1991). The main sign of toxicity of tributyltin is the suppression of immune response (Ghoneum et al. 1990) while MeHg impairs neuron function (Sarafian & Verity 1991). Both compounds occur frequently in fresh water as well as in seawater; e.g. tributyltin was detected at concentrations of 100 to 400 ng ml^{-1} in the waters of southwest Spain (Gomez-Ariza et al. 1992) and MeHg in the Kagoshima Bay (Japan) at concentrations of 6 to 20 ng ml^{-1} (Ando et al. 1992). These compounds accumulate in animal organs and organelles specifically up to a multiple of those concentrations found in the surrounding milieu (Mori & Fujimoto 1991, Schionning et al. 1991).

Apoptosis is a physiological control mechanism, which is characterized by degradation of DNA to oligonucleosomal fragments (multiples of ca 180 base pairs), nuclear condensation and cell death (Williams 1991). Moreover, cells undergo apoptosis during stress conditions, e.g. hyperthermia (Ghibelli et al. 1992). Apoptosis is distinguished from necrosis by a series of characteristics, e.g. changes in cell morphology and cleavage of DNA (Lockshin & Beaulaton 1974). It was the aim of the present study (1) to determine if the 2 water pollutants, TBT and MeHg, can induce apoptosis in not only a vertebrate system (mammalian cell line) but also in an invertebrate model (tissue of the marine

* Addressee for correspondence

sponge *Geodia cydonium*), and (2) to elucidate the accumulative/interference effects of the 2 compounds when added together.

MATERIALS AND METHODS

Materials. The following materials were obtained: Deoxyribonuclease I (DNase I; bovine pancreas) from Boehringer Mannheim (Mannheim, Germany), methyl mercury (MeHg) and tributyltin (TBT, as chloride) from Aldrich (Steinheim, Germany). Monoclonal antibody against heat shock protein-70 [anti-hsp70 antibody (human); N27-F3-4] was provided by Dr H. W. Stürzbecher (Heinrich-Pette-Institut, Hamburg, Germany).

Cell culture. The human T-lymphoblastoid CEM cells (starting concentration: 5×10^5 cells ml^{-1}) were grown in RPMI 1640 medium supplemented with 15 % (v/v) fetal calf serum (Müller et al. 1988); cultures were maintained at 37 °C in a humidified 5 % CO_2 in air atmosphere.

To determine the effect of TBT and MeHg on DNA fragmentation (internucleosomal DNA cleavage) and on cell viability exponentially growing CEM cells were pretreated for 2 h (1) either with tributyltin or with MeHg alone, or (2) with both compounds together. The cells were then incubated in the absence of the compound(s) for up to 10 h. Subsequently cell viability and the percentage of fragmented DNA were determined.

Sponge tissue. Live specimens of the sponge *Geodia cydonium* Jameson (Demospongiae: Tetractinomorpha: Geodiidae) were collected by SCUBA diving from a depth of 20 to 30 m at an unpolluted site in the Mediterranean Sea near Rovinj (Croatia).

Each sponge was cut into cubes (0.12 to 0.15 cm^3) which were kept in a specially devised incubator (Zahn-Daimler et al. 1975) at 16 °C. Samples of sponge cubes (5 g) were put into flasks containing 50 ml of oxygenated seawater and agitated by 15 s pulses from an air compressor. The seawater was continuously oxygenized during these pulses. Under these conditions the sponge cubes can survive for at least 5 wk (Zahn-Daimler et al. 1975). TBT and MeHg were added for 2 h; then the sponge tissue was incubated in the absence of the compound(s) for 0 to 10 h.

DNA fragmentation. DNA fragmentation was determined by agarose gel electrophoresis. DNA was prepared from 4.5×10^7 CEM cells or from 250 mg of *Geodia cydonium* tissue. This material was analyzed by electrophoresis on 1 % horizontal agarose gels as described (Bansal et al. 1991). The gels were stained with ethidium bromide, and the ladders were visualized by UV fluorescence.

In a second approach, the quantitative procedure of Wyllie (1980) was applied where 4.5×10^7 cells were harvested, lysed and centrifuged at $25\,000 \times g$ (20 min). The DNA from both the supernatant (fragmented DNA) and the sediment (intact DNA) was precipitated and measured (Burton 1956). The percentage of fragmented DNA was calculated as described (Bansal et al. 1991).

Cell viability. The viability of all cells was determined microscopically applying the fluorescein diacetate (Sigma F-7378) method (Hahn et al. 1988); a Zeiss Axiophot microscope with epifluorescence optics was used. The cells were stained and inspected; 600 to 700 cells per assay were examined (Clark 1981). The data are given as the percentage of viable cells.

Assay for alkaline-labile sites in DNA. DNA damage analysis was carried out using a fluorometric technique that measures the rate of unwinding of cellular DNA on exposure to alkaline conditions (Birnboim & Jevcak 1981). In brief, 35 ml of CEM-cell suspension (initial concentration: 2.5×10^5 cells ml^{-1}) were treated with different concentrations of MeHg for 2 h and then chilled to 0 °C; the cells were collected by centrifugation, washed, and distributed equally to a set of 10 tubes. Cells were lysed with an urea/detergent solution. An alkaline solution was added, and DNA unwinding was allowed to occur for a 60 min period (22 °C). The samples were neutralized and the amount of residual double-stranded DNA was estimated by using ethidium bromide as the fluorescence dye. After calculating the rate of DNA unwinding, the values were converted to the number of strand breaks per cell by reference to the effect produced by gamma-rays. The results are given in units of Q_d (McWilliams et al. 1983), which is a measure of DNA damage. A Q_d unit corresponds to about 100 strand breaks $cell^{-1}$. Using this approach, single- and double-strand breaks and alkaline-labile lesions are detected (Birnboim & Jevcak 1981).

Gel electrophoresis and western blotting. Sponge cubes were pretreated with 0 or 3 µM tributyltin and then incubated in the absence of the compound. Cell extracts were prepared following the procedure of Hamada & Tsuruo (1988). Gel electrophoresis of extracts (50 µg of protein per slot) was performed in 8 % polyacrylamide gels containing 0.1 % $NaDodSO_4$ as described by Laemmli (1970). The proteins were transferred to nitrocellulose sheets (Towbin et al. 1979) and incubated with monoclonal anti-hsp70 antibody. The immune complexes formed were visualized after an additional incubation with anti-mouse IgG, applying the 4-chloro-1-naphthol/hydrogen peroxide procedure.

Analysis of data. The results were analyzed by paired Student's *t*-test (Sachs 1984).

RESULTS

Induction of apoptosis in CEM cells by TBT

In the first set of experiments CEM cells were used to study the possible induction of apoptosis by TBT. DNA fragmentation was determined by agarose gel electrophoresis. Control experiments without TBT showed no signs of enhanced fragmentation of DNA (Table 1, Fig. 1A, Lane a). Addition of 1 or 3 µM of TBT to the cultures (concentrations used also in a previous study; Aw et al. 1990) caused DNA fragmentation during the 10 h incubation period as visualized qualitatively by migrating the DNA on agarose gels (Fig. 1, Lanes b to d) and quantitatively by the sedimentation technique (22 to 45 %; Table 1). A stepladder-like gel pattern was obtained; the visible bands mark the degradation products in multiples of ca 180 base pairs (Wyllie 1980).

The quantitative measurement for the degree of apoptosis by the sedimentation technique (Table 1, Fig. 2) revealed that DNA fragmentation increased time- and dose-dependently. In the absence of TBT the extent of DNA fragmentation was 4 % (Table 1, Fig. 2); in the presence of this compound (3 µM) the degree of fragmentation significantly increased ($p < 0.01$) to 45 to 50 % during the 10 h incubation period, while no significant increase was observed in the control assays ($p > 0.05$) (Fig. 2A, Table 1). DNA fragmentation was seen already at a concentration of 1 µM TBT (22.1 %; $p < 0.01$). At 10 µM TBT, DNA fragmentation increased to 55 % (Table 1).

Simultaneously with the increase of apoptosis, the percentage of viable cells in the cultures decreased significantly from 97 % (in the absence of TBT) to 64 % (1 µM TBT), 53 % (3 µM), or 51 % (10 µM) (Table 1; $p < 0.01$).

Table 1. Effect of different TBT and MeHg doses on the induction of DNA fragmentation and on cell viability. CEM cells were pre-incubated for 2 h with either one of the compounds and then incubated for 10 h (n = 5)

Pollutant (µM) TBT	MeHg	DNA fragmentation (%)	Viable cells (%)
0	0	4.3 ± 0.4	97.2 ± 1.3
1	0	22.1 ± 1.8	64.4 ± 2.6
3	0	45.5 ± 3.9	53.3 ± 1.9
10	0	54.8 ± 4.6	50.9 ± 1.8
0	0.1	5.2 ± 0.5	96.4 ± 1.2
0	0.3	4.3 ± 0.5	95.8 ± 1.3
0	1	9.5 ± 0.8	89.5 ± 1.6
0	3	18.3 ± 1.9	72.4 ± 1.9
0	10	26.9 ± 2.0	68.1 ± 2.1

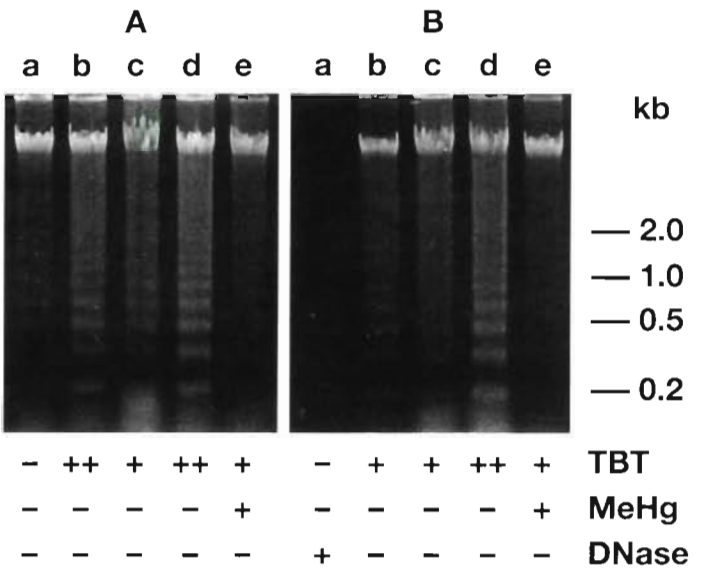

Fig. 1. Effects of TBT and TBT-MeHg treatments on DNA fragmentation in CEM cells and sponge *Geodia cydonium* tissue. (A) Cells treated with 0 (Lane a), 1 (Lane c) or 3 µM TBT (Lanes b and d) for 10 h. In one experiment cells were co-treated with 3 µM TBT in the presence of 0.3 µM MeHg (Lane e). (B) Sponge cubes incubated with 1 µM (Lanes b and c) or 3 µM TBT (Lane d), or 3 µM TBT together with 0.3 µM MeHg (Lane d). In one control experiment DNA preparation was treated with DNase I prior to gel electrophoresis (Lane a)

Induction of apoptosis in CEM cells by methyl mercury

The potency of MeHg to induce apoptosis in CEM cells was determined by the sedimentation technique (Table 1). Addition of increasing concentrations of this compound resulted in a significant ($p < 0.01$) induction of DNA fragmentation from 4 % (absence of the compound; Table 1) to 9.5 % at 1 µM MeHg. At higher concentrations (10 µM) the degree of DNA fragmentation increased to 27 %. The viability of the cells dropped from 97 or 90 % (at 1 µM MeHg) to 68 % (at 10 µM MeHg). At concentrations between 0.1 and 0.3 µM MeHg no significant effect of the compound on DNA fragmentation was measured ($p > 0.05$). Compared to TBT, MeHg induces lower rates of apoptosis.

Production of alkaline-labile sites in DNA by MeHg

To distinguish between direct DNA damage by MeHg and induction of DNA fragmentation by apoptosis, the effect of MeHg on DNA integrity was determined in exponentially growing CEM cells. Cells were inoculated at a density of 2.5×10^5 ml^{-1} and incubated for 2 h in the presence of different concentrations of MeHg. Then the samples were taken and analyzed for DNA strand breaks. MeHg

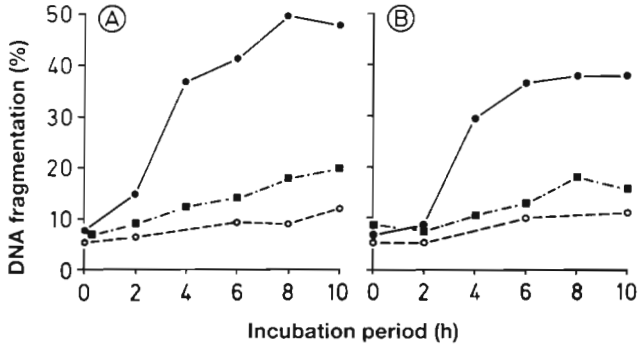

Fig. 2. DNA fragmentation followed by incubation of TBT in the absence or presence of MeHg on either CEM cells (A) or sponge *Geodia cydonium* tissue (B). Samples were pre-incubated with 0 µM (o---o) or 3 µM TBT (●—●), or co-incubated with 0.3 µM MeHg (■·---■), and were taken after a 10 h post-incubation period. n = 5 (SE < 15 %)

at concentrations above 5 µM caused breaks in DNA (Fig. 3). Within the concentration range of 1 to 5 µM MeHg, and under the conditions used, the number of DNA strand breaks was very low, if at all, while apoptosis was clearly observed (Table 1).

Induction of apoptosis in sponge tissue

Sponge cubes were exposed to 1 or 3 µM TBT for 2 h. After 10 h of post-incubation period the pattern of DNA fragments appeared distinctively in a ladder-like form (Fig. 1B, Lanes b to d; Fig. 4, Lane c), similarly to the results obtained in CEM cells (Fig. 1A, Lanes b to d). In the absence of TBT no ladder-like DNA fragmentation was recorded (Fig. 4, Lane a).

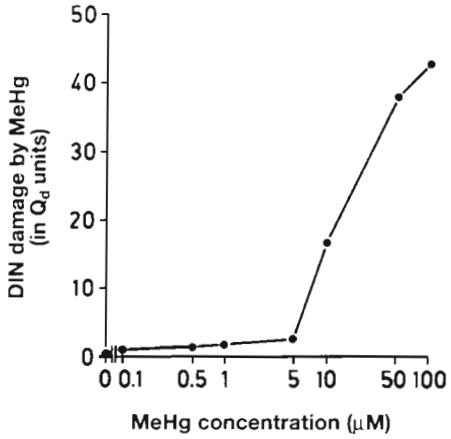

Fig. 3. Effect of MeHg on DNA damage in CEM cells. Cells were inoculated for 2 h in the presence of different concentrations of MeHg. Samples were then taken and analyzed for DNA strand breaks; the extent of DNA damage (alkaline-labile sites) is given in units of Q_d. n = 20 (SE < 20 %)

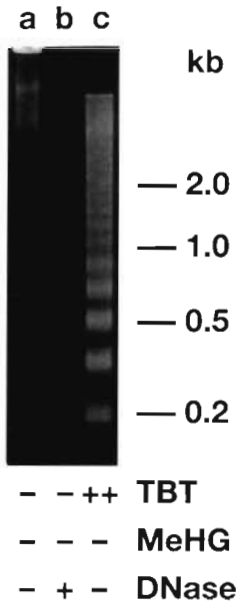

Fig. 4. Effects of TBT on DNA fragmentation in sponge *Geodia cydonium* tissue. Sponge cubes were incubated with 0 (Lane a) or 3 µM TBT (Lane c). In control experiments DNA preparations were treated with DNase I (Lane b)

A quantitative analysis of DNA fragmentation, by using the centrifugation technique, revealed that as early as 4 h after treatment with TBT (3 µM) a significant increase in DNA fragmentation was evident from 5–10 % (controls) to 32 % (3 µM TBT; $p < 0.01$; Fig. 2B).

After incubation of the DNA with DNase I [10 units ml^{-1} in a 50 mM Tris/HCl buffer (pH 7.5; 10 mM $MgCl_2$, 0.1 mM dithiothreitol) at 15 °C for 60 min] prior to gel electrophoretical separation no staining could be detected (Fig. 1B, Lane a; Fig. 4, Lane b).

Expression of heat shock protein in *Geodia cydonium* in response to TBT treatment

Another marker for TBT-induced apoptosis is the simultaneously increasing expression of heat shock protein gene (Cochrane et al. 1991). To further analyze this point in our system, *Geodia cydonium* tissue was extracted after exposure to TBT and analyzed for the presence of heat shock protein on western blotting, by anti-hsp70 antibody (Fig. 5). The proteins from sponges treated with 0 µM (Fig. 5A, B, Lane a) or 3 µM tributyltin (Fig. 5A, B, Lane b) were either stained by Coomassie Brilliant Blue (Fig. 5A) or were transferred to nitrocellulose sheets and incubated first with anti-hsp70 antibody (mouse) and second with anti-mouse IgG (peroxidase conjugated) (Fig. 5B). In the control no immunological cross-reacting protein could be detected (Fig. 5B, Lane a), while a 70 kDa protein could be clearly visualized (Fig. 5, Lane b) in the tissue treated with 3 µM TBT.

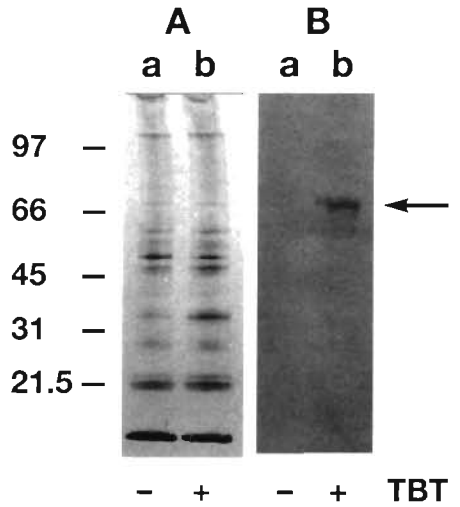

Fig. 5. Expression of hsp70 protein in sponge *Geodia cydonium* tissue after incubation with TBT Sponge cubes were pretreated for 2 h with 0 or 3 µM TBT and then incubated in the absence of the compound for 10 h. Proteins were then extracted and the resulting protein fractions were size-separated by polyacrylamide gel electrophoresis. Proteins from sponges treated with 0 (Lane a) or 3 µM TBT (Lane b) were either stained by Coomassie Brilliant Blue (A) or were analyzed for the presence of hsp70 by western blotting technique (B). Arrow: M_r of the protein species cross-reacting with human anti-hsp70 antibody

Prevention of apoptosis by co-incubation of TBT with MeHg

We also investigated whether apoptosis induction by TBT was modulated by MeHg. Both CEM cells and sponge cubes were pretreated with 3 µM TBT together with 0.3 µM MeHg and then incubated for an additional 0 to 10 h. The typical ladder-like DNA pattern seen in the presence of TBT alone [Fig. 1A, Lanes b to d (CEM); Fig. 1B, Lanes b to d (sponge)] disappeared when assaying simultaneously with TBT and MeHg (Fig. 1A, B, Lane e). These findings are supported also by the application of the sedimentation technique, where the degree of fragmentation dropped significantly ($p < 0.01$) from 48 to 19 % in CEM cells (Fig. 2A) and from 38 to 16 % in sponge tissue (Fig. 2B) after a 10 h post-incubation period.

DISCUSSION

Only very little is known about the potential of heavy metal ions, which are known water pollutants, to induce apoptosis, a process also termed programmed cell death (survey: Fesus et al. 1991). Most frequently the studies with these compounds are concerned with their potential genotoxic, carcinogenic, and teratogenic effects (DiFlora et al. 1991).

Aw et al. (1990) have recently reported that TBT initiates apoptosis in rat thymocytes. Now we extend these findings to an invertebrate model system and demonstrate that this water pollutant causes apoptosis in the marine sponge *Geodia cydonium* as well; in both systems used in the present study (the sponge model and the CEM cells) TBT displayed the apoptotic effect at the same concentration (3 µM). In a series of studies it was previously shown that MeHg causes alkaline-labile sites in DNA (Hamilton-Koch et al. 1986, Fiskesjo 1988). This effect was observed *in vitro* at concentrations above 10 µM. Here we demonstrate that besides this direct effect on the integrity of DNA, MeHg causes apoptosis even at lower concentrations (1 to 10 µM). We applied even lower concentrations of MeHg (0.3 µM) to elucidate that this compound when coincubated with TBT modulates the extent of TBT-induced apoptosis by suppressing this process both in CEM cells and in sponge tissue.

The observation that MeHg abolishes TBT-mediated apoptosis is of importance for the assessment of TBT as a water pollutant. It indicates that the potencies of compounds which cause apoptosis can be reduced by certain – otherwise also harmful – compounds, e.g. MeHg. It can be deduced from the presented data that certain toxic compounds if added together to cells or tissue, e.g. TBT and MeHg, can abolish their abilities to induce DNA fragmentation. At the present state of knowledge we can only speculate about the molecular mechanism by which MeHg abolishes the TBT-induced apoptosis. MeHg is known to bind to organotin; it also binds to sulfhydryl groups of a series of membrane transporters and thereby inhibits the active transport of e.g. Na^+, K^+ and taurine (Anner et al. 1992, Ballatori & Boyer 1992). Based on the latter finding it appears to be likely that MeHg interferes with the cellular uptake of TBT. Another possibility is that the induction of the heat shock protein-70 (hsp70) by MeHg occurring at lower concentrations (or another heavy metal specific stress protein) helps in the reduction of apoptosis.

A further outcome of the present study is the demonstration that in response to TBT the sponge cells not only undergo apoptosis but also express the stress factor, hsp70, to a higher degree. Previously it was found that in the rotifer *Brachionus plicatilis* TBT not only initiates apoptosis but also causes an increased expression of hsp70 gene (Cochrane et al. 1991). The hsp70 proteins are highly conserved in the animal kingdom (Lindquist 1986) and are thought to protect cells by renaturating denatured proteins (Pelham 1988). These results explain the observation that mild heat, another stress factor, augments metal-induced apoptosis (Sellins & Cohen 1991, Takano et al. 1991).

In conclusion, our results show that (1) both TBT and to a lower extent also MeHg alone induce apoptosis in the marine sponge *Geodia cydonium* and (2) MeHg at concentrations below those which cause apoptosis prevents sponge cells from undergoing TBT-mediated apoptosis.

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