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Involvement of mitochondrial and death receptor pathways in tributyltin-induced apoptosis in rat hepatocytes

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Abstract

Tri-*n*-butyltin (TBT), a biocide, is known for its immunotoxicity and hepatotoxicity and is a well-characterised mitochondrial toxin. This report investigates the mechanisms involved in induction of apoptosis by TBT in primary cultures of rat hepatocytes. Release of cytochrome *c* from mitochondria into the cytosol was apparent after 15 min of exposure to 2.5 μM TBT. In addition, activity of initiator caspase-9 increased after 30 min, representing activation of the mitochondrial pathway in hepatocytes. The death receptor pathway was also activated by TBT, as indicated by recruitment of the adaptor protein FADD from the cytosol to the membrane as soon as 15 min after treatment. In addition, levels of the pro-apoptotic protein Bid decreased in the cytosol, while there was an increase in levels of the cleaved form tBid, in TBT-treated hepatocytes. Activity of initiator caspase-8 increased after 30 min. The principal effector caspase-3 was activated following 30 min of treatment with TBT. Activation was confirmed by immunodetection of a 17-kDa cleaved fragment. Apoptotic substrates such as Poly(ADP-ribose) polymerase and DNA fragmentation factor-45 are cleaved by caspase-3 to ensure the dismantlement of the cell. Cleavage of Poly(ADP-ribose) polymerase into a 85-kDa fragment appeared after 30 min of TBT treatment. DNA fragmentation factor-45 disappeared in TBT-exposed rat hepatocytes. This is the first detailed study reporting the involvement of initiator and effector caspases, cleavage of their intracellular substrates and activation of both death receptor and mitochondrial pathways in TBT-induced apoptosis in rat hepatocytes. The comprehension of molecular events of apoptosis is important for the evaluation of the risk to humans and animals.

Keywords: Apoptosis; Tri-*n*-butyltin; Hepatocyte; Caspase; Poly(ADP-ribose) polymerase; Inhibitor of caspase-activated DNase

1. Introduction

Organotin compounds, such as tributyltin (TBT), have bactericidal and fungicidal properties. They are widely used in paper mill and textile industries, as well as in agricultural pesticides and as stabilizers for polyvinyl-chloride plastics. Their presence in the marine environment is mainly due to the use of antifouling paints on ship hulls. Recent reglementation, which restricts use of this organotin compound to larger vessels, has greatly decreased marine pollution. However, TBT still persists in the environment and some studies revealed its presence in daily food products like meat, fish and milk [1–4]. Human exposure arises from consumption of TBT-contaminated food products and occupational exposure of workers during TBT manufacture and use of TBT-containing paints [4–6]. The main effects of TBT on human health are skin and eye irritation and inflammation of the respiratory tract [7,8]. In lower trophic organisms, TBT contamination is responsible for the phenomenon of imposex [9,10]. When aquatic animals and terrestrial mammals are exposed to the organotin, it suppresses their immune system and makes them more vulnerable to infectious diseases [11–19]. Lately, measurable

Abbreviations: TBT, tri-*n*-butyltin; AMP, adenosine monophosphate; MAPKs, mitogen-activated protein kinases; NK, natural killer; XIAP, inhibitors of apoptosis; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; APAF-1, apoptosis activating factor-1; PARP, poly(ADP-ribose) polymerase; ICAD/DFF-45, inhibitor of caspase-activated DNase or DNA fragmentation factor-45; CAD, caspase-activated DNase; WME, Williams' medium E; MEM, minimal essential medium; Temed′ , *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; AMC, 7-amino-4-methylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin; TBE, Tris-borate/EDTA buffer; TBS+T, Tris-buffered saline and Tween; DTT, dithiothreitol

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concentrations of butyltin residues were found in whole-blood samples of volunteer donors [20].

TBT affects many cellular mechanisms. It is known to inhibit oxidative phosphorylation and production of energy [8,21,22], to reduce cyclic AMP production [23], to disturb calcium homeostasis [24–26] and to inhibit cytochrome P450 [27–29]. In CCRF-CEM human T lymphoblastoid cells, TBT was found to activate mitogen-activated protein kinases (MAPKs), leading to apoptosis [30]. TBT-induced DNA fragmentation was observed in freshly isolated rat thymocytes [31]. Furthermore, TBT suppresses human natural killer (NK) cells, preventing them from targeting tumor cells [20]. Nevertheless, its exact molecular mechanism of action is still not established.

Apoptosis is an active form of cell death, which is essential for development and for cellular and tissue homeostasis. Many proteins are involved in the molecular signalisation of apoptosis. The caspase enzymes, for cysteine-aspartyl-proteases, play a central role in the cell death machinery. They are subdivided into initiator (caspase-2, -8, -9 and -10) and effector (caspase-3, -6 and -7) caspases. Caspases are synthesised as pro-enzymes, which are cleaved at internal sites to form an active enzyme [32,33]. Their function in apoptosis is to (1) arrest the cell cycle and inactivate DNA repair; (2) to inactivate inhibitors of apoptosis (XIAP); and (3) to dismantle the cellular cytoskeleton [32]. Usually, initiator caspases, once activated, will activate downstream effector caspases in a cascade-like pattern. Two main pathways are well established in apoptosis: (1) the death receptor pathway and (2) the mitochondrial pathway.

The death receptor pathway requires membrane receptors such as Fas, which will trimerize, then recruit an adaptor molecule, Fas-associated death domain (FADD), and procaspase-8, forming the death-inducing signaling complex (DISC) [34]. At the DISC, procaspase-8 will be processed and caspase-8 is activated, ensuring the direct activation of caspase-3 [35].

Conversely, the mitochondrial pathway is activated upon treatment with neoplasic agents, UV radiation, growth factor withdrawal and DNA damage [35]. Following the assault, the mitochondrial membrane potential is lost, releasing a small apoptogenic molecule, cytochrome *c*, leading to formation of the apoptosome. This is a high molecular weight complex consisting of cytochrome *c*, apoptosis protease activating factor-1 (APAF-1), dATP and procaspase-9, which leads to activation of caspase-9. Subsequently, this initiator caspase will activate downstream caspases, like caspase-3. Both apoptotic pathways converge to caspase-3 activation. Caspase-3 cleaves a panoply of substrates such as Poly (ADP-ribose) polymerase (PARP), a DNA repair enzyme, inhibitor of caspase-activated DNase (ICAD) or DNA fragmentation factor-45 (ICAD/DFF-45), nuclear lamins, gelsolin and fodrin [35].

The two apoptotic pathways can be linked through Bid, a pro-apoptotic member of the Bcl-2 family of proteins [36]. Bid is usually found in the cytosol and is cleaved by caspase-8 to form a truncated protein, tBid, which will translocate to the mitochondria and activate the mitochondrial pathway. This alternative is a cross-talk between the receptor and mitochondrial pathways that can amplify caspase activation necessary for apoptosis.

TBT has been shown to cause apoptosis in several cellular models. In vitro studies revealed the occurrence of apoptosis in rainbow trout hepatocytes [37], rat hepatocytes [38], rat thymocytes [39,40], human lymphoma cells (CCRF-CEM lymphoblastoid, Jurkat T lymphocytes and Hut-78 cell lines) [30,41,42], human neutrophils [43] and neuronal PC12 cells [44]. Studies in rainbow trout hepatocytes [37] revealed the potential implication of cysteine proteases in TBT-induced apoptosis. TBT has been found to increase activity of caspase-3 in Jurkat T-cell and HUT-78 lymphoma cell lines [41,45]. The precise apoptotic mechanism has not yet been determined.

The reported presence of butyltin compounds in blood of healthy human donors [20] and their hepatic deposition [46] suggests widespread human exposure to organotin sources. Thus, there is an increasing need to develop a suitable animal model to monitor and investigate the effects of butyltins and to be able to extrapolate findings to humans. This report investigates the activation of various initiator and executor caspases in primary cultures of rat hepatocytes treated with 2.5 μM TBT and the cleavage of caspase-3 substrates such as PARP and ICAD/DFF-45. Here, we investigate the involvement of the mitochondrial and death receptor pathways in TBT-induced apoptosis. For the mitochondrial pathway, the liberation of cytochrome *c* and activation of caspase-9 are determined. Death-receptor-mediated apoptosis is evaluated by means of translocation of FADD to the cell membrane and activation of caspase-8. In addition, we investigate whether the cleavage of Bid is involved in amplification of the apoptotic response. The understanding of the mode of action of different toxicants is relevant for human and animal risk assessment.

2. Materials and methods

2.1. Materials

All media for cell culture [Williams' medium E (WME), minimal essential medium (MEM), and modified Leibovitz-15 (-L-15) (inspired from Ref. [47])] and gentamicin were purchased from Gibco/Life Technologies (Burlington, ON, CAN). Fetal bovine serum (FBS) was obtained from Immunocorp (Montreal, QC, CAN). TBT, insulin, Hoechst 33258, protease inhibitor cocktail and Ribonuclease A came from Sigma-Aldrich (St. Louis, MO, USA). *N*,*N*,*N*′,*N*′ -tetramethylethylenediamine (Temed), acrylamide, bisacrylamide and protein molecular weight standards were obtained from Bio-Rad (Mississauga, ON, Canada). Proteinase K came from Boehringer Mannheim (Montreal, QC, CAN). The

DNA standard, 100 base pair ladder was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All fluorescent substrates, Ac-Asp-Glu-Val-Asp-(7-amino-4-methylcoumarin) (Ac-DEVD-AMC) for caspase-3, Ac-Leu-Glu-His-Asp-(7-amino-4-trifluoromethylcoumarin) (Ac-LEHD-AFC) for caspase-9 and Z-Ile-Glu-Thr-Asp-(7-amino-4-trifluoromethylcoumarin) (Z-IETD-AFC) for caspase-8, and the caspase-3-specific inhibitor Ac-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO) were purchased from Calbiochem (La Jolla, CA, USA). The anti-caspase-3 rabbit polyclonal antibody (H-277) and anti-Bid rabbit polyclonal (FL-195) antibody came from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while the rabbit anti-inhibitor of caspase-activated DNase (DFF-45/ICAD) polyclonal and anti-FADD rabbit polyclonal antibodies were purchased from Stressgen (Vancouver, BC, CAN). The mouse monoclonal anti-cytochrome *c* (clone 7H8-2C12) antibody was from Pharmingen (San Diego, CA, USA). Anti-PARP monoclonal antibody (Clone c-2-10) was kindly donated by Dr. G. Poirier (University of Laval, QC, CAN).

2.2. Methods

2.2.1. Preparation of primary cultures of rat hepatocytes and treatment with TBT

Hepatocytes from Sprague–Dawley rats weighing 140–180 g were isolated by a two-step perfusion technique [48]. Animals were maintained and handled in accordance with the Canadian Council on Animal Care guidelines for the care and use of experimental animals (CCAC Guide vol. 1, 2nd Ed., 1993). Isodensity Percoll centrifugation was used to purify rat hepatocytes and cell viability was measured using flow cytometry with propidium iodide staining [26]. Cell viability ranged habitually from 80% to 95%. One million isolated hepatocytes were suspended in WME containing 10% FBS and 0.5% gentamicin and were seeded in 60-mm Petri dishes coated with rat tail collagen. After 3 h at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2, cells were washed using MEM to remove dead and unattached cells and 4.5 ml of fresh L-15 medium, supplemented with 0.5% gentamicin and 0.2 μg/ml of insulin, was added and left overnight at 37 °C. Cells were then washed again with MEM and supplied with fresh L-15 medium. Subsequently, they were exposed to TBT (2.5 μM) for different times during 90 min and apoptosis of rat hepatocytes was studied.

2.2.2. Detection of apoptosis

2.2.2.1. DNA fragmentation. Following exposure to TBT(1.5, 2, 2.5, 3 μM for 90 min), 1×10^6 cells were washed twice with phosphate-buffered saline (PBS) and lysed with 50 μl of extraction buffer containing 10 mM Tris pH 8.0, 5 mM EDTA and 0.5% SDS modified from Ref. [36]. Cell lysates were heated for 10 min at 54 °C. Ten microliters of Proteinase K (20 mg/ml) was added to samples, which were incubated overnight at 54 °C. Later, 10 μl of a solution of 0.5 mg/ml of RNase A was added for 1 h at 37 °C. DNA samples were then heated for 10 min at 65 °C, 10 μl of loading buffer (10 mM EDTA, 1% agarose low melting, 0.25% bromophenol blue, 40% sucrose) was added and samples were loaded onto a 2% agarose gel containing ethidium bromide (0.337 μM). Following electrophoresis for 3 h with Tris-Borate/EDTA buffer (TBE), DNA fragmentation was visualised under UV translumination.

2.2.2.2. Condensation of nuclear chromatin. Condensation of nuclear chromatin was visualised using Hoechst 33258 (H-33258) staining. This fluorescent dye complexes with condensed DNA. Cells, which had been exposed to 2.5 μM TBT for 0, 30, 60 or 90 min, were washed three times with PBS and incubated with H-33258 (50 μg/ml in PBS) for 10 min in the dark. Then, H-33258 was replaced with PBS and cells were visualised by fluorescence microscopy. Apoptosis was determined by the intensity of nuclear fluorescence in hepatocytes. Where necessary, hepatocytes were pretreated with the caspase-3 inhibitor (Ac-DEVD-CHO, 6 μM in 50 mM Tris–HCl, pH 7.5), for 1 h, before incubation with the organotin compound.

2.2.3. Enzymatic assays of caspase-3, -8 and -9 activities

Caspase-3 activity was measured by the cleavage of DEVD-AMC, releasing AMC, according to Nicholson et al. [49], with minor modifications. Activities of caspase-8 and -9 were measured by the release of AFC from their respective substrates. Two million hepatocytes, treated with 2.5 μM TBT for different times (0, 15, 30, 45, 60, 75 or 90 min), were washed with PBS and 50 μl of homogenisation buffer (250 mM sucrose, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM tricine, 2 mM $MgCl_2$, pH 8.0) was added. Cells were sonicated for 10 s and homogenates were centrifuged for 1 h at $100\,000\times g$ at 4 °C. Protein concentration was determined in the supernatants using the Bradford assay [50]. Caspase assays were carried out using 200 μg of proteins in each microplate with the appropriate protease assay buffer. The microplate was incubated for 5 min at 37 °C, then the appropriate substrate was added. For caspase-3, Ac-DEVD-AMC (50 μM) was used as substrate in protease buffer [20 mM HEPES pH 7.5, 10% glycerol, 2 mM dithiothreitol (DTT)]. Assays for caspase-8 and -9 were performed in protease assay buffer containing 100 mM HEPES pH 7.5 and 10 mM DTT, using the substrates Z-IETD-AFC (100 μM) and Ac-LEHD-AFC (100 μM), respectively. Cleavage of substrates was monitored over a period of 30 min and corresponding slopes were obtained at appropriate wavelengths of excitation (λ_{Ex}) and emission (λ_{Em}): λ_{Ex} 380 and λ_{Em} 460 for release of AMC and λ_{Ex} 400 and λ_{Em} 505 for AFC release. A SpectraMaxGemini Spectrofluorometer (Molecular Devices, Sunnyvale, CA) was used and the slopes were obtained by the SOFTmax Pro software (Molecular Devices).

2.2.4. Subcellular fractionation and immunodetection of cytochrome c, FADD, caspase-3, Bid, tBid, PARP and DFF-45

Following treatment with 2.5 μM TBT for different times (0, 15, 30, 45, 60 or 90 min), a minimum of 2×10^6 cells were washed with PBS and harvested in homogenisation buffer (250 mM sucrose, 1 mM EDTA-Na_2, 10 mM Na pyrophosphate, 10 mM tricine, 2 mM $MgCl_2$) supplemented with protease inhibitors ([4-(2-aminoethyl)-benzene-sulfonylfluoride] hydrochloride, aprotinin, bestatin, Leupeptin, pepstatin A). Lysates were homogenised using a hand potter (Kontes glass CO, Duall ®22, Fisher, QC, Canada) and were centrifuged at $1500\times g$ for 5 min at 4 °C. Supernatants were then centrifuged at $3640\times g$ for another 5 min at 4 °C. Supernatant samples were then centrifuged for 10 min at $10\,000\times g$ at 4 °C. A final centrifugation at $100\,000\times g$ at 4 °C was performed to isolate the cytosolic fraction (supernatant), which was used for detection of caspase-3, cytochrome *c* and DFF-45 by Western blotting. For immunodetection of PARP, a whole cell lysate was used. For the detection of FADD, Bid and tBid, cells were removed and manually homogenised in a lysis buffer (20 mM Tris–HCl, 5 mM EDTA, 0.3% β-mercapthoethanol, pH 7.5, where protease inhibitors were added freshly) according to Gomez-Angelats and Cidlowski [51]. The homogenates were centrifuged at $280\times g$ for 10 min at 4 °C, and the supernatant was centrifuged at $100\,000\times g$ for 60 min at 4 °C. Membranes were found in the pellet and the supernatant represents the cytosolic fraction.

From each sample, 30–50 μg of proteins in Laemmli's sample buffer [0.5 M Tris–HCl pH 6.8, 10% glycerol, 10% SDS, 0.01% bromophenol blue, 12.5% 2-mercaptoethanol] [52] were heated for 4 min and loaded onto either an 8, 10, 12 or 15% SDS-polyacrylamide gel. Electrophoresis was performed at a constant voltage of 135 V for 1 h. Proteins were transferred to a polyvinylidene difluoride membrane using a semidry blotting apparatus (Millipore, Seattle, USA). Blocking of nonspecific sites was performed under mild agitation, either overnight at 4 °C for caspase-3 and cytochrome *c* or for 1 h at room temperature, for PARP and DFF-45, in Tris-buffered saline with Tween (TBS+T) (2 mM Tris, 13.7 mM NaCl and 0.1% Tween 20, pH 7.4) containing 5% nonfat milk. Then, membranes were washed with TBS+T for 1 h and primary antibody was added either overnight at 4 °C (anti-FADD 1:4000, anti-PARP 1:10 000 and anti-DFF-45 1:1000) or for 1 h at room temperature (anti-Bid 1:1000, anti-caspase-3 1:500 and anti-cytochrome *c* 1:1000). Thereafter, membranes were washed again and incubated 1 h with appropriate secondary antibodies conjugated to horseradish peroxidase. Chemiluminescence was used to detect proteins (ECL kit, Amersham, Bucks, UK). Quantitation of protein bands was achieved by densitometry using a Molecular Dynamics scanner equipped with analytical software IP lab gel and ChemImager.

2.2.5. Statistical analysis

All experiments were performed at least three times with cell preparations obtained from different animals. All data are reported as means and SE. The comparison of the results from the various experimental groups relative to their corresponding controls was carried out using one-way analysis of variance followed by Neuman–Keuls post hoc test. The differences were considered significant when $P < 0.05$.

3. Results

3.1. TBT causes apoptosis in primary cultures of rat hepatocytes

Morphological assessment of apoptosis in rat hepatocytes was analysed using two approaches: DNA fragmentation and condensation of nuclear chromatin. DNA fragmentation is an important hallmark for apoptosis, where DNA is degraded by nucleases such as caspase-activated DNase (CAD), showing a characteristic ladder pattern on an agarose gel. DNA fragmentation was examined to establish the appropriate concentration of TBT, which causes apoptosis in rat hepatocytes. When hepatocytes were exposed to increasing concentrations of TBT for 90 min, the DNA ladder pattern appeared at 2.5 μM TBT (Fig. 1A). When hepatocytes were exposed to 2.5 μM TBT for 90 min, the DNA ladder pattern became apparent at 75 and 90 min (Fig. 1B). Consequently, a concentration of 2.5 μM TBT was selected for further analysis of apoptosis in rat hepatocytes. Ethanol (EtOH), the control vehicle, did not induce apoptosis.

Nuclear chromatin condensation is a morphological change, which is characteristic of apoptosis and can be monitored using the fluorescent dye Hoechst 33258. Condensation of nuclear chromatin was apparent after 30 min of treatment with 2.5 μM TBT, reaching a maximum level after 90 min. This was shown by an increase in nuclear fluorescence intensity compared to untreated control hepatocytes (Fig. 2). Pretreatment of hepatocytes with an inhibitor of caspase-3 (Ac-DEVD-CHO) inhibited TBT-induced chromatin condensation (Fig. 2). This indicates that the caspase cascade is involved in the molecular mechanisms of action of TBT toxicity in rat hepatocytes.

3.2. TBT-induced apoptosis occurs through activation of the mitochondrial pathway

The mitochondrial pathway of apoptosis is characterised by the release of the apoptogenic molecule cytochrome *c* from mitochondria into the cytosol, leading to activation of the initiator caspase-9. Caspase-9 activity began to increase after 30 min of treatment with 2.5 μM TBT (Fig. 3A), and reached a 19-fold increase after 75 min, compared to control hepatocytes. As illustrated in Fig. 3B,C, cytochrome *c* release into the cytosol occurred after 15 min

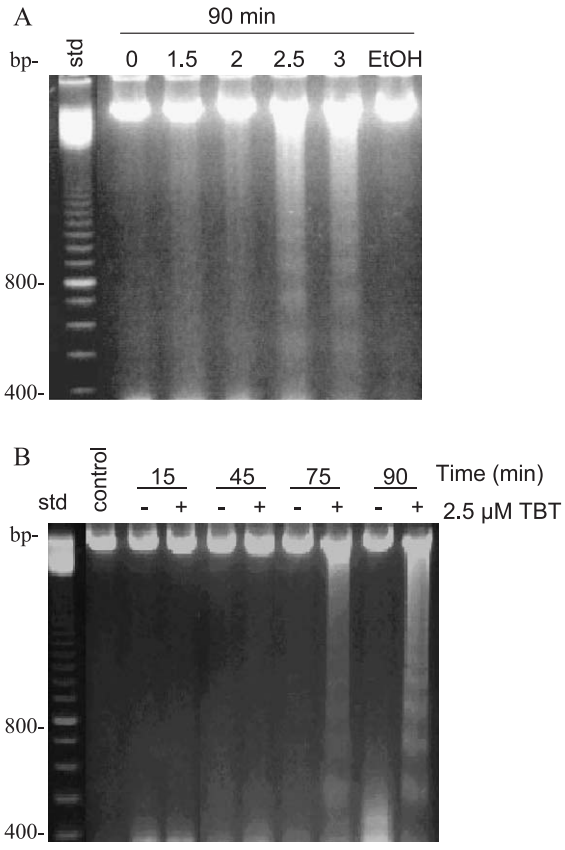

Fig. 1. DNA fragmentation of rat hepatocytes treated with TBT. (A) Hepatocytes were subjected to various concentrations of TBT (0, 1.5, 2, 2.5, 3 μM) for 90 min. (B) Hepatocytes were treated with 2.5 μM TBT for 90 min. A plus (+) sign represents the TBT-treated sample and a minus (−) sign represents the vehicle [ethanol (EtOH)]. DNA fragmentation was assessed on a 2% agarose gel containing ethidium bromide. Std is the 100-base-pair ladder standard. The data are representative of three experiments using different cell preparations.

of treatment with 2.5 μM TBT, reaching a maximum level after 75–90 min. Cytochrome *c* release after 15 min preceded the increase in caspase-9 activity, which occurred after 30 min.

3.3. TBT-induced apoptosis occurs through activation of the death receptor pathway

The death receptor pathway involves activation of the initiator caspase-8 through death receptors and their associated proteins. Following treatment with 2.5 μM TBT, the adaptor protein FADD was detected at the cellular membrane level after 15 min (Fig. 4A,B). Levels of FADD increased gradually with exposure time up to 90 min (Fig. 4B). There was a corresponding gradual decrease in levels of FADD in the cytosol from 10 to 90 min, following treatment with 2.5 μM TBT (Fig. 4C,D). There was no change in cytosolic levels of FADD in control hepatocytes. Enzymatic activity of caspase-8 was measured using a specific fluorescent substrate Z-IETD-AFC that, upon cleavage by active caspase-8, releases the fluorochrome AFC. The activation of caspase-8 in rat hepatocytes was apparent after 30 min of exposure to 2.5 μM TBT, relative to untreated control cells (Fig. 4E). A maximum level was reached after 75 min, with a 6-fold increase in activity.

3.4. Cleavage of pro-apoptotic protein Bid in TBT-induced apoptosis

Caspase-8 is able to cleave Bid to form tBid, which can amplify the activation of caspases. There was a gradual decrease in levels of Bid in the cytosol after 30–90 min of treatment with 2.5 μM TBT (Fig. 5A,B). There was a corresponding increase in levels of tBid in the noncytosolic fraction (Fig. 5C,D). Levels of tBid gradually increased from 15 to 90 min after treatment with 2.5 μM TBT. tBid was not detected in untreated controls (data not shown).

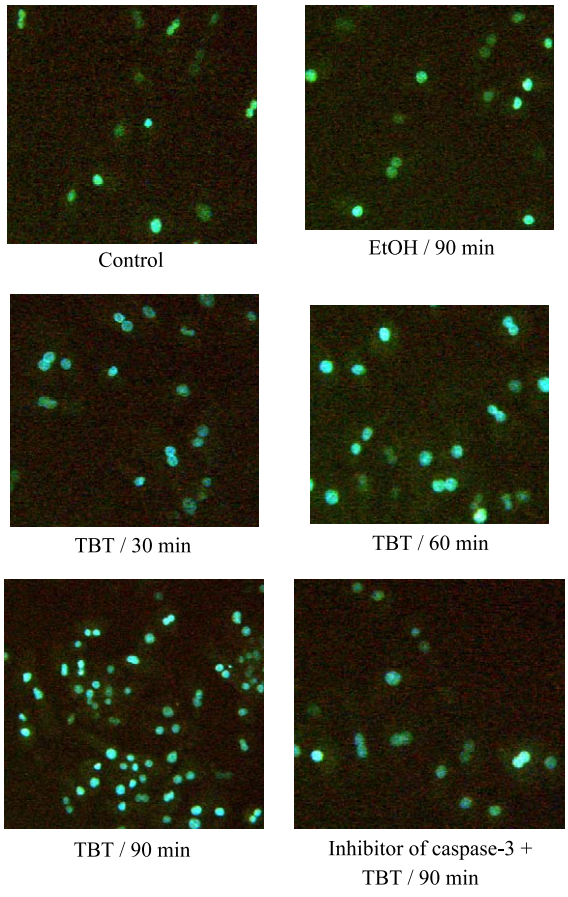

Fig. 2. Morphological analysis of nuclear chromatin condensation after exposure of rat hepatocytes to 2.5 μM TBT. A time course of treatment of hepatocytes with 2.5 μM TBT was followed for 90 min. Inhibition of nuclear chromatin condensation was assessed following pretreatment for 1 h with a caspase-3 inhibitor (Ac-DEVD-CHO, 6 μM) before exposure to 2.5 μM TBT for 90 min. Cells were stained with Hoechst 33258 dye and all images were taken at a magnification of 320×. Images are representative of four different experiments.

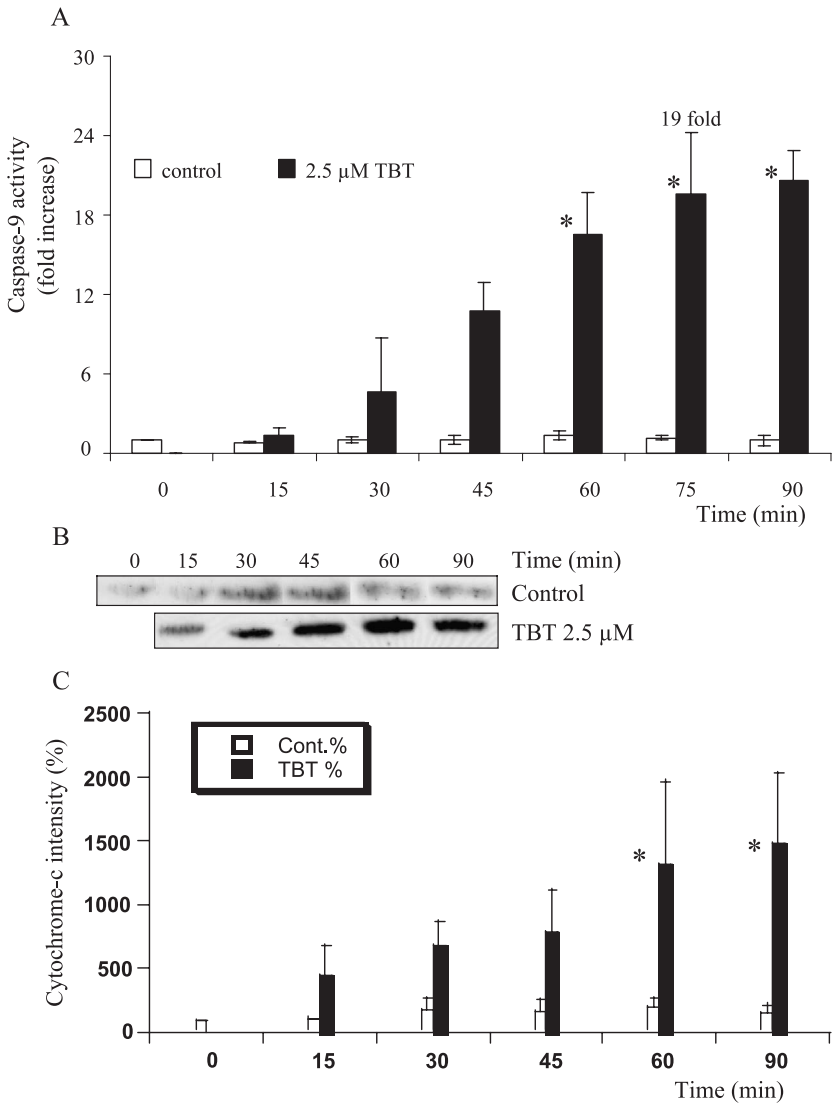

Fig. 3. Activation of the apoptotic mitochondrial pathway by TBT in rat hepatocytes. (A) Measurement of enzymatic activity of caspase-9 in control vs. 2.5 μM TBT-treated cells using the substrate Ac-LEHD-AFC. (B) Western blotting of cytochrome *c* (15 kDa) in the cytosol of control vs. 2.5 μM TBT-treated hepatocytes. (C) Autoradiograms of cytochrome *c* were analysed using a scanning laser densitometer. All data are representative of a compilation of three distinct experiments. Error bars represent SE.

3.5. TBT-induced apoptosis involves activation of caspase-3 and cleavage of its substrates

Caspase-3 is the main effector caspase involved in apoptosis. It plays a critical role in the dismantlement of the cell during apoptosis. Following exposure of rat hepatocytes to 2.5 μM TBT, there was an increase in caspase-3 enzymatic activity after 30 min. Maximum activation occurred after 75 min, with a 107-fold increase compared to control cells (Fig. 6A). Caspases are activated upon cleavage at internal sites and association of the large and small subunits occurs to form an active tetramer. The native form of procaspase-3 appears at 32 kDa and the cleaved fragment (17 kDa) represents the active form of caspase-3 (Fig. 6B). A small amount of active caspase-3 was observed by immunodetection after 60 min of 2.5 μM TBT treatment (Fig. 6B,C), but higher levels were present after 90 min. The 17-kDa fragment was not detectable before 60 min (Fig. 6B), despite activation of the enzyme after 30 min (Fig. 6A). Differing levels of sensitivity of detection for the two techniques could explain this.

Once activated, caspase-3 cleaves many substrates, eventually leading to destruction of the cell. PARP (116 kDa) is cleaved by caspase-3 into two fragments of 85 and 24 kDa. The immunoblot indicates cleavage of PARP as soon as 30 min after treatment with 2.5 μM TBT, relative to control cells (Fig. 7A,B). Another caspase-3 substrate is DFF-45/ICAD (47 kDa). This protein is the chaperone of CAD. Caspase-3 will cleave the inhibitor molecule (ICAD) releasing the CAD, which degrades DNA. The immunodetection of DFF-45 shows disappearance of the molecule with increasing time of exposure to 2.5 μM TBT, compared to control hepatocytes

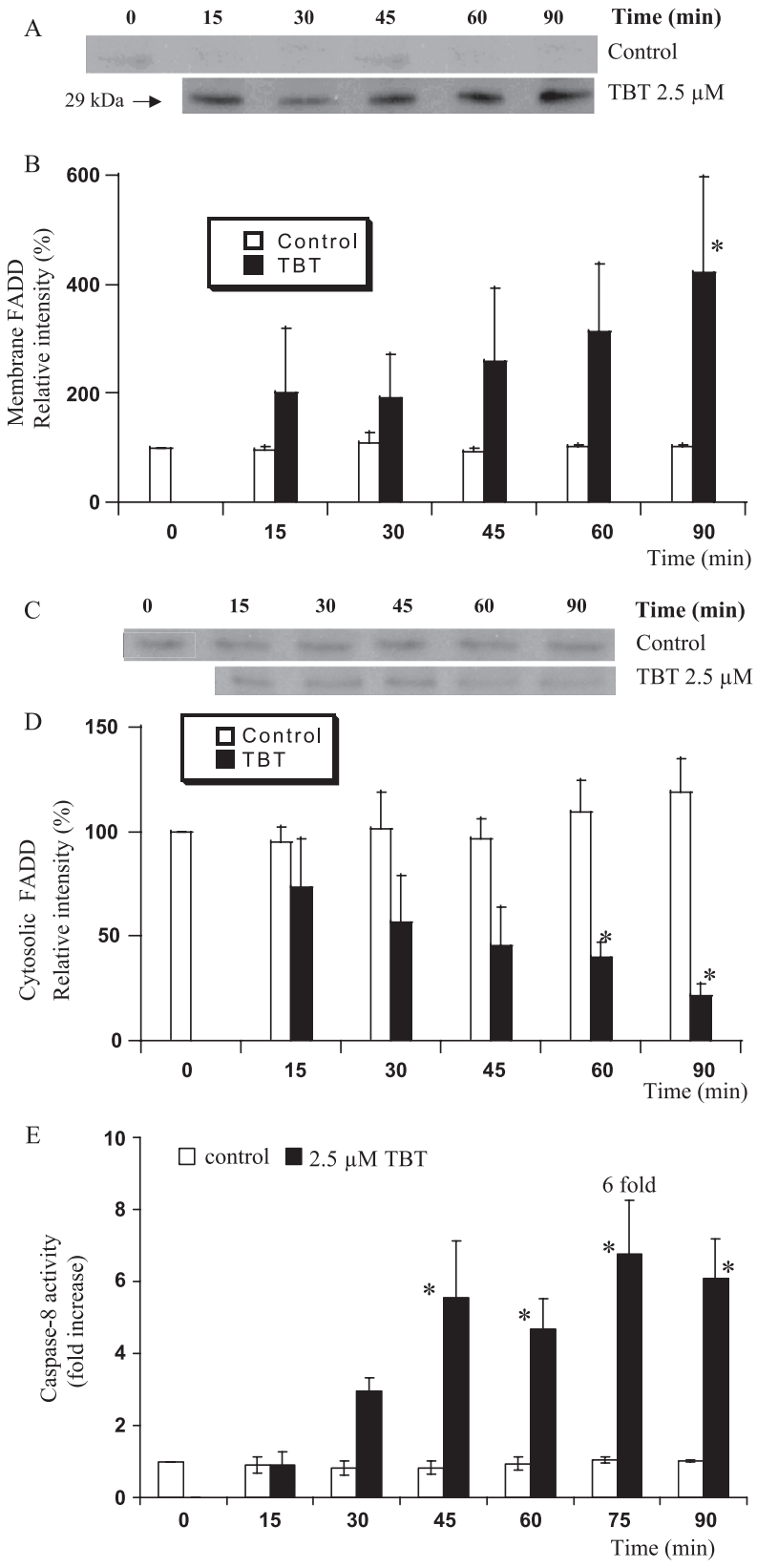

Fig. 4. Activation of the apoptotic death receptor pathway by TBT in rat hepatocytes. (A) Western blotting of FADD (29 kDa) at the membrane following treatment with 2.5 μM TBT in rat hepatocytes. (B) Autoradiograms of FADD were analysed using a scanning densitometer. (C) Detection of cytosolic FADD following treatment with 2.5 μM TBT in rat hepatocytes. (D) Autoradiograms of cytosolic FADD were analysed using a scanning densitometer. (E) Enzymatic activity of caspase-8 in control vs. 2.5 μM TBT-treated cells was assessed with fluorescent substrate Z-IETD-AFC. All data represent a compilation of three separate experiments. Error bars represent±SE.

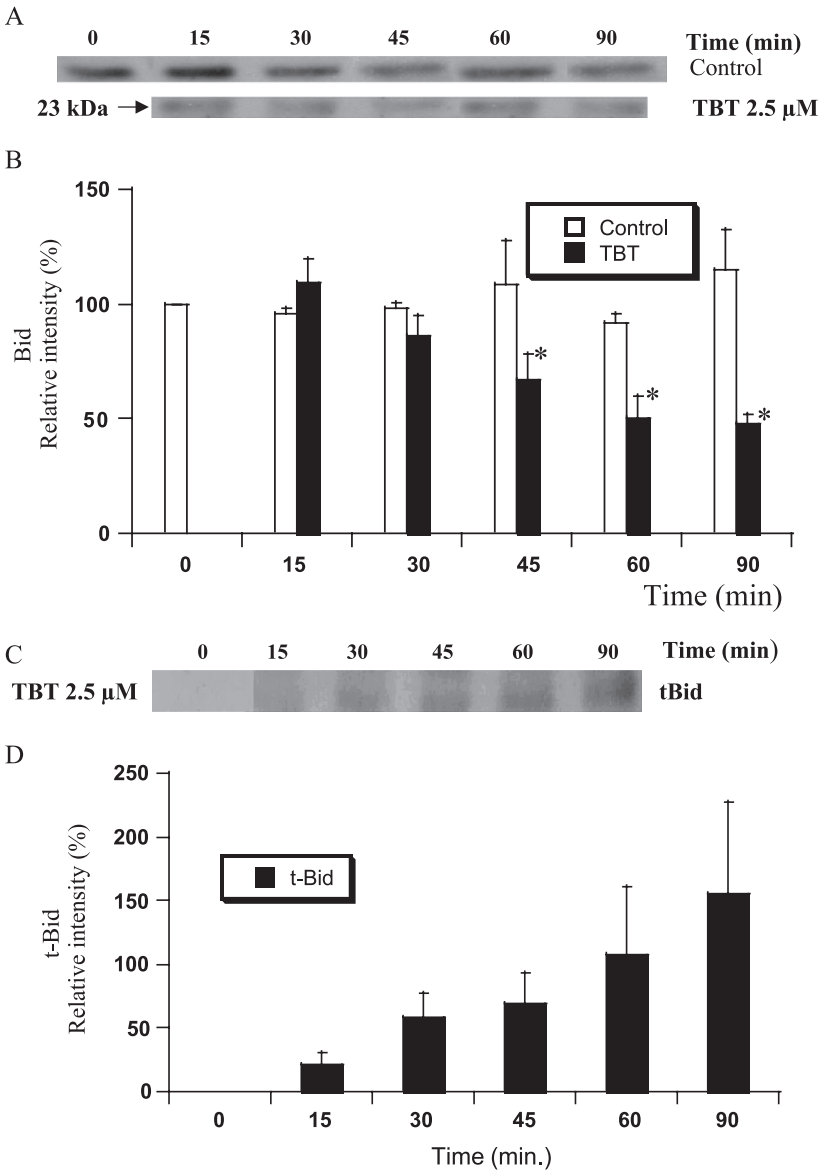

Fig. 5. TBT activates the cross-talk pathway between the death receptor and mitochondrial pathways. (A) Western blotting of Bid (23 kDa) in the cytosolic fraction following treatment with 2.5 μM of TBT. Bid was not detected in the noncytosolic fraction (data not shown). (B) Autoradiograms of Bid were analysed using a laser scanning densitometer. (C) Western blot of tBid in the noncytosolic fraction of 2.5 μM TBT-treated hepatocytes. (D) Autoradiograms of tBid were analysed using a scanning densitometer. tBid was not detected in the cytosolic fraction of TBT-treated hepatocytes nor in untreated controls (data not shown). Data represent means±SE from three separate experiments.

(Fig. 7C,D). The loss of DFF-45/ICAD was apparent after 30 min, reaching a minimum level after 60 min.

4. Discussion

The aim of this study was to investigate the molecular mechanisms of TBT-induced apoptosis in rat hepatocytes. We used a detailed approach to investigate the involvement of both the mitochondrial and death receptor pathways in TBT-induced apoptosis. We also determined the involvement of different caspases in the TBT-induced apoptotic cascade. In rat hepatocytes, TBT caused DNA fragmentation at concentrations of 2.5–3 μM after 75–90 min, confirming that the apoptotic phenomenon is present. In comparison with other cellular models, a concentration of 3 μM TBT increased DNA fragmentation in freshly isolated rat thymocytes after a 10-min exposure [31]. Following TBT assault in rainbow trout hepatocytes, the ladder pattern was apparent at a concentration of 2 μM after 90 min [37]. Indeed, at 2.5 μM TBT, rainbow trout hepatocytes did not produce DNA fragmentation, but their viability was greatly reduced due to necrotic cell death.

Chromatin condensation in rat hepatocytes preceded the appearance of the DNA ladder pattern, which is to be expected. A specific caspase-3 inhibitor prevented the con-

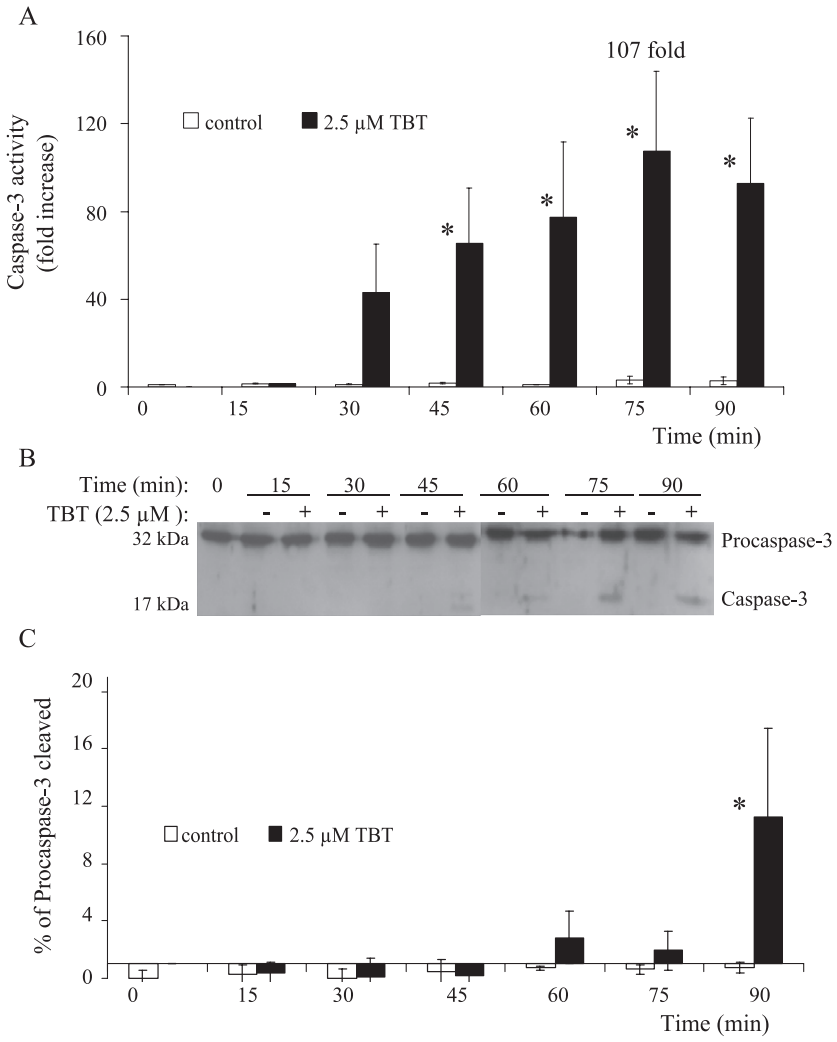

Fig. 6. Activation of the execution phase of apoptosis by TBT in rat hepatocytes. (A) Increase in enzymatic activity of caspase-3 when hepatocytes are subjected to 2.5 μM TBT. (B) Immunoblot of procaspase-3 and its cleaved fragment (17 kDa) after exposure to 2.5 μM TBT, relative to untreated controls. A plus (+) sign represents the TBT-treated sample and a minus (−) sign represents the vehicle (EtOH). (C) Autoradiograms of the 17-kDa fragment were analysed relative to total bands (procaspase-3 and 17-kDa fragment) using a scanning laser densitometer. Data represents means±SE from three separate experiments.

densation of nuclear chromatin, suggesting the involvement of caspase-3 in TBT-induced apoptosis. The broad-spectrum caspase inhibitor, z-VAD-fmk was a potent inhibitor of TBT-induced DNA fragmentation in rainbow trout hepatocytes [37]. In the T cell line HUT-78, DNA fragmentation and membrane blebbing was apparent following exposure to 2 μM TBT for 3 h [41]. Following pretreatment with z-VAD-fmk, these morphological changes were blocked.

Cytochrome *c* was detected in the cytosol after 15 min of TBT-exposure in isolated rat hepatocytes. This corresponds to an early event in apoptosis and is in agreement with previous studies. The upstream caspase-9 was activated in TBT-induced apoptosis in rat hepatocytes. Caspase-9 activation was preceded by cytochrome *c* release. These findings demonstrate that TBT activates the mitochondrial pathway in hepatocytes. It was demonstrated that low concentrations of TBT caused cytochrome *c* release from isolated mitochondria [53]. In Jurkat lymphoma cells, cytochrome *c* liberation was observed as soon as 5 min after exposure to 2 μM TBT and continued until 60 min [54]. TBT was shown to activate the caspase-9, but not caspase-8, in human platelets [55].

The present study is the first to demonstrate the involvement of a death receptor pathway in TBT-induced apoptosis. Our study shows translocation of FADD from the cytosol to the cell membrane in hepatocytes treated with 2.5 μM TBT, as well as the activation of caspase-8. Caspase-8 activation is usually related to the death receptor pathway; however, to our knowledge, no membrane-bound specific receptor has been yet associated with TBT. However, Fas receptors are expressed in hepatocytes and have been shown to be upregulated in hepatitis [56]. Hepatocytes also express the receptors TNF-R1, TRAIL-R1 and TRAIL-R2 [57]. Recently, a lipocaline-like protein was found to bind to TBT in serum of the Japanese flounder [58]. In addition, we demonstrate the involvement of the

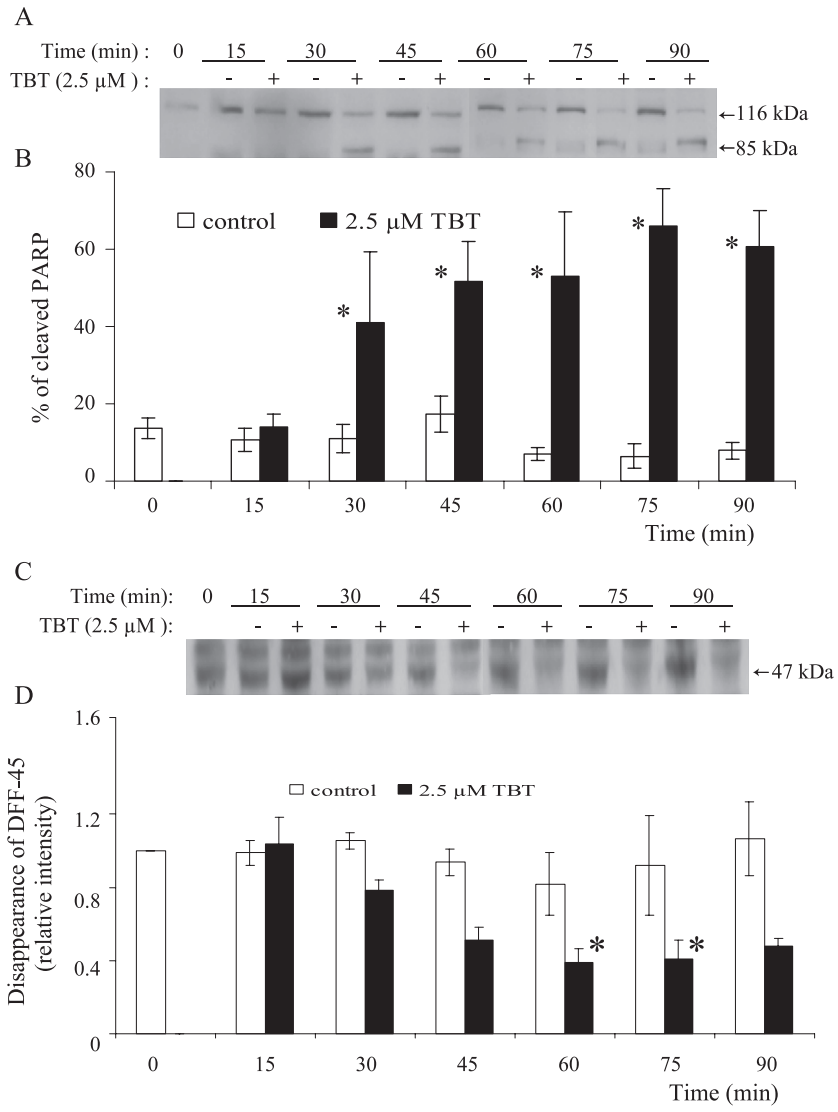

Fig. 7. Inactivation of caspase-3 substrates by TBT in rat hepatocytes. (A) Immunoblot of 2.5 μM TBT-induced cleavage of Poly(ADP-ribose)Polymerase was analysed using a whole cell lysate. The band at 116 kDa represents native PARP and its cleaved fragment is at 85 kDa. (B) Laser scanning densitometry of cleaved fragment of PARP relative to the native form. (C) Immunoblot representing the disappearance of DNA fragmentation factor-45 (DFF-45) (47 kDa) from the cytosol of 2.5 μM TBT-treated hepatocytes. The higher molecular weight band represents nonspecific antibody binding. (D) Autoradiograms of DFF-45 were analysed using a laser scanning densitometer. Data represent means±SE from three separate experiments.

pro-apoptotic protein Bid as a cross-talk between the death receptor and mitochondrial pathways in TBT-induced apoptosis in hepatocytes. Bid is known to be processed by caspase-8 to form truncated Bid (tBid). tBid can enter mitochondria and play a role in the release of cytochrome *c* [57,59]. The mechanism involving tBid is a way to amplify apoptosis via activation of the mitochondrial pathway [22].

Caspases are activated upon cleavage of the proenzyme at an internal aspartate residue. Immunodetection of caspase-3 showed cleavage of the pro-enzyme after 60 min of TBT treatment in rat hepatocytes. Caspase-3 activity peaked at 107 times that of control hepatocytes. TBT increased catalytic caspase-3 activity in HUT-78 cells by 30 times that of controls, after 3 h of treatment [41]. However, in platelets, 2 μM TBT caused very rapid activation of caspase-3 after 2–4 min [55].

The list of intracellular caspase substrates is still growing and caspase-3 is known to cleave a large number of them [34]. PARP is a nuclear enzyme that is responsible for catalysing the transfer of ADP-ribose polymers onto itself and other nuclear proteins in response to DNA strand breaks [60]. PARP cleavage is considered as a signature event for apoptosis and is immunodetected in primary cultures of rat hepatocytes exposed to 2.5 μM TBT. Another target of caspase-3 is DFF-45/ICAD. CAD is responsible for DNA degradation in apoptosis [61]. Disappearance of DFF-45/ICAD preceded DNA fragmentation that was noticeable after 75–90 min of TBT treatment. The cleavage of key caspase-3 substrates is reported here

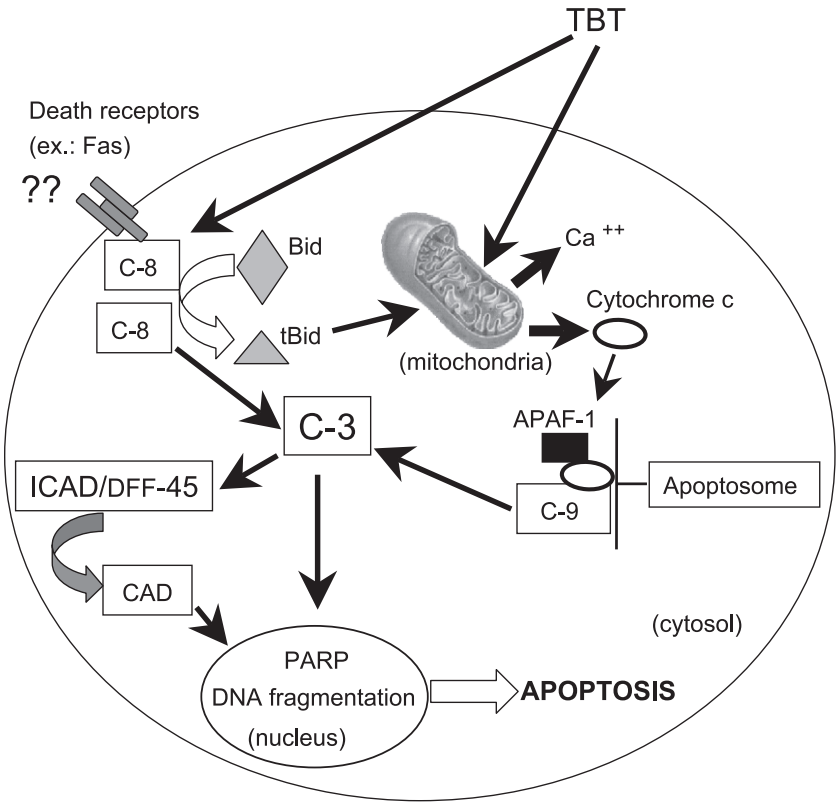

Fig. 8. Pathway of TBT-induced apoptosis in rat hepatocytes. C-3: caspase-3, C-8: caspase-8, C-9: caspase-9, Bid: pro-apoptotic protein, tBid: truncated form of Bid, PARP: Poly(ADP-ribose)Polymerase, ICAD/DFF-45: inhibitor of CAD or DNA fragmentation factor-45, CAD: caspase-activated DNase.

for the first time, to our knowledge, in rat hepatocytes following TBT-induced apoptosis.

In this report, we demonstrated the involvement of both the mitochondrial and death receptor pathways, as well as tBid as a linking step, in TBT-induced apoptosis of cultured rat hepatocytes. Based on these data and information available in the literature, we can propose a mechanistic model of TBT-induced apoptosis in cultured rat hepatocytes (Fig. 8). TBT could trigger apoptosis via caspase-dependent pathways, involving both death receptor(s) and mitochondria. TBT-induced changes in calcium homeostasis are well established [37]. TBT could cause calcium release from intracellular stores, such as the mitochondria and endoplasmic reticulum. This could lead to the release of cytochrome *c* from mitochondria. Cytochrome *c* would participate in the apoptosome complex leading to activation of caspase-9 and the subsequent activation of caspase-3. On the other hand, TBT could directly activate caspase-3, via translocation of FADD to a death receptor complex at the cell membrane and subsequent activation of caspase-8. Caspase-8 is able to truncate Bid to form tBid, and the latter can translocate to the mitochondria and be involved in the release of cytochrome *c* into the cytosol. This pathway can further amplify the mitochondrial pathway and the activation of caspases. The effector caspase-3 will dismantle its substrates such as PARP and DFF-45/ICAD, causing DNA fragmentation and ultimately apoptotic cell death in cultured rat hepatocytes.

TBT is ubiquitous in our environment and it is known to cause much damage at the cellular level. Measurable levels of TBT have been detected in whole blood and livers of human volunteers [20,46]. These findings suggest that the human population is being exposed to TBT sources without being aware of it. A potential risk of human contamination can arise from different sources such as antifouling paints and fish. Due to the persistence of organotin compounds in the environment, human risk should be assessed very closely.

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