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Cortical Astrocytes Exposed to Tributyltin Undergo Morphological Changes In Vitro

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ABSTRACT—We investigated the effect of tributyltin (TBT), an endocrine-disrupting chemical, on the morphology and viability of cultured rat cortical astrocytes. Cultured astrocytes exhibited smooth and planiform morphology under normal conditions. Following exposure to TBT, however, they showed rapid morphological changes that are characterized by asteriated cell bodies and process formation in a time- and concentration-dependent manner. Higher concentrations of TBT produced progressive cell death of the astrocytes. In serum-free medium, TBT at a concentration as low as 200 nM induced the stellation. Pharmacological studies revealed that the morphological changes were alleviated by application of diverse free radical scavengers or antioxidants such as catalase, superoxide dismutase, Trolox, ascorbic acid and *N*-acetyl-L-cysteine, suggesting that TBT-induced stellation is caused by oxidative stress involving free radicals, particularly reactive oxygen species. Furthermore, we found that the astrocyte stellation was abolished by treatment with inhibitors of phospholipase C, mitogen-activated protein kinase kinase or tyrosine phosphatase. The data suggest that TBT causes the stellation through intracellular signaling cascades rather than its non-specific toxicity. These findings provide an important insight for reconciling the problems in assumed aversive actions of this environmental pollutant for mammals.

Keywords: Organotin, Tributyltin, Astrocyte, Stellation, Morphology

Tributyltin (TBT), one of organotin compounds, has been utilized widely in the stabilization of polyvinyl chloride and in antifouling paints on vessels for a long period of time (1). It has recently been receiving much attention as an endocrine-disrupting chemical because the compound develops 'imposex', which consists of superimposition of male characteristics onto a reproductive anatomy in female snails (2, 3). This anatomical aberration is possibly a consequence of the inhibition by TBT of aromatase cytochrome P450, which catalyzes the aromatization of androgens to estrogens (4, 5).

Although there are several reports indicating that intoxication of humans and experimental animals with organotin compounds causes epilepsy and amnesia (6 – 8), their toxicity for mammals has not been completely elucidated. Actually, it is plausible that TBT brings about an unidentified effect on humans because they might accumulate TBT in the body through intake of food such as fish and shellfish inhabiting in a TBT-polluted environment (9). Our recent study showed that TBT has both potent vasoconstrictor and vasorelaxant activities that were likely mediated by α_1-adrenoceptors on the vascular smooth muscle and muscarinic receptors on the vascular endothelium, respectively (10). This finding might give a warning about the yet unconfirmed toxicity of this environmental pollutant for mammals.

As for the central nervous system, we have demonstrated that TBT induces massive neuronal death in a concentration- and time-dependent manner with CA3 > CA1 > dentate gyrus ranking of vulnerability of the hippocampal subfields using organotypic slice cultures of immature rat hippocampus (11). The neurotoxicity was attenuated by free radical scavengers or antioxidants such as catalase, superoxide dismutase, Trolox and α-tocopherol, which suggests an involvement of free radicals including reactive oxygen species. Except for our previous study, however, little is known about the effects of TBT on the central nervous system. Because astrocytes are well known to become reactive in association with brain tissue damage caused by hypoxia, ischemia or seizures (12, 13), we investigated the effects of TBT on cultured astrocytes of rat cerebral cortex and

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found that TBT causes morphological changes of astrocytes in a concentration- and time-dependent manner. The phenomenon was further investigated pharmacologically.

MATERIALS AND METHODS

Cell culture

Primary cultures of astrocytes were prepared from the cerebral cortexes of postnatal 2-day-old Wistar rats (SLC, Shizuoka) as described else (14). In brief, dissociated cortical cells were suspended in modified Eagle's medium containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% fetal bovine serum (Cell Culture Lab., Cleveland, OH, USA) and then plated on uncoated 75-cm^2 flasks at a density of 600,000 cells/cm^2. Non-astrocytic cells including neurons and microglia were detached from the bottom of the flask using a shaker and then removed by a change of the medium. A monolayer of type I astrocytes was obtained 14 – 18 days after the plating. The astrocytes were again dissociated by trypsinization and reseeded on uncoated 48-well plates or 96-well plates at a density of 20,000 cells/cm^2. The cultures were routinely kept at 37°C in a humidified and 5%-CO_2 atmosphere, and the culture medium was changed every 3 days. The cultures were used in the following experiments 10 – 11 days after the passage, the period of time which allowed the cells to become confluent again.

Exposure to TBT

Cultures were exposed for 5 – 96 h to fresh TBT (tributyltin chloride; Wako Pure Chemical Industries, Ltd., Saitama) dissolved in <0.02% dimethyl sulfoxide (DMSO, Wako) in concentrations ranging from 0.03 to 3 μM. In control cultures, astrocytes were treated with medium containing 0.02% DMSO for the same time as TBT exposure.

Morphological observation and immunocytochemistry

Cell morphology was assessed by light microscopic examination. To identify astrocytes, the immunohistochemical staining for glial fibrillary acidic protein (GFAP) was performed. At 5 – 96 h after the cultures were transferred to the medium containing TBT, the cells were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde at 4°C for 30 min. After being washed with phosphate-buffered saline at room temperature, the cultures were incubated overnight with monoclonal antibodies for GFAP (Boehringer Mannheim Biochemica, Mannheim, Germany) and stained with a Vectastain ABC kit (Vector, Burlingame, CA, USA) (15). The percentage of stellate cells was determined by counting cells in four randomly selected 1-mm^2 fields per well.

Determination of cell viability and redox activity

Damages of the plasma membrane were evaluated by measuring release of the cytosolic enzyme lactate dehydrogenase (LDH) into the bathing medium. At 24 h after the cultures were transferred to the serum-free medium, 50 μl of culture supernatant was collected from each well, and LDH activity were determined with a colorimetric LDH assay kit (Promega, Madison, WI, USA). Total cellular LDH activity was determined after cell solubilization with 0.3% Triton X-100. To evaluate the viability of cells, cellular redox activity was measured by modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay (16). MTT (0.5 mg/ml) was added to each well and then incubation continued for 4 h at 37°C. The dye MTT is converted into a purple formazan by the redox activity of living cells. After the incubation, the cells were solubilized by adding a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.8), and the amount of formazan product was determined by measuring absorbance at 570 and 630 nm.

Drugs

Catalase from bovine liver, *N*-acetyl-L-cysteine (NAC) and SQ22536 (9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine) were purchased from Sigma Chemical Co., St. Louis, MO, USA; and superoxide dismutase (SOD) from bovine erythocyte, ascorbic acid and sodium orthovanadate were obtained from Wako. Trolox, U-73122 (1-[6-([(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino)hexyl]-1*H*-pyrrole-2,5-dione), U-0126 (1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenylmercapto)butadiene) and genistein were purchased from Aldrich Chem., Co., Inc. (Milwaukee, WI, USA), RBI (Natick, MA, USA), Promega and Calbiochem (La Jolla, CA, USA), respectively.

Statistics

The same experiments were repeated four times using separate cultures prepared from different litters. All data are expressed as the means ± S.E.M. Statistical significance was assessed with Student's *t*-test, the Welch test or Tukey's multiple range test following one-way analysis of variance (ANOVA). The half-maximal effective concentration (EC_{50}) and lethal concentration (LC_{50}) was determined by the least squares method.

RESULTS

Effect of TBT on the morphology and viability of astrocytes

A 24-h incubation in control medium containing 0.02% DMSO caused neither morphological changes nor cytotoxicity in cultured astrocytes (Fig. 1A). However, 1 μM TBT induced a remarkable change in cell morphology, which

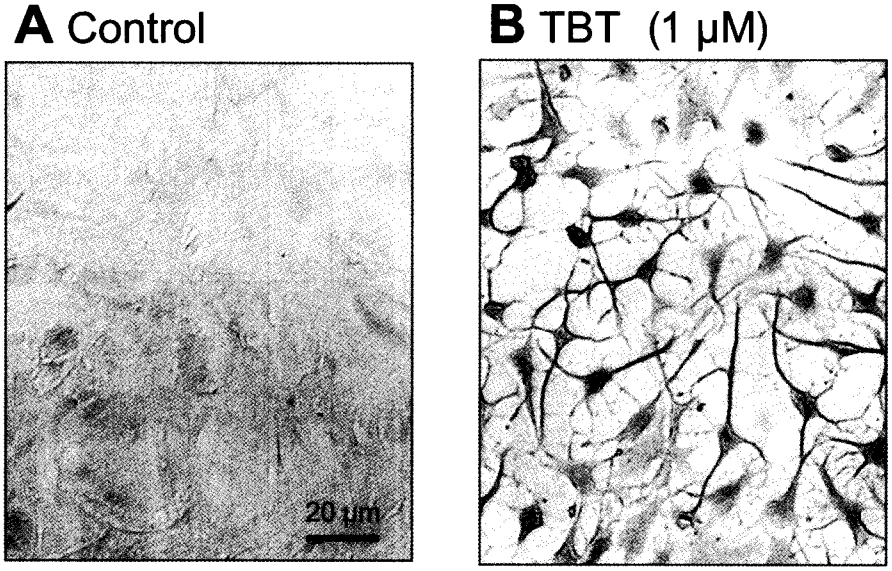

Fig. 1. Representative photomicrographs showing TBT-induced morphological changes of rat cortical astrocytes in 10% FBS-containing medium. After treatment with TBT at 0 μM (A) or 1 μM (B) for 24 h, the cells were fixed and stained with a monoclonal antibody for GFAP.

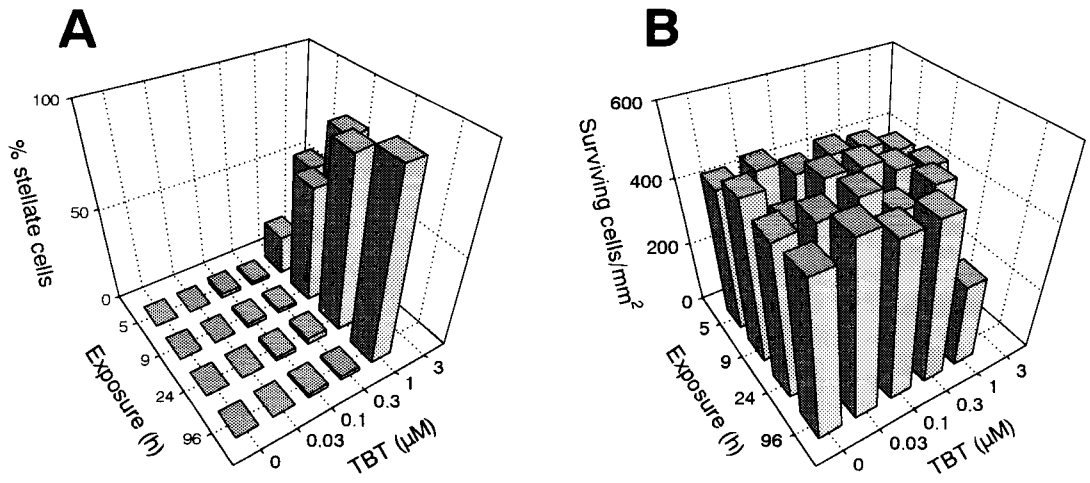

Fig. 2. Time course and concentration-dependency of TBT-induced stellation (A) and survivability (B) of astrocytes exposed to TBT at 0.03, 0.1, 1 and 3 μM for 5, 9, 24 and 96 h. The numbers of planiform cells and asteriated cells were measured under a light microscope, and the percentage of the stellate cells to the total astrocytes was calculated (A). The density of surviving cells is defined as the total number of surviving astrocytes divided by the bottom area of the flask (B). The data are shown as the means of 4 cases tested.

was characterized by asteriated cell body and process formation (stellation) (Fig. 1B). To evaluate the concentration dependency and time course of the morphological changes, the number of asteriated cells was measured after TBT treatment at concentrations of 0.03, 0.1, 0.3, 1 and 3 μM for 5, 9, 24 and 96 h (Fig. 2A). Although TBT did not change astrocyte morphology at concentrations of <0.3 μM, the exposure to TBT at 1 or 3 μM for >5 h markedly provoked morphological changes. As a result, a concentration- and time-dependent profile was confirmed in TBT-induced stellation. EC_{50} was calculated to be 0.610 ± 0.003 μM (n = 4). In this series of experiments, the total number of surviving astrocytes was simultaneously monitored to determine the cytotoxicity of TBT (Fig. 2B). We found that TBT at 1 μM for 96 h and at 3 μM for >24 h decreased the number of astrocytes. LC_{50} was 1.046 ± 0.130 μM (n = 4). Because there is a significant difference between EC_{50} and LC_{50} ($t(6) = 3.3486$, $P = 0.016$, Student's t-test), the cell

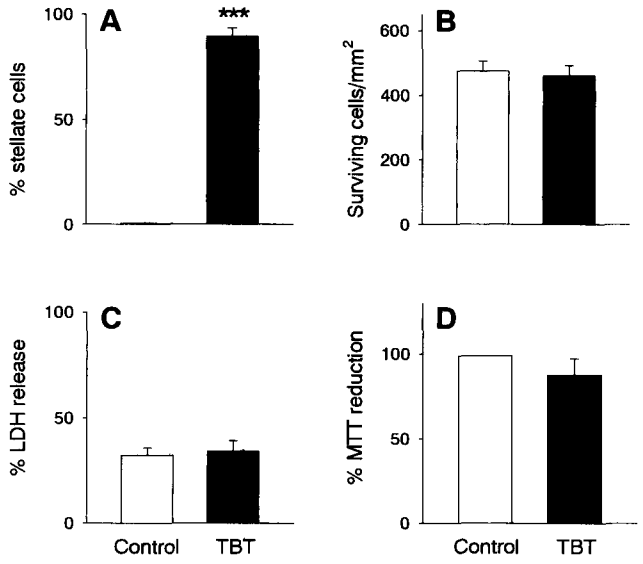

Fig. 3. Effects of TBT at 1 μM for 24 h on astrocytic morphology and viability. The ratio of stellate cells (A), the number of surviving astrocytes (B), LDH release (C) and MTT reduction ability (D) were measured in the culture incubated in the absence (□) or presence (■) of 1 μM TBT for 24 h. ***$P<0.001$ vs Control: Welch test. The data represent the means ± S.E.M. of 4 cases.

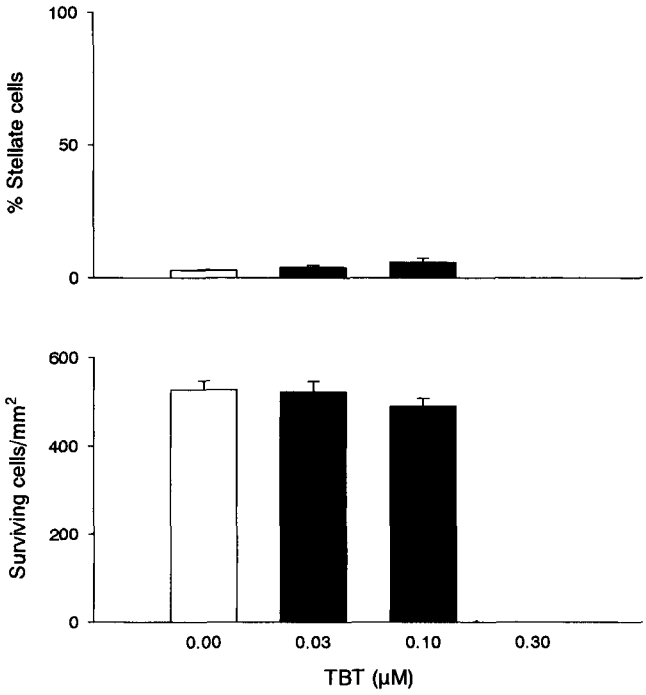

Fig. 4. Effects of long-term exposure of TBT at low concentrations on astrocytic morphology and viability. The ratio of stellate cells and the number of surviving astrocytes exposed to TBT at 0.03, 0.1 or 0.3 μM for 2 weeks. The percentage of the stellate cells to the total astrocytes was calculated. The density of surviving cells is defined as the total number of surviving astrocytes divided by the bottom area of the flask. The data represent the means ± S.E.M. of 6 cases.

viability was further evaluated by LHD release and MTT reduction assays. TBT at 1 μM for 24 h induced the stellation of astrocytes (Fig. 3A) without affecting the total number of surviving cells (Fig. 3B). The same conditioning did not change LDH release (Fig. 3C) or MTT reduction activities (Fig. 3D). These results suggest that the mild stimulation of astrocytes with TBT affects the morphology but not the survivability or the cell vigor. It is also supposed that different cascades underlie TBT-induced stellation and cell death. This idea was further supported by the following experiment, in which we investigated the influence of an agent that disrupts microtubules and inhibits tubulin polymerization, colchicine, on astrocyte stellation because the morphological changes of astrocytes are well known to reflect cytoskeletal rearrangements (17 – 21). Cotreatment with 25 μM colchicine almost completely abolished TBT-induced morphological changes, but it did not inhibit TBT-induced cell death (data not shown). This result indicates that TBT-induced cell death is not a consequence of the stellation and suggests that the mechanism of the stellation was probably different from that of cell death.

To investigate the chronic effects of TBT on astrocytes, we observed the morphology and survival of astrocytes exposed to TBT at lower concentrations for 2 weeks. TBT at 0.3 μM for 2 weeks induced cell death without affecting cell morphology, while TBT at concentrations of <0.1 μM for 2 weeks did not change astrocyte morphology nor the number of surviving cells (Fig. 4).

Pharmacological characterization of TBT-induced astrocyte stellation

To exclude the possible disturbance of pharmacological characterization of TBT-induced stellation by serum contained in the culture medium, the following experiment was performed in serum-free medium. First, we examined the effect of serum-containing and serum-free medium on TBT-induced stellation and cell death. Twelve hours after culture medium containing 10% FBS was changed to serum-free medium, the cells were treated with TBT, and then the ratio of asteriated cells and the number of surviving cells were gauged (Fig. 5A). Although a concentration-response relationship of the survivability was virtually unaffected by the medium conditions, TBT even at low concentrations of >0.2 μM induced the stellation in serum-free medium. However, because the effect of TBT at 1 μM was not different between FBS-containing medium and serum-free medium, the following pharmacological analysis was conducted on the stellation induced by 1 μM TBT.

The processes of cell injury or damages often involve free radical generation or oxidative stress (22 – 24). Indeed,

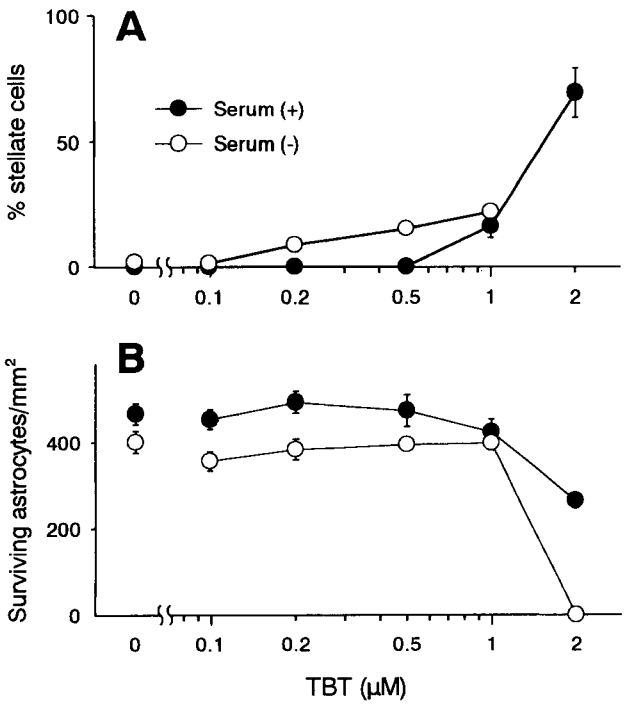

Fig. 5. Effect of serum-containing and serum-free medium on TBT-induced stellation and cell death of astrocytes. The ratio of stellate cells (A) and the number of surviving astrocytes was measured in the cultures which had received 12-h exposure to TBT in 10% FBS-containing medium (●) and serum-free medium (○). The data represent the means ± S.E.M. of 4 cases.

in hippocampal slice cultures, we have previously shown that TBT cytotoxicity for neurons was abolished by free radical scavengers (11). Therefore, we investigated the effect of lipid-soluble antioxidant (Trolox) or water-soluble antioxidant (ascorbic acid or NAC) on TBT-induced morphological changes (Fig. 6). When the antioxidants were applied during the 24-h exposure to 1 μM TBT, 200 μM Trolox inhibited TBT-induced morphological changes by about 70%. Ascorbic acid (0.1 mM) also lowered the stellation by about 50%. However, higher concentrations of ascorbic acid did not completely antagonize the stellation, which suggests that TBT-induced stellation was mediated, at least in part, by radical species that can not be quenched by ascorbic acid. On the other hand, another water-soluble antioxidant NAC (50 μM) almost completely abolished TBT-induced morphological changes. Therefore, in order to clarify what kinds of radicals were involved, we inspected the effect of the oxygen radical scavenger protein catalase or SOD on TBT-induced morphological changes. Either 1 U/ml catalase or 10 U/ml SOD inhibited the morphological changes by about 60%. The result suggests that the stellation is mediated partially by one or more reactive oxygen species, probably like H_2O_2 and O_2^-.

Several previous papers indicated that TBT induces consecutive $[Ca^{2+}]_i$ increase possibly through Ca^{2+} release from intracellular stores (11, 25). To investigate whether TBT-induced stellation was mediated by the phospholipase C (PLC) product inositol trisphosphate (IP_3), which is a stimulant of intracellular Ca^{2+} stores, the effect of the PLC inhibitor U-73122 (26) was examined. U-73122 (10 μM) reduced TBT-induced morphological changes by about 70% (Fig. 7). Furthermore, because diacylglycerol (DAG), another PLC product, activates protein kinase C (PKC), which subsequently activates the mitogen-activated protein kinase (MAPK) cascade, the effect of the MAPK kinase (MEK) inhibitor U-0126 (27) was investigated. U-0126 (250 μM) attenuated the stellation by about 70% (Fig. 7). It is well known that an increase in cellular cyclic AMP level and subsegment activation of cyclic AMP-dependent protein kinase (PKA) caused astrocyte stellation (20, 28). Therefore the effect of the adenylyl cyclase inhibitor

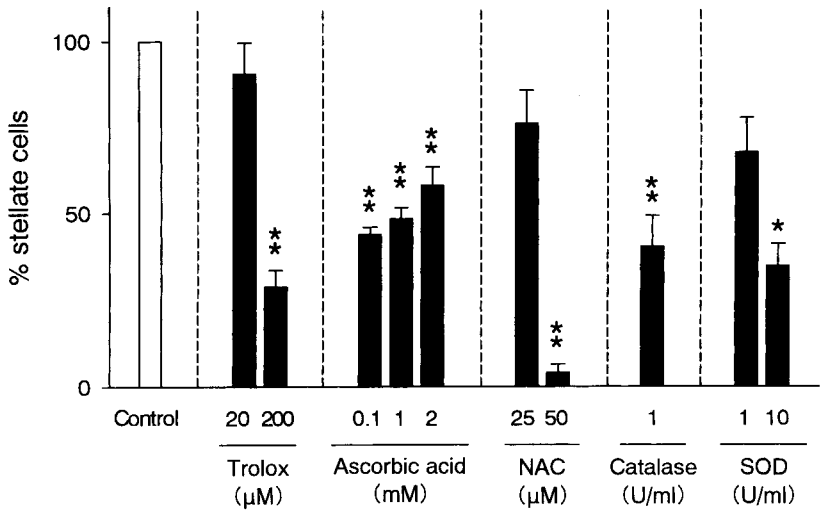

Fig. 6. Effects of various antioxidants on TBT-induced morphological changes. Astrocytes cultured in serum-free medium for 24 h were treated with TBT at 1 μM for 24 h in the presence of catalase, SOD, Trolox, ascorbic acid or NAC. Each bar is expressed as a percentage the to control culture: (the data of drug-treated cultures) / (the data of the cultures exposed to TBT alone) × 100. *$P<0.05$, **$P<0.01$ vs Control, Tukey's test following ANOVA. The data represent the means ± S.E.M. of 4 cases. All antioxidants used here alone had no effects on astrocyte morphology at the same concentrations (data not shown).

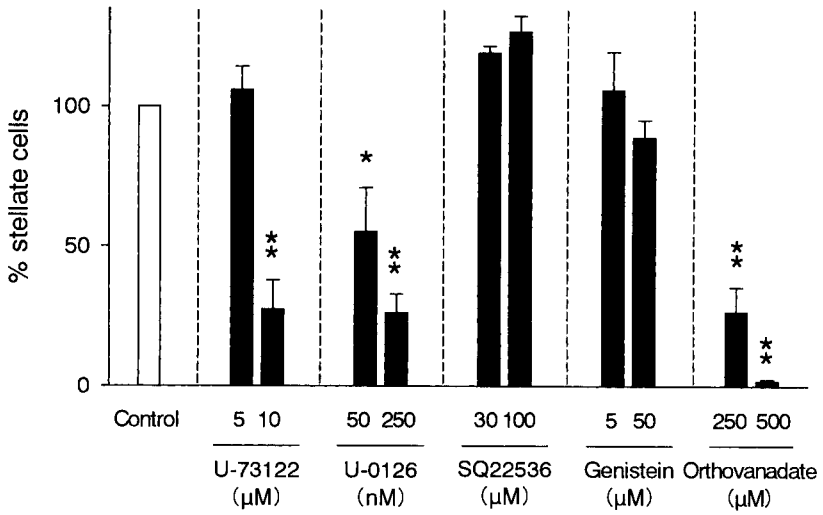

Fig. 7. Effects of inhibitors of various signal transductions on TBT-induced morphological changes. Astrocytes were treated with 1 μM TBT in the presence of U-73122, U-0126, SQ22536, genistein or orthovanadate. Each bar is expressed as a percentage to the control culture, which was treated with TBT alone. $^{*}P<0.05$, $^{**}P<0.01$ vs Control, Tukey's test following ANOVA. The data represent the means ± S.E.M. of 4 cases. All inhibitors used here alone had no effects on astrocyte morphology at the same concentrations (data not shown).

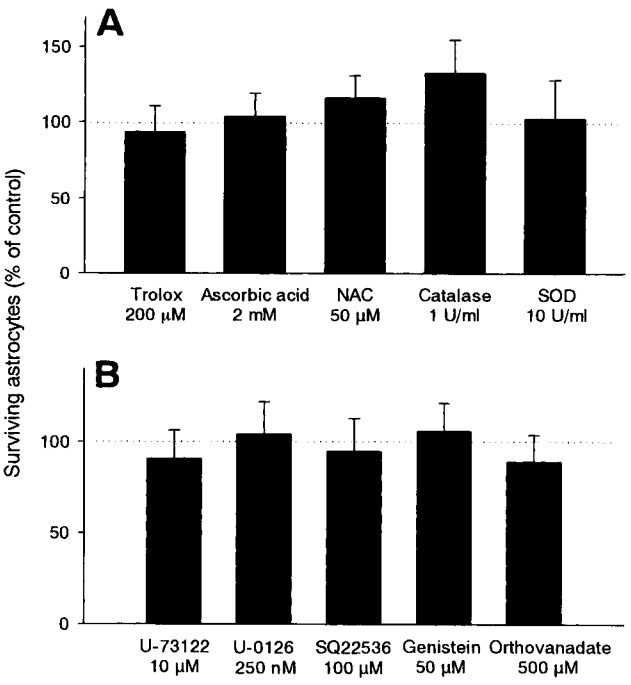

Fig. 8. Effects of various antioxidants (A) or inhibitors of various signal transductions (B) on viability astrocytes exposed to 1 μM TBT. Astrocytes were treated with 1 μM TBT in the presence of Trolox, ascorbic acid, NAC, catalase, SOD, U-73122, U-0126, SQ22536, genistein or orthovanadate. Each bar is expressed as a percentage to the control culture: (the data of drug-treated cultures) / (the data of the cultures exposed to TBT alone) × 100. The data represent the means ± S.E.M. of 6 cases.

SQ22536 (29, 30) was examined, although SQ22536 at 100 μM did not affect TBT-induced stellation (Fig. 7). Finally, since several reports indicate that tyrosine phosphorylation and dephosphorylation regulate the morphology of astrocytes (31–34), we examined the effects of the tyrosine kinase inhibitor genistein and the tyrosine phosphatase inhibitor orthovanadate. Although TBT-induced astrocyte stellation was not affected by genistein, it was almost completely eliminated by 500 μM orthovanadate (Fig. 7). Incidentally, none of the drugs used in this pharmacological study affected the viability of astrocytes (Fig. 8).

DISCUSSION

In the present study, we have shown for the first time that TBT, known as an endocrine-disrupting chemical, induces morphological changes of astrocytes in a concentration- and time-dependent manner. We previously found that TBT causes severe neuronal damage in organotypic cultures of hippocampal slices (11). Therefore, we examined the effect of TBT on astrocyte viability, but the measurement of the number of cells, LDH release and MTT reduction ability revealed that acute treatment of TBT at <1 μM induced no significant cell damage, while higher concentrations of TBT reduced the viability. On the other hand, chronic treatment of TBT at a low concentration of 0.3 μM reduced the viability of astrocytes without morphological changes. Because there is a significant difference between EC_{50} for the stellation and LC_{50} and also because the antimicrotubule agent colchicine abolished TBT-induced morphological changes but not the cell death, different intracellular cascades possibly work between the stellation and cell death. Particularly, this action of colchicine indicates as well that cell death is not a consequence of the stellation.

Astrocytes displayed morphological changes at lower concentrations of TBT in serum-free medium than that in FBS-containing medium. Serum is known to inhibit astrocyte stellation induced by various stimuli. The serum constituents, probably such as serine protease thrombin and

the bioactive lipid lysophosphatidic acids, were responsible for serum-inhibited stellation (15, 35–37). Therefore, TBT-induced stellation might also be alleviated in the presence of serum. However, it should be noted that TBT produced stellation even at low concentration of 0.2 μM. Horiguchi et al. (38) reported that TBT concentrations in rock shells living in the polluted sea area in Japan reach 5–10 μM. Therefore, TBT may be accumulated also in mammals as a result of ecological magnification. Indeed, Kannan and Falandysz (39) indicated that a significant concentration (about 0.4 μM) of butyltin was detected in human liver. Although there are no data available for its concentration in human brain, it is plausible that TBT was accumulated in the brain at a similar or higher level, as compared with the liver, because of the lipophilicity of TBT. Thus the effect of 0.2 μM TBT observed in the present study might feasibly occur in mammals.

The result that Trolox, NAC or ascorbic acid blocked the TBT-induced morphological changes of astrocytes suggests an involvement of free radical. In particular, reactive oxygen species including H_2O_2 and O_2^- may play some role because TBT toxicity was abolished by catalase as well as SOD. H_2O_2 is freely diffusible across the plasma membrane, whereas catalase and SOD are membrane-impermeable. Therefore, exogenously applied catalase and SOD can only reduce extracellular H_2O_2 and O_2^-, respectively. Thus, the effectiveness of these scavenger proteins might indicate that TBT produces reactive oxygen species around the cells or near the inside of the plasma membrane. However, another possibility can also be raised. Rivera et al. (40) reported that TBT penetrates the plasma membrane only in the presence of endogenous O_2^- and then induces morphological changes in erythrocytes and hemolysis. Therefore, TBT-induced stellation may require endogenous H_2O_2 and O_2^- rather than TBT itself producing harmful reactive oxygen species.

Because inhibitors of PLC and MAPK prevented TBT-induced stellation, PLC activation and the MAPK cascade might play roles in the stellation. Our previous study indicated that neurons exposed to TBT exhibited $[Ca^{2+}]_i$ increase, possibly through Ca^{2+} release from the intracellular store. Taken together, we think that IP_3 and DAG produced by PLC evoke a $[Ca^{2+}]_i$ rise and thereby activates MAPK and then stellation is subsequently induced.

Because the inhibitor of adenylyl cyclase did not prevent TBT-induced stellation, TBT unlikely affected the cascade of cyclic AMP. Further studies would be necessary to verify these possibilities.

Phosphorylation and dephosphorylation of protein tyrosine residues, mediated by tyrosine kinase and tyrosine phosphatase, respectively, are also known to regulate the morphology of fibroblasts and astrocytes (31–33). TBT-induced stellation was not affected by the tyrosine kinase inhibitor, but it was almost completely eliminated by the tyrosine phosphatase inhibitor. Therefore, these results suggest that tyrosine phosphatase activity is necessary for TBT-induced astrocyte stellation. However, it remains to be understood how TBT activates the tyrosine phosphatase and what molecules are the direct targets of TBT for activation of this enzyme. Because Padmanabhan et al. (34) indicated that focal adhesion kinase (FAK) phosphatase is an important molecule for astrocyte stellation, TBT might be associated with the FAK phosphatase cascade. The clarification of signal transduction between TBT and tyrosine phosphatases would be a key for understanding molecular mechanisms of TBT-induced stellation.

In conclusion, our present study has shown that TBT induced morphological changes of rat cortical astrocytes even at relatively low concentrations, but higher concentrations of TBT provoke massive death of astrocytes. Our data suggest that TBT renders oxidative stress to astrocytes, which might be mediated, at least in part, by H_2O_2 and O_2^-, and that PLC, MEK and tyrosine phosphatase are involved in TBT-induced stellation. Our previous study described TBT-induced neurotoxicity in hippocampal slice cultures, whereas the present study has shown that TBT affects astrocytes at lower concentrations. Because the neuronal physiology is tightly regulated by astrocytes, these results suggest that low concentrations of TBT, which do not produce rigid neurotoxicity, change the functions of neurons. Further investigation on functional changes of neurons induced by TBT is now underway. The broad toxicity of TBT predicts aversive influences on physiological functions of the central nervous system in mammals. Our finding on the signaling cascade activated by TBT provides important insight for the problems in such presumed aversive actions of this environmental pollutant.

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