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Original Article

Triphenyltin inhibits GA-binding protein α nuclear translocation

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ABSTRACT — Organotin compounds such as triphenyltin (TPT), which are common environmental pollutants, had been widely used as antifouling agents for ship bottoms. Although toxic effects of organotins through nuclear receptors such as retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ have been well demonstrated, other mechanisms underlying organotin-induced toxicity have hardly been reported. In the present study, we focused on the transcription factor GA-binding protein (GABP), which regulates the expression of various housekeeping genes, as a novel target of TPT toxicity. We investigated the change of GABPα subunit protein expression induced by TPT. Although 100-500 nM concentration of TPT was not found to affect the total protein expression of GABPα, TPT significantly decreased nuclear translocation of GABPα in human embryonic kidney (HEK) 293T cells. In addition, TPT increased intracellular reactive oxygen species (ROS) levels. Both inhibition of GABPα nuclear translocation and the increase in ROS levels were observed in menadione (an ROS inducer)-treated HEK293T cells. Our results indicate that TPT causes inhibition of GABPα nuclear translocation, which may be triggered by ROS production. This might have serious implications in cellular physiology, thereby affecting cell survival.

Key words: Triphenyltin, GA-binding protein, Reactive oxygen species, Menadione, Nuclear translocation

INTRODUCTION

Organotin compounds, in particular triphenyltin (TPT), had been widely used as antifouling agents for ship bottoms or fishing nets, and agricultural pesticides. Since the 1980s, their irreversible effects on sexual abnormalities in some female gastropods, known as “imposex”, have been reported, and thus, the use of organotins including TPT is internationally restricted. However, the concentrations detected in the seabed remain flat and there are concerns about secondary exposure to humans through seawater and seafoods polluted with organotins. In fact, 0.038-2.6 mg TPT/kg was detected in fish (Stab *et al*., 1996), and detection at nanomolar levels in human blood have also been reported (Rantakokko *et al*., 2008). Therefore, it is necessary to consider the potential risks of organotins to humans.

Several studies have reported the toxicities of TPT (Kotake, 2012); for example, TPT causes premature apoptosis through activation of the transcription factor

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NF-κB (Marinovich *et al*., 1996). Moreover, most of toxic effects of TPT are likely to be induced through nuclear receptors. TPT at 10-100 nM concentration directly binds and activates the retinoid X receptor (RXR) and peroxisome proliferator-activated receptor (PPAR) γ in mammalian cells (Kanayama *et al*., 2005; Nakanishi *et al*., 2005; Hiromori *et al*., 2009). In addition, 9-cis retinoic acid, known as RXR ligand, induces imposex in gastropods (Castro *et al*., 2007), and is therefore an important mechanism underlying TPT-induced imposex. Furthermore, TPT exposure changes aromatase levels by altering the expression of CYP19 through RXR activation in humans (Nakanishi, 2006). TPT is also known to promote the differentiation of preadipocyte 3T3-L1 cells, an *in vitro* murine preadipocyte model, to adipocytes, through the PPARγ and RXR signaling pathways by upregulating the expression of adipocyte marker genes (Kanayama *et al*., 2005). Although the toxic effects of TPT through RXR and PPARγ have been well demonstrated, they do not, however, account for all the toxicity.

In this study, we focused on GABP, an Ets transcription factor, which regulates several biological essential genes. It consists of two subunits (α and β), and forms a transcriptionally active heterotetrameric complex. GABPα subunit contains the DNA binding domain, while the GABPβ subunit contains a transcriptional activation domain and a carrier protein binding domain, to which the carrier proteins importin-α and β, bind and gets transferred to the nucleus (Hayashi *et al*., 2013). It is known that GABP regulates the expression of various genes that are essential for normal cellular physiology such as mitochondrial (Virbasius *et al*., 1993) and ribosomal (Curcic *et al*., 1997) related genes. GABP also plays an important role in the control of immunity (Luo *et al*., 2017) and cell cycle (Sowa *et al*., 1997). Furthermore, the dramatic importance of GABP was shown in a study when GABPα-deficient mice exhibited embryonic lethality (Ristevski *et al.*, 2004). This indicates the biological essentiality of GABP and a dysfunction of GABP is therefore expected to cause adverse effects on cell survival.

It is reported that reactive oxygen species (ROS) inhibits its nuclear translocation of GABP and subsequently disrupts GABP function (Martin *et al*., 1996; Chinenov *et al*., 1997). TPT is known to increase intracellular ROS levels, and various toxicities due to TPT-induced ROS have been reported (Xu *et al*., 2011). In the current study, we investigated the effects of TPT on GABP nuclear translocation and its relationship to intracellular ROS levels.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney (HEK) 293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in complete DMEM (Nissi Pharmaceuticals, Tokyo, Japan) supplemented with 0.58 g/L L-glutamine, 100 units/mL penicillin G, 100 μg/mL streptomycin, 0.2% $NaHCO_3$, 10% FBS (Biosera, Nuaille, France), and 4500 mg/L glucose and incubated at 37°C in a humidified 5% CO_2 incubator.

Cell viability assay

HEK293T cells were seeded at a density of 1.0×10^5 cells in 24 well culture plate and incubated overnight. After TPT treatment, cell viability was determined by the water-soluble tetrazolium salt (WST)-1 assay (DOJINDO, Kumamoto, Japan), according to the protocols described by Miyara *et al*. (2016) with minor modifications, the cell culture medium was replaced with WST-1 reagent diluted 1:24 in cell culture medium.

Protein isolation

After TPT or menadione treatment, cytosolic, nuclear and whole-cell extracts were isolated individually following the protocols outlined in another study (Ishida *et al*., 2017).

Western blotting

The protocol described in a published study was followed (Umeda *et al*., 2016). Anti-GABPα antibody (sc-28312, Santa Cruz Biotechnology, Dallas, TX, USA), anti-lamin B1 antibody (sc-377000, Santa Cruz Biotechnology), anti-β-tubulin antibody (018-25044, FUJIFILM Wako Pure Chemicals, Osaka, Japan), and anti-GAPDH antibody (016-25523, FUJIFILM Wako Pure Chemicals) were used to detect the proteins. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (A9044, Sigma-Aldrich, St. Louis, MO, USA) were used as a secondary antibody.

Plasmid constructions

Full-length Myc-tagged GABPα and V5-tagged GABPβ1 was generated using the primers summarized in Table 1. The PCR products were digested with Hind III, Not I and Xho I, and cloned into the pcDNA3.1/Zeo (+) (Invitrogen, Carlsbad, CA, USA) vector cut with these two enzymes. All clones generated by PCR were confirmed by sequencing.

Table 1. Sequences of primer used for plasmid construction.

Plasmid	Sequence (5' to 3')
Myc-GABPα	1st F: TGATCTCAGAGGAGGACCTGATGACTAAAAGAGAAGCAGA
	2nd F: GAGTAAGCTTACCATGGAGCAGAAGCTGATCTCAGAGGAGGACCT
	R: AATCCGCGGCCGCTCAATTATCCTTTTCCGTTTGC
V5-GABPβ1	1st F: TCCTCGGTCTCGATTCTACGATGTCCCTGGTAGATTTGGG
	2nd F: GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTA
	3rd F: AGTATA GCGGCCGCACCATGGGTAAGCCTATCCCTAAC
	R: GCTGGCCTCGAGTTAAACAGCTTCTTTATTAG

Transfection

HEK293T cells were seeded at a density of 1.5×10^6 cells in 60 mm culture dish and incubated overnight. Plasmids were transfected by using Polyethylenimine (PEI) (Polysciences, Inc., Warrington, PA, USA). Plasmids and PEI were mixed in Opti-MEM® I Reduced-Serum Medium (Thermo Fisher Scientific, Waltham, MA, USA), and the mixture was immediately vortexed and incubated for 10 min at room temperature. After replacing with the fresh medium, the mixture was added and incubated overnight at 37°C. Both Myc-tagged GABPα expression vectors and V5-tagged GABPβ1 expression vectors were co-transfected in HEK293T cells to investigate the intracellular localization of GABPα, because it was considered that co-expression of GABPβ would be also necessary for appropriate intracellular localization of GABPα.

Immunocytochemical analysis

After transfection of Myc-tagged GABPα and V5-tagged GABPβ1 for 24 hr, HEK293T cells were seeded at a density of 1.0×10^5 cells in poly-D-lysine-coated four-well chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) and incubated overnight at 37°C. The cells were exposed to 100, 250 or 500 nM concentrations of TPT for 6 hr or 20 μM menadione for 3 hr, and immunostained, according to the protocols as described previously (Ishida *et al*., 2013) with minor modifications. Mouse anti-Myc antibody (M192-3, Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) and rabbit anti-β-actin antibody were used in the primary antibody reaction, and Alexa Fluor® 488-conjugated goat anti-mouse IgG (A11001, Thermo Fisher Scientific) and Alexa Fluor® 555-conjugated goat anti-rabbit IgG (A21428, Thermo Fisher Scientific) were used in the secondary antibody reaction. DAPI (D1306, Thermo Fisher Scientific) solution was used as the counterstain.

ROS detection

HEK293T cells were seeded at a density of 3.5×10^5 cells in collagen-coated 35 mm glass base dish (AGC TECHNO GLASS CO., LTD., Shizuoka, Japan) and incubated overnight at 37°C. The cells were exposed to 50, 100 or 250 nM concentrations of TPT for 6 hr or 20 μM menadione for 3 hr. Following TPT treatment, the cells were washed with PBS (-), and incubated with 10 μM Dihydroethidium (DHE) (Thermo Fisher Scientific) in Live Cell Imaging Solution (Thermo Fisher Scientific) for 30 min. Then, DHE solution was replaced with Live Cell Imaging Solution, and fluorescence was evaluated with the FV-1000D Laser confocal microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

Data are expressed as means ± SD for at least three independent experiments. Statistical evaluation of the data was performed with ANOVA followed by Dunnett's test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of TPT exposure on cell viability in HEK293T cells

In order to investigate the cytotoxic effects of TPT, HEK293T cells were exposed to 50, 100, 250 or 500 nM concentrations of TPT for either 24 or 48 hr, and the cell viability was then measured by WST-1 assay. As shown Fig. 1a, after TPT exposure for 24 hr, cell viability was significantly decreased to 88.4%, 74.5%, and 54.7% at 100, 250, and 500 nM respectively, compared to control. When exposed for 48 hr, cell viability was significantly decreased to 68.9% and 30.7% at 250 and 500 nM respectively, compared to control (Fig. 1b).

Effect of TPT exposure on nuclear translocation of GABPα

To investigate the effect of TPT exposure on GABP, HEK293T cells were exposed to 100, 250 or 500 nM concentrations of TPT for 6 hr, after which the nuclear translocation of GABPα was evaluated. GABPα protein expression in nuclear fraction was significantly decreased to 62%, 51% and 65% respectively relative to

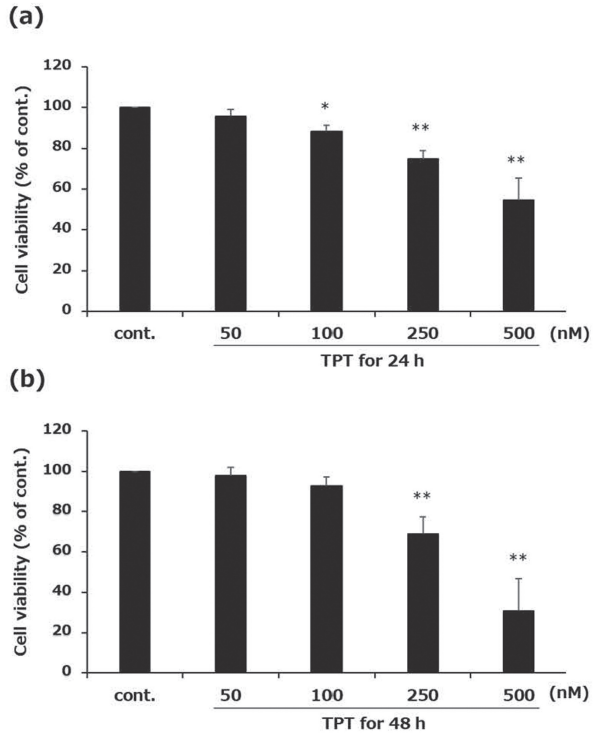

Fig. 1. Effect of TPT on the cell viability in HEK 293T cells. HEK 293T cells were exposed to DMSO (as a control), 50, 100, 250, and 500 nM TPT for 24 hr (a) and 48 hr (b), and then the cell viability was measured by WST-1 assay. Data are expressed as the means ± SD (n=3), *$p < 0.05$ vs. cont., **$p < 0.01$ vs. cont.

controls, whereas the protein expression in cytosolic fraction tended to increase relative to control post-exposure (Fig. 2a, 2b). To investigate the intracellular localization of GABPα, immunostaining experiment was performed on HEK293T cells overexpressing GABPα and GABPβ1. As shown in Fig. 3a, GABPα was co-localized with DAPI, a nuclear marker, in the control cells. In contrast, a part of GABPα was co-localized with β-actin, a cytosolic marker, in the TPT-treated cells (Fig. 3a), which supported the results of Fig. 2a and 2b. Moreover, TPT exposure did not affect GABPα protein expression in whole cells (Fig. 2c). These results suggest that TPT inhibits GABPα nuclear translocation without affecting the protein levels of GABPα.

Effects of ROS production on nuclear translocation of GABPα

To investigate whether ROS production inhibits GABP nuclear translocation, we evaluated GABPα protein expression in the nucleus of menadione (an ROS inducer)-treated HEK293T cells. After 20 μM menadione treatment, GABPα protein expression in the nuclear fraction was significantly decreased to 36.9% relative to control, whereas that in the cytosolic fraction tended to increase relative to control (Fig. 2d, 2e). In addition, a part of GABPα was co-localized with β-actin in the 20 μM menadione-treated cells (Fig. 3a). Next, we measured intracellular ROS levels using DHE staining. As shown in Fig. 3b, intracellular ROS levels were markedly increased after 20 μM menadione treatment. In addition, intracellular ROS levels were modestly increased after 50, 100, and 250 nM concentrations of TPT exposure (Fig. 3b). The results indicate that increased ROS in the cells may interfere with nuclear translocation of GABPα.

DISCUSSION

In this study, we clarified the toxic effects of TPT on GABPα and the underlying mechanism. Firstly, we measured cell viability after TPT exposure to HEK293T cells. Nanomolar levels of TPT exposure for 24 and 48 hr caused cell death in a concentration-dependent manner (Fig. 1). We determined TPT exposure time to 6 hr (shorter than 24 hr) to investigate their effects on nuclear translocation of GABP or ROS production.

We evaluated GABPα protein expression after TPT exposure, because GABPα, but not GABPβ, has DNA-binding domain, and the decrease in GABPα expression is directly linked to GABP dysfunction. Although TPT exposure did not affect GABPα protein expression in whole cells (Fig. 2c), nuclear translocation of GABPα protein was significantly decreased when exposed to 100-500 nM of TPT (Fig. 2a, 2b, and 3a), which would lead to GABP dysfunction. There are some studies that investigated the activation mechanisms of GABP. For example, GABPα has several redox-sensitive cysteine residues; Cys^{388} and Cys^{401} are important for the DNA binding activity of GABPα, and Cys^{421} in GABPβ-binding domain plays an important role in heterodimerization (Martin *et al*., 1996; Chinenov *et al*., 1997). The carrier protein, importin-α, transports GABP transcriptional complex from cytosol to nuclei through direct binding to GABPβ subunit, and so heterodimerization is necessary for GABP nuclear translocation. Prevention of heterodimerization has been confirmed to inhibit GABP nuclear translocation (Hayashi *et al*., 2013). Cys^{319} in GABPβ plays an important role for binding to importin-α, and this cysteine residue may also be redox-sensitive (Hayashi *et al*., 2013). Thus, nuclear translocation of GABP is dependent on both its heterodimerization and binding of carrier proteins.

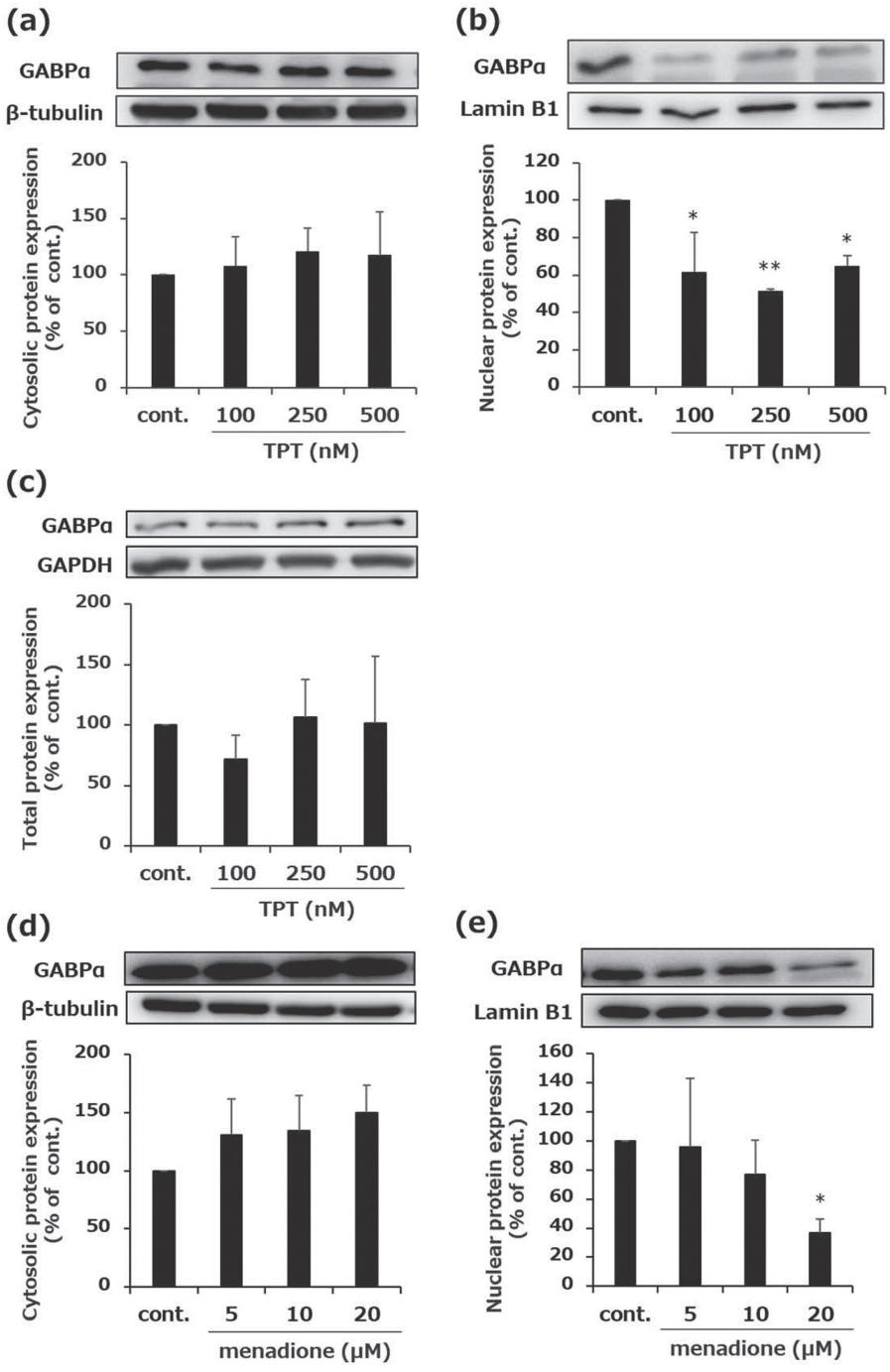

Fig. 2. Effect of TPT and menadione on nuclear translocation of GABPα. HEK293T cells were exposed to DMSO, 100, 250, and 500 nM TPT for 6 hr, and then the protein expression of GABPα in cytosol fraction (a), nuclear fraction (b), and whole-cells (c) was measured by western blotting. Date are expressed as the means ± SD (n=3), *$p < 0.05$ vs. cont., **$p < 0.01$ vs. cont. HEK293T cells were exposed to DMSO, 5, 10, and 20 μM menadione for 3 hr, and then the protein expression of GABPα in cytosol fraction (d) and nuclear fraction (e) was measured by western blotting. Date are expressed as the means ± SD (n=3), *$p < 0.05$ vs. cont.

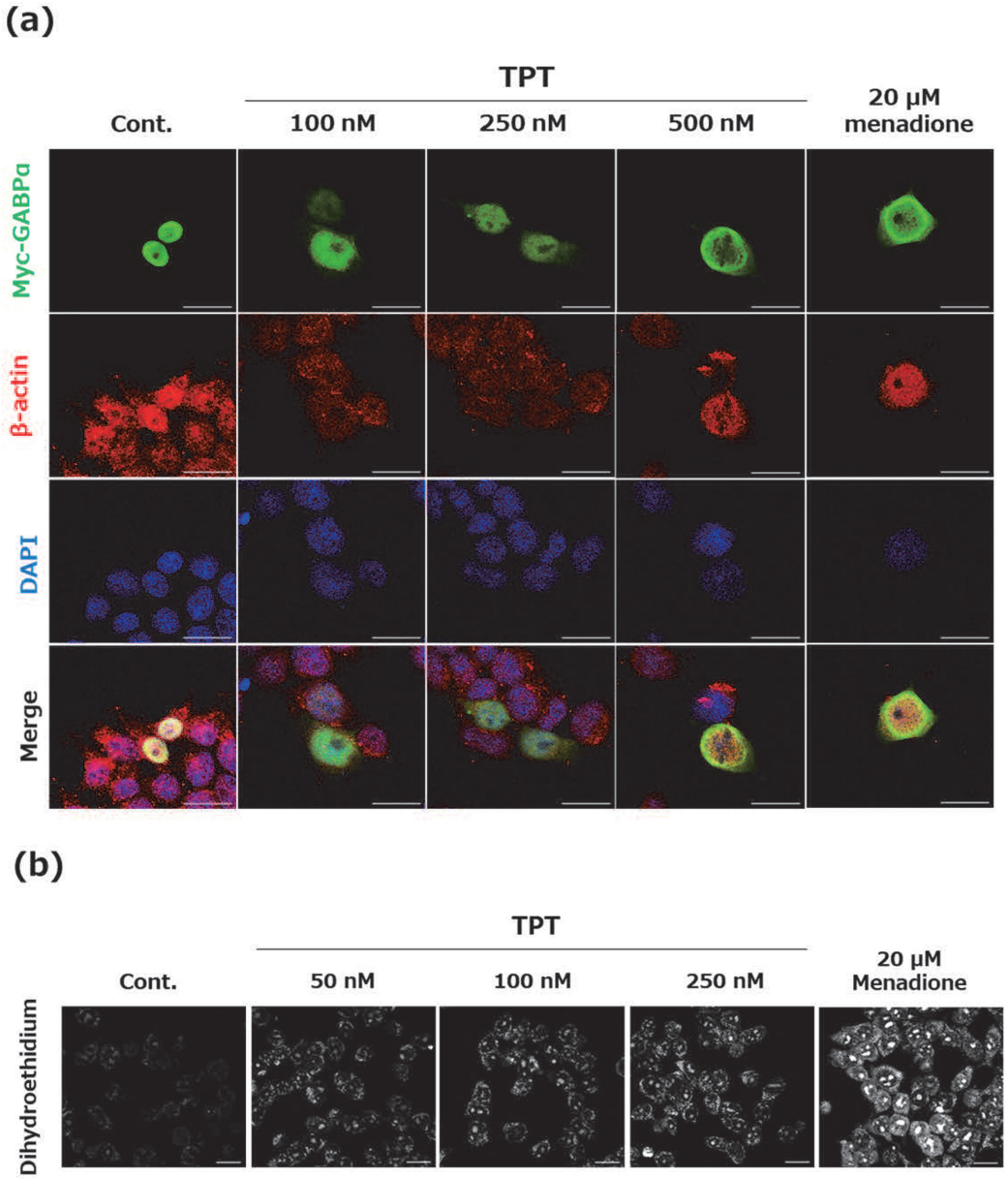

Fig. 3. Immunostaining of GABPα and DHE staining in HEK293T cells treated with TPT and menadione. (a) HEK293T cells were transfected with Myc-tagged GABPα expression vectors and V5-tagged GABPβ1 expression vectors, and then 100, 250, and 500 nM TPT was exposed for 6 hr, or 20 μM menadione was exposed for 3 hr. Cells were immunostained with a mouse anti-Myc antibody (green) and a rabbit anti-β-actin antibody (red) as a cytosol marker. 4',6-Diamidino-2-phenylindole (DAPI) is used for nuclear staining (blue). Scale bar = 20 μm. (b) HEK293T cells were exposed to DMSO, 50, 100, and 250 nM TPT for 6 hr, or 20 μM menadione for 3 hr, and then intracellular ROS levels was detected by DHE staining. After DHE probe treatment for 30 min, cells were observed by live cell imaging with the FV-1000D Laser confocal microscope.

It is reported that ROS inhibits nuclear translocation of GABP and subsequently disrupts GABP function (Martin *et al*., 1996; Chinenov *et al.*, 1997). In fact, we also confirmed that both inhibition of GABPα nuclear translocation (Figs. 2d, 2e, and 3a) and ROS production (Fig. 3b) was caused by treatment of 20 μM menadione, an ROS inducer, in HEK293T cells. In addition, intracellular ROS levels increased when exposed to TPT (Fig. 3b), although

the induction level was not as much as that caused by menadione. These results raise the possibility that the increase in intracellular ROS levels caused by menadione or TPT inhibits GABP heterodimerization and/or its binding to carrier proteins, which causes inhibition of nuclear translocation of GABP.

GABP is also known as nuclear respiratory factor (NRF) -2. We have previously reported that NRF-1, a member of the NRF family, is a target for tributyltin (TBT) toxicity. TBT reduces NRF-1 expression and subsequently decreases α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid-type glutamate receptor subunit 2 (GluA2) expression at the transcriptional level, leading to neurotoxicity (Ishida *et al.*, 2017; Hanaoka *et al*., 2018). Therefore, NRFs may be novel targets in organotin toxicities.

Luo *et al*. have reported that GABP plays an important role in regulation of T cell homeostasis and immunity (Luo *et al*., 2017), and immunotoxicity has been reported as one of the main effects of TPT. (Dacasto *et al*., 2001). Thus, GABP dysfunction may be involved in TPT-induced immunotoxicity. Many compounds, including TPT, can produce ROS. Although GABP is considered to be usually affected by these compounds, very few reports actually describe the relationship between the two. In addition, the physiological functions of GABP are not fully understood, and further studies are needed to clarify not only the involvement of GABP in chemical toxicity and but also their physiological functions.

In conclusion, we clarified that TPT exerts toxic effects on GABPα through inhibition of their nuclear translocation, which may be triggered by ROS production. Although there are many studies about TPT toxicities focusing on its RXR or PPARγ agonistic actions, our data provides insight into a novel toxic mechanism of TPT.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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