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Critical role of hydrogen peroxide in the differential susceptibility of Th1 and Th2 cells to tributyltin-induced apoptosis

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ABSTRACT

Tributyltin (TBT), an environmental pollutant, debilitates immune responses via induction of apoptosis in $CD4^+$ T cells through an undefined mechanism of action. Accumulating evidence indicates that the susceptibility of Th1 and Th2 cells to TBT-induced apoptosis differs. In this study, by using HL-60 cell model, we show that hydrogen peroxide (H_2O_2) plays a critical role in TBT-induced apoptosis. Generation of H_2O_2 induced by TBT resulted in a change in mitochondrial membrane potential that proceed apoptotic pathway where, at least in part, involved activation of caspase-3. We also demonstrated that Th1 clones appear to be more vulnerable to apoptosis induction than Th2 clones following exposure to TBT, which was well correlated with increased H_2O_2 generation in Th1 clones than Th2 clones. There was an inverse correlation between TBT-induced apoptosis and the basal levels of intracellular GSH, a major cellular antioxidant. Furthermore, the addition of NAC that replenish intracellular GSH levels inhibited generation of H_2O_2 and apoptosis in Th1 clones. These results suggest that TBT selectively induces apoptosis via generation of H_2O_2 in Th1 cells because of their low GSH levels, which may contribute to the Th2 predominance induced by TBT.

1. Introduction

Tributyltin (TBT) has been used worldwide since the mid-1960s in antifouling paints for ships and fishing nets; its release into the marine environment has resulted in world-wide pollution [1]. Although the production and use of TBT have now become regulated, environmental contamination still persists. Human exposure to TBT occurs via consumption of TBT-containing meat and fish products [2,3]. Several reports have indicated possible neurological, developmental, and immunologic toxic effects on mammals [4–8].

Adaptive immune responses are initiated by the activation of antigen-specific naive $CD4^+$ T cells, which recognize specific peptides on antigen presenting cells (APC) and subsequently differentiate into T helper (Th) cells. Th cells are classified into at least two subsets, Th1 and Th2 subsets. Th1 cells secrete IFN-γ, IL-2, and TNF-β to stimulate cell-mediated immunity, while Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 to stimulate

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humoral immunity and are prominent mediators of allergic responses [9]. Th1 cells have been shown to be more susceptible than Th2 cells to apoptotic cell death [10,11]. The documented immunotoxicities of TBT include induction of T-cell apoptosis via generation of reactive oxygen species (ROS) [12], leading to the suppression of the T-cell arm of immune responses [8,13]. In addition to the induction of immune suppression by TBT, our recent studies indicate that TBT, especially at low doses, promotes Th2 differentiation and suppresses Th1 differentiation from naive $CD4^+$ T cells, resulting in the Th2-predominant state associated with the exacerbation of Th2-driven allergic airway inflammation [14,15]. This effect of TBT on Th2 polarization was mediated by depletion of the antioxidant glutathione (GSH) within APC, which modulated cytokine production responsible for Th1/Th2 differentiation [15]. These results suggest that the effect of TBT on the dysregulation of immune responses is mediated by oxidative stress, secondary to increases in oxidants and/or decreases in antioxidants.

Although oxidative stress is critical in the induction of apoptosis by TBT, a precise determination of the molecular mechanism by which ROS production is upregulated remains unidentified. In addition, the contribution of preferential induction of apoptosis in Th1 cells in the generation of Th2 predominance by TBT remains unclear. Using HL-60 and HP100 cells, a subline of HL-60 expressing extremely high catalase activity [16], here we show that hydrogen peroxide (H_2O_2) plays a critical role in TBT-induced apoptosis. Generation of H_2O_2 induced by TBT resulted in a change in mitochondrial membrane potential ($\Delta\Psi_m$) that proceed apoptotic pathway where, at least in part, involved activation of caspase-3. Furthermore, using several independent Th1 and Th2 clones, we also showed that TBT preferentially induced apoptosis in Th1 cells rather than Th2 cells. The increased susceptibility of Th1 cells to TBT-induced apoptosis resulted from lower GSH levels, which may detoxify the H_2O_2 produced by TBT treatment. Our results suggest that unequal induction of apoptosis in Th1 over Th2 cells contributes to the Th2 predominance induced by TBT.

2. Materials and methods

2.1. Reagents

Tri-*n*-butyltin chloride was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Dihydrorhodamine 123 (DHR), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) were obtained from Molecular Probes, Inc. (Eugene, OR). 3-Aminotriazol and *N*-acetylcysteine (NAC) were acquired from Nacalai Tesque, Inc. (Kyoto, Japan). *N*-Carbobenzoxy-Asp-Glu-Val-Asp fluoromethyl ketone (z-DEVD-fmk) was purchased from MBL Co. Ltd. (Nagoya, Japan).

2.2. Cells

HL-60 cells and HP100 cells were grown in RPMI 1640 medium supplemented with 6% fetal calf serum at 37 °C under 5% CO_2 in a humidified atmosphere. HP100 cells, which are derived from HL-60 cells, are 340-fold more resistant to H_2O_2 than parental HL-60 cells, due to an 18-fold higher catalase activity [16]. In the indicated experiments, HL-60 cells were preincubated with 175 U/ml of catalase (Sigma, St. Louis, MO) with occasional swirling of the culture flask for 1 h to introduce calatase into the intracellular environment as described by Ikeda et al. [17].

Ovalbumin (OVA)-specific Th1 and Th2 clones were established from the lymph node cells of OVA-immunized BDF1 mice, as described [18]. Briefly, splenic $CD4^+$ T cells (1×10^5 cells) isolated from BDF1 mice immunized 11 days previously with 100 μg OVA in complete Freund's adjuvant (CFA) were cultured with 200 μg/ml of OVA and irradiated (35 Gy) syngeneic spleen cells either in the presence of 0.1 μg/ml IFN-γ (PeproTech EC) plus 5 μg/ml anti-IL-4 (11B11, BD PharMingen) (Th1 condition) or 0.1 μg/ml IL-4 (PeproTech EC) plus 5 μg/ml anti-IFN-γ (R4-6A2, BD PharMingen) (Th2 condition) followed by limiting dilution under Th1 or Th2 condition, respectively. The functional phenotypes of established respective Th clones were confirmed by IFN-γ or IL-4 detected in culture supernatant and by intracellular IFN-γ/IL-4 staining of Th clones stimulated with OVA and splenic APC. Th clones were maintained in 2.5 ng/ml IL-2 (recombinant human IL-2; Ajinomoto, Yokohama, Japan) with biweekly stimulation with OVA and splenic APC. Cells were used for experimentation at least 10 days after the last stimulation.

2.3. TBT treatment

TBT, prepared in DMSO, was diluted in culture medium to produce working solutions of 0.1 and 0.001% for HL-60 cells and Th clones, respectively. Cells were seeded at a density of 1×10^6 cells/ml in 24-well plates, and then treated with TBT at the indicated concentrations for 5 min to 6 h. When indicated, 25 μM z-DEVD-fmk, 50 or 100 μM NAC, or 100 mM 3-aminotriazol were included in these cultures.

2.4. Detection of DNA ladder formation

Apoptosis was evaluated by examining the pattern of DNA laddering characteristically generated in apoptotic cells on gel electrophoresis as described previously [19]. Briefly, cells were disrupted in ice-cold cytoplasm extraction buffer [10 mM Tris (pH 7.6), 0.15 M NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100]. After centrifugation at 1000 × *g* for 5 min at 4 °C, the pelleted intact nuclei were resuspended in lysis buffer [10 mM Tris (pH 7.6), 0.4 M NaCl, 1 mM EDTA, and 1% Triton X-100]. Samples were then left on ice for 10 min and centrifuged at 12,000 × *g* at 4 °C to separate nucleoplasm from high-molecular-weight chromatin. After incubation with 20 μg/ml ribonuclease A for 1 h at room temperature, samples were treated with 100 μg/ml proteinase K for 2 h at 37 °C. DNA was extracted with phenol/chloroform and precipitated with ethanol. DNA fragments were electrophoresed on a 1.4% agarose gel containing 0.375 μg/ml ethidium bromide.

2.5. Annexin V staining

Plasma membrane asymmetry, which is characteristic of apoptosis, was assessed by Annexin V staining [20]. Briefly, Th

clones treated with TBT as above, then stained with Annexin V-FITC using a TACS™ Annexin V Kit (Trevigen, Inc., Gaithersburg, MD) according to the manufacturer's instruction. Cells were stained with propidium iodide to exclude dead cells and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA).

2.6. Assay for intracellular ROS generation

To evaluate intracellular ROS generation, we used a non-fluorescent cell-diffusible DHR to detect superoxide (O_2^-) and H_2O_2 [21,22] and DCFH-DA to detect peroxides and H_2O_2 [23], both of which can be trapped intracellularly and converted to fluorescent rhodamine 123 (R123) and 2′,7′-dichlorofluorescein (DCF), respectively, in the presence of ROS. HL-60 cells were loaded with 5 μM DHR or 5 μM DCFH-DA for 15 min at 37 °C; loaded cells were incubated with 0.4 μM TBT at 37 °C for the indicated times and immediately analyzed by flow cytometry as described [24,25]. Ten thousand cells per sample were acquired in histograms using CELLQuest software. Dead cells and debris were excluded from analysis by electronic gating using forward and side scatter measurements.

2.7. Assay for mitochondrial membrane potential ($\Delta\Psi_m$)

To assess changes in $\Delta\Psi_m$, TBT-treated cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37 °C and immediately analyzed by flow cytometry as described [26]. $DiOC_6(3)$ incorporates into mitochondria with a nonlinear dependence on $\Delta\Psi_m$, emitting exclusively within the spectrum of green light [27]. Ten thousand cells per sample were acquired in histograms using CELLQuest software. Dead cells and debris were excluded from analysis by electronic gating using forward and side scatter measurements.

2.8. Assay for intracellular GSH level

Intracellular GSH levels were evaluated using high performance liquid chromatography (HPLC) with a gold electrode as described [28]. Briefly, cells were suspended in ice-cold PBS containing 0.1% Triton X-100, then homogenized using a microhomogenizer with a Teflon-coated pestle. Cell lysates were centrifuged at 18,500 × *g* for 5 min at 4 °C. An aliquot of the supernatant was assessed for protein concentration using a BCA protein assay kit (Pierce, Rockford, IL). After the addition of 5% trichloroacetic acid (TCA) (Wako Pure Chemical Industries, Osaka, Japan) to the supernatant, samples were centrifuged at 18,500 × *g* for 10 min at 4 °C. Supernatants were diluted in 0.1N HCl. The total GSH concentration was determined using an electrochemical detector coupled to an HPLC (HPLC-ECD) (Eicom, Kyoto, Japan) using authentic GSH (Kohjin Co. Ltd., Tokyo, Japan) as a standard. Data were presented as the means of all samples in nmole GSH/mg protein.

2.9. Statistics

Statistic analyses were performed with one-way analysis of variance (ANOVA) followed by Scheffe's test and Student's *t*-test with Welch correction. *p*-Values less than 0.05 were considered to be statistically significant. In some experiments, the results were presented as a representative gel, when at least three independent experiments showed similar trend.

3. Results

3.1. TBT induces H_2O_2 generation and subsequent DNA ladder formation

In the first series of experiments, using HL-60 cell model we sought to identify the molecular species of ROS that involved in the induction of apoptosis by TBT. We treated HL-60 cells with 0.2–0.5 μM of TBT that had been shown to induce apoptosis in human PBMC or Jurkat cells [29,30]. TBT at 0.3 μM or more induced DNA ladder formation in a concentration dependent manner (Fig. 1A). When intracellular catalase that specifically decomposes H_2O_2 was increased in HL-60 cells by preincubation with catalase [17], the DNA ladder formation induced by 0.4 μM TBT was significantly suppressed (Fig. 1B). We then compared the susceptibilities to TBT-induced apoptosis of HL-60 cells and HP100 cells, a subline of HL-60 expressing 18-fold higher catalase activity than HL-60 cells [16]. Following treatment of both lines with 0.4 μM TBT, we observed a significant decrease in the induction of DNA ladder formation in HP100, which was significantly accelerated by the addition of 3-aminotriazol, an inhibitor of catalase (Fig. 1C). Furthermore, this DNA ladder formation was also inhibited by the addition of NAC at the concentration that inhibited ROS-mediated apoptosis [31,32] (Fig. 1D).

We then examined the effect of TBT treatment at concentrations that induced apoptosis on the generation of H_2O_2 in HL-60 cells. After loading with either DHR to detect O_2^- and H_2O_2 [21,22] or DCFH-DA to detect peroxides and H_2O_2 [23], HL-60 cells were treated with 0.4 μM TBT for indicated times. ROS generation was assessed by measurement of the increases in the fluorescence of R123, an oxidized product of DHR that was detectable at 5 min and 15 min after TBT treatment with approaching significance ($p = 0.061$) and significance ($p = 0.021$), respectively (Fig. 2A, gray bar). On the other hand, fluorescence of DCF, an oxidized product of DCFH-DA was detectable at 15 min after TBT treatment with significance (Fig. 2A, black bar). Preincubation of HL-60 cells with catalase reduced the levels of ROS detected by DCF (Fig. 2B), indicating that the ROS generated by TBT-treatment were predominantly H_2O_2. Addition of NAC also inhibited the increases in DCF fluorescence intensity seen over time (Fig. 2B). Taken together, these results indicate that TBT induces H_2O_2 generation that involves critically in the TBT-induced apoptosis.

3.2. Change in $\Delta\Psi_m$ follows after H_2O_2 generation in cells treated with TBT undergoing apoptosis

Loss of $\Delta\Psi_m$ is associated with the release of cytochrome *c* from mitochondria [30,33]. We investigated if TBT-induced H_2O_2 production participated in the change in $\Delta\Psi_m$. HL-60 and HP100 cells were treated with 0.4 μM TBT for the indicated times. Cells were then loaded with $DiOC_6(3)$ to facilitate the measurement of $\Delta\Psi_m$ by changes in fluorescence. HL-60 cells

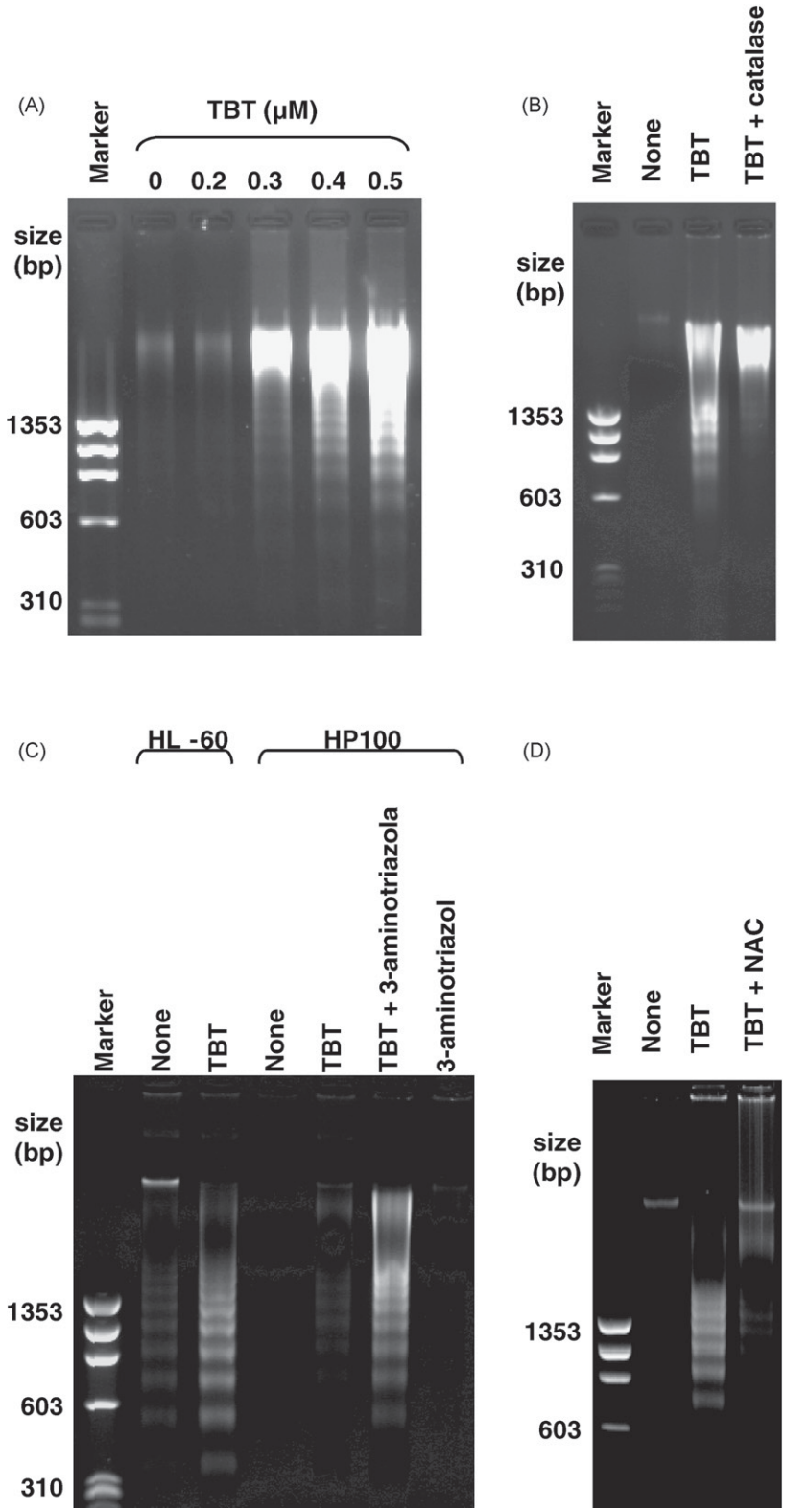

Fig. 1 – DNA ladder formation in HL-60 cells treated with TBT. (A) HL-60 cells (1×10^6 cells/ml) were treated with increasing concentrations of TBT for 6 h at 37 °C. (B) Catalase-introduced HL-60 cells were treated with 0.4 μM TBT for 4 h at 37 °C. (C) HL-60 cells and HP100 cells (1×10^6 cells/ml) were treated with 0.4 μM TBT in the presence or absence of 100 mM 3-aminotriazol for 4 h at 37 °C. (D) HL-60 cells were treated with 0.4 μM TBT in the presence or absence of 50 μM NAC for 4 h at 37 °C. The cells treated with vehicle alone (0.1% DMSO) served as a control. DNA was extracted from cells and analyzed by agarose gel electrophoresis. Data shown are representative of three independent experiments with similar results. Marker: Size marker DNA (ΦX 174/Hae III digest).

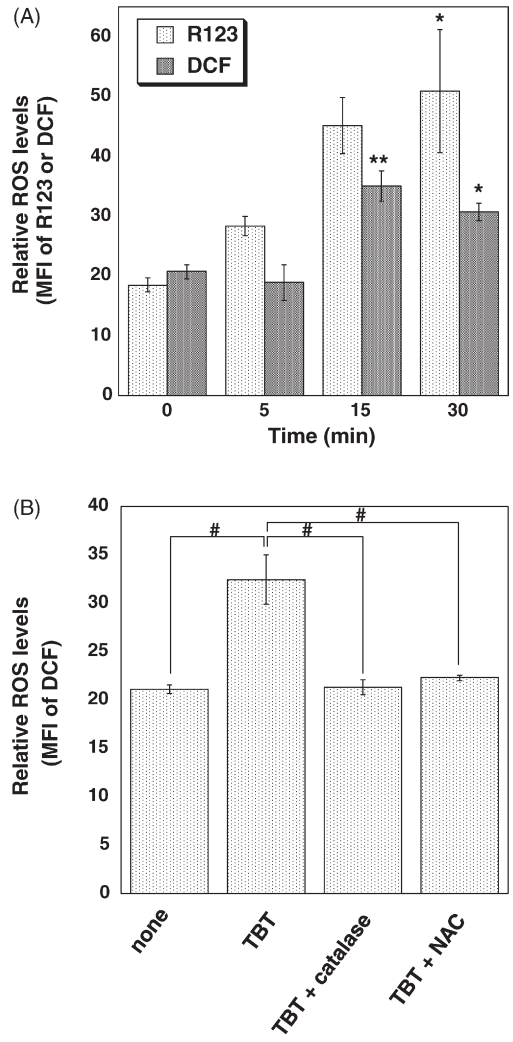

Fig. 2 – Generation of O_2^- and H_2O_2 in HL-60 cells treated with TBT. (A) HL-60 (1×10^6 cells/ml) loaded with DHR or DCFH-DA were incubated in the presence or absence of 0.4 μM TBT at 37 °C for the indicated times. Cells were analyzed by flow cytometry (FACScan). The numbers on the histogram vertical axis show the mean fluorescence intensities (MFI) of cells. Results are expressed as the means ± S.E. of triplicate samples. $^{**}p < 0.01$; $^{*}p < 0.05$; significant differences from controls were determined using one-way ANOVA followed by Scheffe's test. (B) HL-60 cells and catalase-introduced HL-60 cells were treated with 0.4 μM TBT in the presence or absence of 50 μM NAC for 15 min at 37 °C. Results are expressed as the means ± S.E. of triplicate samples. $^{\#}p < 0.01$; significant differences from TBT treatment were determined using a Student's t-test with Welch correction.

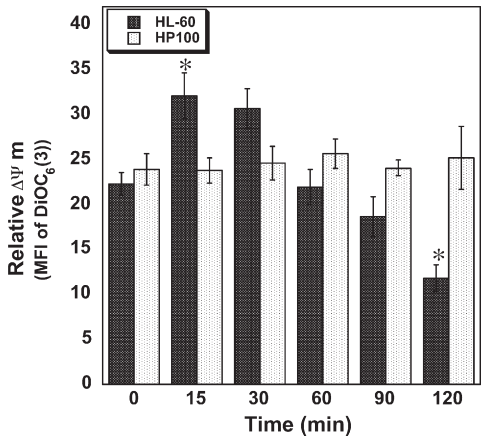

Fig. 3 – Changes in $\Delta\Psi_m$ of HL-60 and HP100 cells treated with TBT. HL-60 and HP100 cells (1×10^6 cells/ml) were treated with 0.4 μM TBT at 37 °C for the indicated times. After treatment, cells were incubated for 15 min with 40 nM $DiOC_6(3)$ at 37 °C. Cells were analyzed by flow cytometry (FACScan). The numbers on the histogram vertical axis show the mean fluorescence intensities (MFI) of cells. Results are expressed as the means ± S.E. of triplicate samples. $^{*}p < 0.05$; significant differences from controls were determined using one-way ANOVA followed by Scheffe's test.

treated with TBT exhibited a transient increase in $\Delta\Psi_m$ at 15 min after treatment followed by a significant decrease at 120 min, as indicated by parallel changes in $DiOC_6(3)$ fluorescence (Fig. 3). In contrast, $\Delta\Psi_m$ did not change in HP100 cells treated with TBT. As a transient increase in $\Delta\Psi_m$ followed by loss of $\Delta\Psi_m$ is known to be a key event in the apoptotic pathway, preceding cytochrome *c* release and the activation of caspase-3 [34–36], we examined the involvement of caspase-3 activation in TBT-induced apoptosis. HL-60 cells were treated with a cell permeable caspase-3 specific inhibitor z-DEVD-fmk that had been shown to inhibit caspase-3 activity almost completely at 30 μM in HL-60 cells [37]. Treatment of HL-60 cells with 25 μM (Fig. 4) or more (data not shown) of z-DEVD-fmk significantly inhibited the TBT-induced DNA ladder formation in HL-60 cells, though the larger DNA fragments (>1351 bp) were not inhibited by z-DEVD-fmk. In conjunction with the results demonstrating that TBT induced H_2O_2 generation, these results suggest that the H_2O_2 induces a loss of $\Delta\Psi_m$, leading to the release of cytochrome *c* from mitochondria and subsequent activation of caspase-3, which, at least in part, might contribute TBT-induced apoptosis.

3.3. *Differential sensitivity of Th1 and Th2 clones to TBT-induced apoptosis*

Using HL-60 cell model, we showed that H_2O_2 is critically involved in the TBT-induced apoptosis. It has been reported that Th1 and Th2 cells display differential abilities to undergo activation-induced cell death (AICD) in process of which ROS plays a critical role [10,11,38]. Therefore, in the next series of experiments, we examined whether Th1 and Th2 cells differ in their susceptibility to TBT-induced apoptosis that possibly contribute to the Th2 polarization [14,15]. To this end, we established OVA-specific Th1 and Th2 clones from mice immunized with OVA by restimulating these cells *in vitro* with OVA in the presence of anti-IL-4 plus IFN-γ or anti-IFN-γ plus IL-4, respectively. Thirteen independent Th1 clones used in the present experiment produced 1579.4 ± 213.8 ng/ml IFN-γ

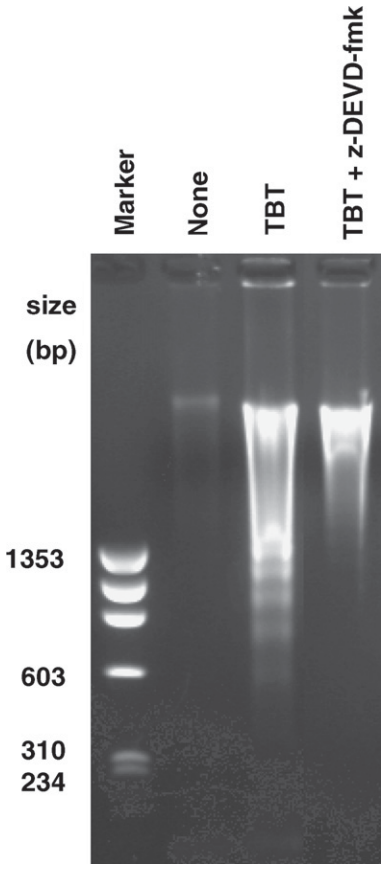

Fig. 4 – Effect of a caspase-3 inhibitor on TBT-induced apoptosis. HL-60 cells (1×10^6 cells/ml) were treated with 0.4 μM TBT in the presence or absence of 25 μM z-DEVD-fmk for 4 h at 37 °C. HL-60 cells treated with vehicle alone (0.1% DMSO) served as a control. DNA was extracted from the cells, and DNA ladder formation was analyzed by agarose gel electrophoresis. Data shown are representative of three independent experiments with similar results. Marker: Size marker DNA (ΦX 174/Hae III digest).

and no detectable IL-4, and contained 72.9 ± 9.1% IFN-γ^+ cells and no detectable IL-4^+ cells upon stimulation with OVA in the presence of splenic APC. Fourteen independent Th2 clones produced 2.8 ± 0.9 ng/ml of IL-4 and no detectable IFN-γ, and contained 50.4 ± 9.8% IL-4^+ cells and no detectable IFN-γ^+ cells. These Th1 and Th2 clones were exposed to varying concentrations of TBT for 4 h. Cells were then subjected to FACS analysis after staining with Annexin V, which identifies membrane phosphatidylserine externalization in apoptotic cells. Although equal levels of basal apoptosis were observed in Th1 and Th2 clones, the percentage of Annexin V-positive cells was significantly higher in Th1 clones following inclusion of 1.0 μM or greater concentrations of TBT into cultures (Fig. 5A). When assayed for apoptosis by DNA ladder formation, TBT even at 0.3 μM selectively induced apoptosis in Th1 clones but not in Th2 clones (Fig. 5B). We then examined whether Th1 and Th2 clones generate H_2O_2 upon TBT exposure. As shown in Fig. 5C, Th1 clones treated with 1 μM TBT generated significantly higher levels of ROS as indicated by the increase in fluorescence intensity of DCF, which was cancelled by the introduction of catalase into cells or by the presence of NAC. In contrast, TBT exposure did not induce ROS generation in Th2 clones (Fig. 5C). Finally, the cancellation of H_2O_2 generation by NAC was associated with suppression of apoptosis in Th1 clones induced by TBT (Fig. 5D). These results indicate that Th1 clones generate higher levels of H_2O_2 than Th2 clones upon TBT exposure, which contributes to the higher susceptibility of Th1 clones to TBT-induced apoptosis.

3.4. *Low intracellular GSH levels in Th1 clones confer higher susceptibility to TBT-induced apoptosis*

The addition of NAC, at the concentration that replenishes intracellular GSH [39] provided protection against the apoptosis induced in Th1 clones by TBT (Fig. 5C). As this result suggested that the intracellular levels of GSH differed between Th1 and Th2 clones, we examined the basal levels of GSH in Th1 and Th2 clones. The basal levels of GSH in Th1 clones were significantly lower than those in Th2 clones (Fig. 6, $p < 0.001$ by Student's *t*-test with Welch correction), indicating that the higher susceptibility of Th1 clones to TBT-induced apoptosis may be due to low levels of GSH, which can detoxify H_2O_2 generated by TBT treatment.

4. Discussion

In this study, using HL-60 cell model we demonstrated that H_2O_2 plays a critical role in TBT-induced apoptosis. We also indicated that TBT preferentially induces apoptosis in Th1 cells rather than Th2 cells, which may exacerbate Th2-driven allergic diseases. The higher susceptibility of Th1 cells to TBT-induced apoptosis is due to lower intracellular GSH levels in Th1 cells in comparison to Th2 cells.

The concentrations of TBT that induced HL-60 cells and Th1 cells (>0.3 μM) seemed to be environmentally relevant, since it has been reported that 0.12 ± 0.09 μM of TBT along with its metabolite dibutyltin and monobutyltin can be detected in human blood [40]. It is likely reasonably that the relatively higher concentration of TBT is needed to detect various biochemical changes in cells and apoptosis during short period of time than that detected in serum.

The inhibitory effects of NAC and catalase indicate that H_2O_2 generated by TBT treatment plays a critical role in TBT-induced apoptosis. In the presence of glutathione peroxidase, NAC can replenish intracellular GSH that can detoxificate H_2O_2 [39,41,42]. Therefore, the addition of NAC or preincubation with catalase specifically detoxified H_2O_2 generated by TBT treatment, preventing the induction of apoptosis. In addition, HP100, a subline of HL-60 that possesses 18-fold higher catalase activity, displayed resistance to TBT-induced apoptosis. Suppression of catalase activity in these cells by the addition of 3-aminotriazole restored their susceptibility to TBT-induced apoptosis. These results collectively indicate that H_2O_2 plays a central role in TBT-induced apoptosis.

The preferential generation of H_2O_2 following TBT treatment is substantiated by the observation that TBT binds a component of the mitochondrial F_0F_1-ATP synthase complex,

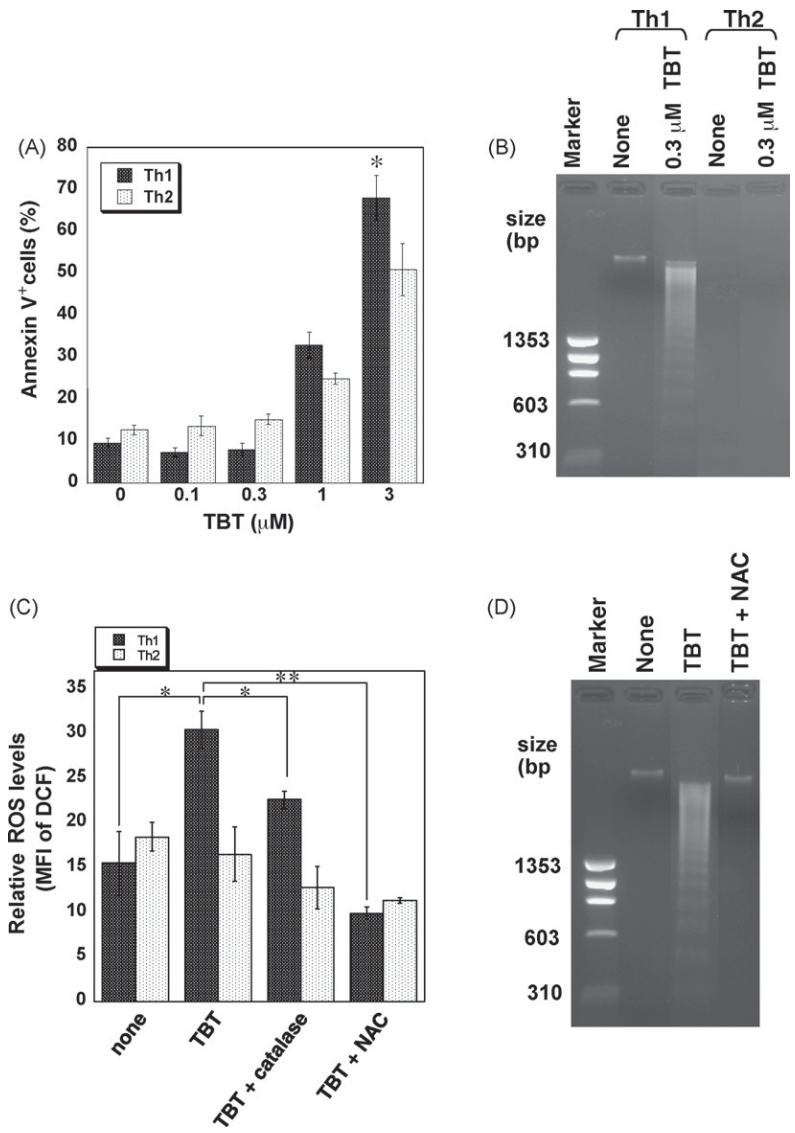

Fig. 5 – TBT-induced apoptosis in Th1 and Th2 clones and the generation of H_2O_2 in Th1 and Th2 clones treated with TBT. (A) Th1 and Th2 clones were treated with indicated concentrations of TBT for 4 h at 37 °C. Results, expressed as the mean percentage of apoptotic cells ± S.E., were obtained from five Th1 and eight Th2 clones. One representative experiment of five independent iterations is shown. $^{*}p < 0.05$; significant differences between Th1 and Th2 clones were determined using a Student's t-test with Welch correction. (B) Th1 and Th2 clones were treated with 0.3 μM TBT for 18 h at 37 °C. DNA was extracted from these cells, and DNA ladder formation was analyzed by agarose gel electrophoresis. (C) Th1 and Th2 clones loaded with DCFH-DA were incubated in the presence or absence of 1.0 μM TBT at 37 °C for 5 min. The numbers on the histogram vertical axis show the mean fluorescence intensities (MFI) of cells. Results are expressed as the means ± S.E. of triplicate samples. $^{**}p < 0.01$; $^{*}p<0.05$; significant differences from TBT treatment were determined using a Student's t-test with Welch correction. (D) Th1 clones were treated with 0.5 μM TBT in the presence or absence of 100 μM NAC for 18 h at 37 °C. DNA ladder formation was analyzed as (B). Data shown are representative of three independent experiments with similar results. Marker: Size marker DNA (ΦX 174/Hae III digest).

leading to the depletion of intracellular ATP stores [43] via an oligomycin-like effect [44]. These decreases in intracellular ATP result in a reduced state of the mitochondrial respiratory chain and subsequent mitochondrial production of O_2^-, which undergoes spontaneous or superoxide dismutase (SOD)-catalyzed dismutation to H_2O_2 [45,46].

It has been reported that mitochondrial-dependent intrinsic apoptosis involves two redundant parallel pathways that lead to DNA fragmentation and chromatin condensation. One of these pathways involves activation caspase-3 and subsequent activation of caspase-activated DNase (CAD) by activated caspase-3 that ultimately leads to oligonucleosomal DNA fragmentation [47]. It is well documented that ROS induced change in mitochondrial membrane potential resulted in the release of cytochrome c from mitochondria, which induces activation of caspase-3 [12,48]. The second pathway involves

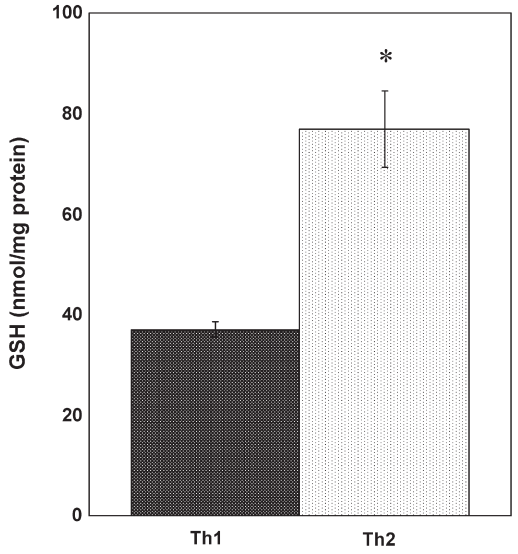

Fig. 6 – Basal intracellular GSH content in Th1 and Th2 clones. The GSH content of cells was assessed by high performance liquid chromatography with a gold electrode as described in Section 2. Data represent the means ± S.E. of five Th1 and eight Th2 clones. $^{*}p < 0.001$; significant differences between Th1 and Th2 cells were assessed by Student's *t*-test with Welch corrections.

apoptosis-inducing factor (AIF) released from mitochondria to nucleus and leads to large-scale DNA fragmentation and peripheral chromatin condensation in a caspase-independent manner [49]. Recently, it also has been shown that ROS induces the release of AIF from mitochondria via activation of poly-(ADP-ribose) polymerase-1 (PARP-1) [50]. Therefore, ROS plays critical role in the initiation of both caspase-dependent and -independent apoptotic pathways. Our results that caspase-3 inhibitor, z-DEVD-fmk, significantly suppressed TBT-induced DNA fragmentation with molecular weight of less than 1.3 kbp suggest that activation of caspase-3 is, at least in part, involved in the TBT-induced apoptosis. However, z-DEVD-fmk did not suppress generation of high-molecular-weight DNA fragment induced by TBT. Therefore, it is possible that AIF is also involved in the TBT-induced apoptosis.

Our results that H_2O_2 plays a critical role in TBT-induced apoptosis of HL-60 cells together with the reported ability of H_2O_2 to augment Th2 immune responses [51] and difference between Th1 and Th2 cells in the susceptibility to AICD [38] led us to examine if Th1 cells were more susceptible to TBT-induced apoptosis than Th2 cells. The results obtained in this study clearly indicate that Th1 clones are more susceptible to TBT-induced apoptosis than Th2 clones, which is in line with the observation that bronchoalveolar lymphocytes from asthmatic patients containing large number of Th2 cells [52] displayed resistance to TBT-induced apoptosis [53]. The higher susceptibility of Th1 clones to TBT-induced apoptosis was well correlated with higher H_2O_2 levels generated in Th1 than Th2 clones. Furthermore, we discovered that the addition of NAC, which can replenish intracellular GSH [39,41], inhibits TBT-induced apoptosis via cancellation of H_2O_2 generation. In addition, the GSH levels of Th1 clones were significantly lower than those of Th2 clones. As H_2O_2 functioned in TBT-induced apoptosis and GSH can catalyze the reductive detoxification of ROS [31,32], it is likely that the higher susceptibility of Th1 clones to TBT-induced apoptosis was due to lower levels of intracellular GSH.

In previous studies, we demonstrated that TBT induces Th2 predominance via promotion of Th2 development and the suppression of Th1 development from naive $CD4^+$ T cells, which exacerbates airway inflammation [14,15]. These results suggest that the higher susceptibility of Th1 cells and the increased resistance of Th2 cells to TBT-induced apoptosis contributes to the Th2 predominance induced by TBT. Relevantly, the Th2 predominance seen in atopic patients was shown to be due to the preferential apoptosis of circulating Th1 cells [54]. Previous results also indicate that the effect of TBT on Th1/Th2 development was mediated by depletion of the antioxidant GSH in APC, leading to suppression of IL-12 production, which is responsible for Th1 development, and augmentation of IL-10 production, which is responsible for Th2 development [15]. Thus, oxidative stress occurring secondary to the depletion of intracellular GSH and/or generation of additional H_2O_2 may be the common mechanism underlying TBT-induced Th2 predominance.

Allergic diseases are caused by exaggerated Th2-type immune responses toward common, typically innocuous environmental antigens. Unregulated Th2 development and the preferential apoptosis of Th1 cells have been suggested to contribute to such Th2-predominant immune responses toward allergens [54,55]. Accumulating evidence indicates that oxidative stress, resulting from an oxidant/antioxidant imbalance secondary to either production of excess oxidants or depletion of antioxidants, such as GSH, plays a critical role in the pathogenesis of allergic diseases [56]. In the past few decades, there have been significant increases in both the incidence and severity of allergic diseases [57]. Environmental factors, such as pollutants and food additives, which do not have allergic potential but exert proallergic adjuvant effect, may play crucial roles in the induction and/or exacerbation of allergic diseases. In this respect, TBT can be regarded as one of the important environmental pollutant that might affect human health, since TBT along with its decomposition products have been reported to be still detectable in substantial amounts in seawater, sediment, marine fish, and even in human blood [40,58], though TBT production and use are now under regulation. The ability of TBT at environmentally relevant concentration to promote Th2 development [14,15] and preferentially induce apoptosis in Th1 cells provides a plausible mechanism whereby a significant environmental pollutant TBT may contribute to these increase in the prevalence of Th2-driven allergic diseases.

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REFERENCES

[1] Ueno D, Inoue S, Takahashi S, Ikeda K, Tanaka H, Subramanian AN, et al. Global pollution monitoring of butyltin compounds using skipjack tuna as a bioindicator. Environ Pollut 2004;127:1–12.

[2] Kannan K, Senthilkumar K, Giesy JP. Occurrence of butyltin compounds in human blood. Environ Sci Technol 1999;33:1776–9.

[3] Takahashi S, Mukai H, Tanabe S, Sakayama K, Miyazaki T, Masuno H. Butyltin residues in livers of humans and wild terrestrial mammals and in plastic products. Environ Pollut 1999;106:213–8.

[4] Mizuhashi S, Ikegaya Y, Matsuki N. Cytotoxicity of tributyltin in rat hippocampal slice cultures. Neurosci Res 2000;38:35–42.

[5] Davis A, Barale R, Brun G, Forster R, Gunther T, Hautefeuille H, et al. Evaluation of the genetic and embryotoxic effects of bis(tri-*n*-butyltin)oxide (TBTO), a broad-spectrum pesticide, in multiple in vivo and in vitro short-term tests. Mutat Res 1987;188:65–95.

[6] Snoeij NJ, Penninks AH, Seinen W. Dibutyltin and tributyltin compounds induce thymus atrophy in rats due to a selective action on thymic lymphoblasts. Int J Immunopharmacol 1988;10:891–9.

[7] Bressa G, Hinton RH, Price SC, Isbir M, Ahmed RS, Grasso P. Immunotoxicity of tri-*n*-butyltin oxide (TBTO) and tri-*n*-butyltin chloride (TBTC) in the rat. J Appl Toxicol 1991;11:397–402.

[8] Aw TY, Nicotera P, Manzo L, Orrenius S. Tributyltin stimulates apoptosis in rat thymocytes. Arch Biochem Biophys 1990;283:46–50.

[9] Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996;383:787–93.

[10] Varadhachary AS, Perdow SN, Hu C, Ramanarayanan M, Salgame P. Differential ability of T cell subsets to undergo activation-induced cell death. Proc Natl Acad Sci USA 1997;94:5778–83.

[11] Roozendaal R, Vellenga E, de Jong MA, Traanberg KF, Postma DS, de Monchy JG, et al. Resistance of activated human Th2 cells to NO-induced apoptosis is mediated by gamma-glutamyltranspeptidase. Int Immunol 2001;13:519–28.

[12] Gennari A, Viviani B, Galli CL, Marinovich M, Pieters R, Corsini E. Organotins induce apoptosis by disturbance of $[Ca^{2+}]_i$ and mitochondrial activity, causing oxidative stress and activation of caspases in rat thymocytes. Toxicol Appl Pharmacol 2000;169:185–90.

[13] Raffray M, McCarthy D, Snowden RT, Cohen GM. Apoptosis as a mechanism of tributyltin cytotoxicity to thymocytes: relationship of apoptotic markers to biochemical and cellular effects. Toxicol Appl Pharmacol 1993;119:122–30.

[14] Kato T, Uchikawa R, Yamada M, Arizono N, Oikawa S, Kawanishi S, et al. Environmental pollutant tributyltin promotes Th2 polarization and exacerbates airway inflammation. Eur J Immunol 2004;34:1312–21.

[15] Kato T, Tada-Oikawa S, Takahashi K, Saito K, Wang L, Nishio A, et al. Endocrine disruptors that deplete glutathione levels in APC promote Th2 polarization in mice leading to the exacerbation of airway inflammation. Eur J Immunol 2006;36:1199–209.

[16] Kasugai I, Yamada M. High production of catalase in hydrogen peroxide-resistant human leukemia HL-60 cell lines. Leuk Res 1992;16:173–9.

[17] Ikeda K, Kajiwara K, Tanabe E, Tokumaru S, Kishida E, Masuzawa Y, et al. Involvement of hydrogen peroxide and hydroxyl radical in chemically induced apoptosis of HL-60 cells. Biochem Pharmacol 1999;57:1361–5.

[18] Kato T, Hakamada R, Yamane H, Nariuchi H. Induction of IL-12 p40 messenger RNA expression and IL-12 production of macrophages via CD40-CD40 ligand interaction. J Immunol 1996;156:3932–8.

[19] Hiraku Y, Kawanishi S. Oxidative DNA damage and apoptosis induced by benzene metabolites. Cancer Res 1996;56:5172–8.

[20] Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995;184:39–51.

[21] Bueb JL, Gallois A, Schneider JC, Parini JP, Tschirhart E. A double-labelling fluorescent assay for concomitant measurements of $[Ca^{2+}]_i$ and $O_2^{\bullet}$ production in human macrophages. Biochim Biophys Acta 1995;1244:79–84.

[22] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993;302:348–55.

[23] Bass DA, Parce JW, Dechatelet LR, Szejda P, Seeds MC, Thomas M. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J Immunol 1983;130:1910–7.

[24] Tada-Oikawa S, Oikawa S, Kawanishi M, Yamada M, Kawanishi S. Generation of hydrogen peroxide precedes loss of mitochondrial membrane potential during DNA alkylation-induced apoptosis. FEBS Lett 1999;442:65–9.

[25] Mizutani H, Tada-Oikawa S, Hiraku Y, Oikawa S, Kojima M, Kawanishi S. Mechanism of apoptosis induced by a new topoisomerase inhibitor through the generation of hydrogen peroxide. J Biol Chem 2002;277:30684–9.

[26] Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 1995;181:1661–72.

[27] Petit PX, O'Connor JE, Grunwald D, Brown SC. Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. Eur J Biochem 1990;194:389–97.

[28] Hiraku Y, Murata M, Kawanishi S. Determination of intracellular glutathione and thiols by high performance liquid chromatography with a gold electrode at the femtomole level: comparison with a spectroscopic assay. Biochim Biophys Acta 2002;1570:47–52.

[29] Stridh H, Fava E, Single B, Nicotera P, Orrenius S, Leist M. Tributyltin-induced apoptosis requires glycolytic adenosine trisphosphate production. Chem Res Toxicol 1999;12:874–82.

[30] Stridh H, Cotgreave I, Muller M, Orrenius S, Gigliotti D. Organotin-induced caspase activation and apoptosis in human peripheral blood lymphocytes. Chem Res Toxicol 2001;14:791–8.

[31] Mahipal SV, Subhashini J, Reddy MC, Reddy MM, Anilkumar K, Roy KR, et al. Effect of 15-lipoxygenase metabolites, 15-(S)-HPETE and 15-(S)-HETE on chronic myelogenous leukemia cell line K-562: Reactive oxygen species (ROS) mediate caspase-dependent apoptosis. Biochem Pharmacol 2007;74:202–14.

[32] Shenoy S, Vasania VS, Gopal M, Mehta A. 8-Methly-4-(3-diethylaminopropylamino) pyrimido [4′,5′;4,5] thieno (2,3-b) quinoline (MDPTQ), a quinoline derivate that causes ROS-mediated apoptosis in leukemia cell lines. Toxicol Appl Pharmacol 2007;222:80–8.

[33] Nishikimi A, Kira Y, Kasahara E, Sato EF, Kanno T, Utsumi K, et al. Tributyltin interacts with mitochondria and induces cytochrome c release. Biochem J 2001;356:621–6.

[34] Stridh H, Gigliotti D, Orrenius S, Cotgreave I. The role of calcium in pre- and postmitochondrial events in tributyltin-induced T-cell apoptosis. Biochem Biophys Res Commun 1999;266:460–5.
[35] Banki K, Hutter E, Gonchoroff NJ, Perl A. Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. J Immunol 1999;162:1466–79.
[36] Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. Cell 1997;91:627–37.
[37] Virag L, Marmer DJ, Szabo C. Crucial role of apopain in the peroxynitrite-induced apoptotic DNA fragmentation. Free Radic Biol Med 1998;25:1075–82.
[38] Hildeman DA, Mitchell T, Teague TK, Henson P, Day BJ, Kappler J, et al. Reactive oxygen species regulate activation-induced T cell apoptosis. Immunity 1999;10: 735–44.
[39] Molina-Jimenez MF, Sanchez-Reus MI, Andres D, Cascales M, Benedi J. Neuroprotective effect of fraxetin and myricetin against rotenone-induced apoptosis in neuroblastoma cells. Brain Res 2004;1009:9–16.
[40] Whalen MM, Loganathan BG, Kannan K. Immunotoxicity of environmentally relevant concentrations of butyltins on human natural killer cells in vitro. Environ Res 1999;81: 108–16.
[41] Cotgreave IA. *N*-Acetylcysteine: pharmacological considerations and experimental and clinical applications. Adv Pharmacol 1997;38:205–27.
[42] Hancock JT, Desikan R, Neill SJ. Does the redox status of cytochrome *c* act as a fail-safe mechanism in the regulation of programmed cell death? Free Radic Biol Med 2001;31:697–703.
[43] Snoeij NJ, Bol-Schoenmakers M, Penninks AH, Seinen W. Differential effects of tri-*n*-butyltin chloride on macromolecular synthesis and ATP levels of rat thymocyte subpopulations obtained by centrifugal elutriation. Int J Immunopharmacol 1988;10:29–37.
[44] Aldridge WN, Street BW. Oxidative phosphorylation. The specific binding of trimethyltin and triethyltin to rat liver mitochondria. Biochem J 1970;118:171–9.
[45] Kushnareva Y, Murphy AN, Andreyev A. Complex I-mediated reactive oxygen species generation: modulation by cytochrome *c* and $NAD(P)^+$ oxidation-reduction state. Biochem J 2002;368:545–53.
[46] Di Meo S, Venditti P. Mitochondria in exercise-induced oxidative stress. Biol Signals Recept 2001;10:125–40.
[47] Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 1998;391:43–50.
[48] Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome *c*. Biochim Biophys Acta 1998;1366:139–49.
[49] Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, et al. Two distinct pathways leading to nuclear apoptosis. J Exp Med 2000;192:571–80.
[50] Kang YH, Yi MJ, Kim MJ, Park MT, Bae S, Kang CM, et al. Caspase-independent cell death by arsenic trioxide in human cervical cancer cells: reactive oxygen species-mediated poly(ADP-ribose) polymerase-1 activation signals apoptosis-inducing factor release from mitochondria. Cancer Res 2004;64:8960–7.
[51] Obata F, Hoshino A, Toyama A. Hydrogen peroxide increases interleukin-12 p40/p70 molecular ratio and induces Th2-predominant responses in mice. Scand J Immunol 2006;63:125–30.
[52] Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326:298–304.
[53] Muller M, Grunewald J, Olgart Hoglund C, Dahlen B, Eklund A, Stridh H. Altered apoptosis in bronchoalveolar lavage lymphocytes after allergen exposure of atopic asthmatic subjects. Eur Respir J 2006;28:513–22.
[54] Akdis M, Trautmann A, Klunker S, Daigle I, Kucuksezer UC, Deglmann W, et al. T helper (Th) 2 predominance in atopic diseases is due to preferential apoptosis of circulating memory/effector Th1 cells. FASEB J 2003;17:1026–35.
[55] Romagnani S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol 2000;105:399–408.
[56] Dworski R. Oxidant stress in asthma. Thorax 2000;55(Suppl. 2):S51–3.
[57] Holgate ST. The epidemic of allergy and asthma. Nature 1999;402:B2–4.
[58] Bhosle NB, Garg A, Jadhav S, Harjee R, Sawant SS, Venkat K, et al. Butyltins in water, biofilm, animals and sediments of the west coast of India. Chemosphere 2004;57:897–907.