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Change in plasma membrane potential of rat thymocytes by *tert*-butylhydroquinone, a food additive: Possible risk on lymphocytes

Maki Takeda [a], Keisuke Oyama [b], Norio Kamemura [c], Kaori Kanemaru [a, c], Keizo Yuasa [c], Kumio Yokoigawa [a, c], Yasuo Oyama [a, c, *]

[a] Faculty of Integrated Arts and Sciences, Tokushima University, Tokushima 770-8502, Japan
[b] Osaka University Hospital, Suita, Osaka 565-0871, Japan
[c] Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan

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ABSTRACT

Tertiary butylhydroquinone (TBHQ) is a food additive and has various beneficial actions under in vitro and in vivo experimental conditions. Therefore, it is necessary to collect additional data on the toxicity of TBHQ in order to avoid adverse effects during clinical applications. Changes in plasma membrane potential are associated with changes in physiological functions even in non-excitable cells such as lymphocytes. Thus, compounds that affect membrane potential may modify some lymphocytic functions. The effect of TBHQ on plasma membrane potential was examined in rat thymocytes using flow cytometric techniques. Treatment of rat thymocytes with TBHQ caused hyperpolarization and then depolarization. The TBHQ-induced hyperpolarization was due to the activation of Ca^{2+}-dependent K^+ channels. TBHQ elevated intracellular Ca^{2+} levels. The depolarization by TBHQ was caused by a nonspecific increase in membrane ionic permeability. Both the sustained depolarization and elevation of intracellular Ca^{2+} level by TBHQ are thought to be adverse for thymocytes because such changes disturb membrane and intracellular signaling. The thymus is most active during neonatal and pre-adolescent periods. If TBHQ exerts adverse actions on thymocytes, it may result in an immunotoxic effect in neonates and adolescents.

1. Introduction

Tertiary butylhydroquinone (TBHQ, Chemical Abstracts, 1948-33-0) is a food additive that has antioxidant activity (World Health Organization, 1999). The European Food Safety Authority has evaluated the toxicity of TBHQ and determined safe levels of TBHQ as a food additive (European Food Safety Authority, 2004). Antioxidative TBHQ exerts various beneficial actions under in vitro and in vivo experimental conditions (Jin et al., 2011; Mittal et al., 2014; Duan et al., 2016) because reactive oxygen species are involved in many diseases (Uttara et al., 2009; Brieger et al., 2012). Various major diseases such as atherosclerosis, arthritis, ischemia, Alzheimer's, Parkinson's, diabetes, sclerosis, gastritis, aging, liver diseases, cancer and AIDS are due to the generation of free radicals (Halliwell and Gutteridge, 1984). Since TBHQ has been already approved as a food additive, it is plausible that it will be used as a preventive and/or therapeutic medicinal agent. However, TBHQ also exhibits diverse toxic actions under experimental conditions (Imhoff and Hansen, 2010; Braeuning et al., 2012; Shibuya et al., 2012; Eskandani et al., 2014). For example, TBHQ induces mitochondrial oxidative stress causing Nrf2 activation. The oxidation of TBHQ produces more cytotoxic *tert*-butyl-*p*-benzoquinone. Therefore, it is necessary to collect additional data on the toxicity of TBHQ in order to avoid adverse actions during medical applications.

Plasma membrane potential is determined by membrane ionic permeability and transmembrane ionic gradient. Both are modulated by various physiological, pharmacological, and toxicological factors. The ionic gradient across membranes is maintained by the functional integration of cellular energy metabolism and a membrane with selective permeability. Changes in membrane potential are associated with altered physiological functions even in non-excitable cells, such as lymphocytes (Tsien et al., 1982; Han and Kang, 2009; Varga et al., 2010; Lam and Wulff, 2011; Feske et al., 2012). Opening and closing of K^+ channels change membrane K^+

* Corresponding author. Faculty of Bioscience and Bioindustry, Tokushima University, Tokushima 770-8513, Japan.
E-mail address: oyamay@tokushima-u.ac.jp (Y. Oyama).

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permeability, one of the important determinants of membrane potential. Charybdotoxin, a specific blocker of Ca^{2+}-dependent K^+ channels (Miller et al., 1985), inhibits the proliferation of T lymphocytes (Price et al., 1989). Non-specific blockers of K^+ channels inhibit the activation of B lymphocytes, resulting in an attenuation of lymphocytic progression through the cell cycle (Amigorena et al., 1990). Furthermore, the activity of phosphoinositide phosphatase is tuned within physiological range of plasma membrane potential (Murata et al., 2005). Thus, membrane potential can function beyond channel proteins. Based on the evidence, it is likely that the compounds that affect membrane potentials modify some functions in lymphocytes.

In this study, we examined the effect of TBHQ on the plasma membrane potential in rat thymocytes. Voltages were assessed using a voltage-sensitive fluorescent dye and the cells were studied using flow-cytometry, which revealed the mechanisms of TBHQ-induced changes in plasma membrane potential. One may argue that membrane potential is not a target for the adverse effects of food additives, including preservatives. However, the depolarization modulates plasma membrane phospholipid dynamics and K-Ras signaling in fibroblasts, excitable neuroblastoma cells, and Drosophila neurons (Zhou et al., 2015). Thus, the changes in membrane potentials seem to affect membrane and cellular functions. Furthermore, the thymus is most active during neonatal and pre-adolescent periods. Since there is a major concern about the adverse effect of food additives on children's health, any adverse change in thymocytes may be clinically important.

2. Materials and methods

2.1. Chemicals

TBHQ (99.1% purity) was purchased from Tokyo Chemical Ind. (Tokyo, Japan). *bis*-(1,3-Dibutylbarbituric acid)trimethine oxonol (Oxonol), Fluo-3-AM, and propidium iodide (PI) were supplied by Invitrogen (Eugene, OR, USA). Chelators for extracellular Ca^{2+} and intracellular Zn^{2+} were, respectively, disodium salt of ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid and *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (EDTA and TPEN; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Unless mentioned, all other reagents were obtained from Wako Pure Chemicals (Osaka, Japan).

2.2. Cell preparation

Experiments were performed under the approval (No. 05279) of Tokushima University Committee for Animal Experiments. Thymus glands were obtained from 6 to 8 week old Wistar rats that were anesthetized with ether. To dissociate individual cells, the glands were triturated in chilled Tyrode's solution (2–4 °C). The solution containing dissociated cells (cell suspension) was incubated at 36–37 °C for 1 h before experiments. The cell suspension contained trace amounts (216.9 ± 14.4 nM) of zinc from the cell preparation.

Thymocytes were used for this study of chemical cytotoxicity due to the following experimental reasons. First, single dissociated cells with intact membranes are easily obtained since no enzymatic treatment is required to isolate individual cells. Second, several types of chemical and biological substances induce cell death (Corsini et al., 2013; Kuchler et al., 2014). Third, the process of cell death (apoptosis, necrosis, and autophagy) is well studied (Klein et al., 2014; Poon et al., 2014; Shimizu et al., 2014). However, there is an experimental limitation for dissociated thymocytes. Thymocytes spontaneously undergo apoptosis during prolonged incubation. All experiments, therefore, including the preparation of the cell suspension, were completed within 8 h after the dissection of thymus from rats to avoid possible contribution of spontaneous apoptosis. Therefore, the time for TBHQ treatment should be less than 5 h. Cell shrinkage is one of parameters observed during an early stage of apoptosis. We employed forward scatter as a parameter of cell size. The intensity of forward scatter in control cells was not changed during the experiment. The cell viability of control cells was also unchanged during the experiment.

TBHQ (10–300 mM in 2 μL dimethyl sulfoxide) was added to 2 mL of cell suspension to achieve final concentrations of 10–300 μM, and the cells were treated with TBHQ for 1–4 h, depending on respective experimental procedures. A sample (100 μL) from each cell suspension was analyzed by flow cytometry. Data acquisition from 2000 to 2500 cells took approximately 10–15 s. The sheath flow rate was manually adjusted to measure 195–205 cells/s with an interval of 180 μs between measurements of forward and side scatters. The measurement started after achieving a constant flow of cells. Cell lethality estimated from 25,00 cells was quite similar to that estimated from 10,000 cells. Therefore, 2,500 cells were deemed sufficient to examine cellular actions of TBHQ.

2.3. Fluorescence measurement

Fluorescence was measured and analyzed using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) and JASCO software package (Chikahisa et al., 1996). Chemical reagents, except for fluorescent probes, exhibited no fluorescence under the experimental conditions used. PI (5 μM), a fluorescent probe that stains the DNA of dead cells, was added to the cell suspension to assess cell lethality. Measurements of membrane potentials were made using 500 nM Oxonol. Cells were incubated with 1 μM Fluo-3-AM for 50–60 min before fluorescence measurement in order to monitor the change in $[Ca^{2+}]_i$ (Kao et al., 1989). Fluo-3 fluorescence was monitored in the cells treated with 10 μM TPEN to avoid the contribution of intracellular Zn^{2+} to Fluo-3 fluorescence. Oxonol and Fluo-3 fluorescence were monitored in cells that did not show PI fluorescence (living cells with intact membranes). The excitation wavelength for fluorescent probes was 488 nm, and emissions were detected at 530 ± 20 nm for Oxonol and Fluo-3 fluorescence, and at 600 ± 20 nm for PI fluorescence. Oxonol and Fluo-3-AM were dissolved in DMSO. Therefore, control cell suspension contained 0.1% DMSO. The intensities of Oxonol and Fluo-3 fluorescence in control cells were similar to those observed in cell suspension containing 0.3% DMSO. Cell viability of the suspension containing 0.1% DMSO was also similar to that of cell suspension containing 0.3% DMSO. Thus, DMSO (up to 0.3%) did not affect cellular parameters examined in this study.

2.4. Statistical analysis and presentation

Statistical analysis was carried out using Tukey's multivariate analysis. *P*-values of <0.05 indicate statistical significance. Values are expressed as mean and standard deviation of 4–8 samples. Each experimental series was conducted three times unless stated otherwise.

3. Results

3.1. Changes in oxonol fluorescence by TBHQ

TBHQ at a concentration of 300 μM decreased the intensity of Oxonol fluorescence immediately after application, which was maintained for 30–60 min (Fig. 1A). An augmentation was observed at 90 min after the start of application (Fig. 1A). Although the attenuation of Oxonol fluorescence always preceded the