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Comparative Cytotoxicity between Butylated Hydroxytoluene and Its Methylcarbamate Derivative, Terbucarb, on Isolated Rat Hepatocytes

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Butylated hydroxytoluene (3,5-di-tert-butyl-4-hydroxytoluene; BHT) is widely used as phenolic antioxidant in processed foods, cosmetics and petroleum products. It is well known that high doses of BHT cause acute hepatic damage accompanied by centrilobular necrosis in rats (Nakagawa et al. 1984). The hepatic damage is associated with prolonged depletion of glutathione (GSH). Terbucarb (2,6-di-tert-butyl-para-tolyl-methylcarbamate), which has a methylcarbamate group substituted for the phenol group on BHT, was developed as an insecticide and is also presently used as a herbicide on turfgrass (Jagschitz 1973). Despite the metabolic and toxicological details known about BHT in vivo (WHO Technical Report Series 1987) and in vitro (Nakagawa and Tayama 1988),no extensive studies have been reported on the metabolism and toxicity of Terbucarb. The isolated hepatocyte system provides a very useful system for the study of the temporal sequences leading to cell damage caused by chemicals and drugs. Here, using freshly isolated rat hepatocytes, we report on the comparative toxic effects of BHT and its methylcarbamate derivative, Terbucarb.

MATERIALS AND METHODS

Chemicals were purchased from the following companies: BHT (purity > 98%) from the Tokyo Kasei Co. (Tokyo Japan); Terbucarb (purity > 98%) from the GL Science (Tokyo Japan); reduced glutathione and bovine serum albumin from the Sigma Chemical Co. (St Louis, MO, U.S.A); and collagenase from Wako Pure Chemicals Ind. (Osaka, Japan). Chemical structures of BHT and Terbucarb are shown in Figure 1.

Male Fischer-344 rats (220-260 g) were used in all experiments. Hepatocytes were isolated by collagenase perfusion of liver as described by Moldéus et al. (1978) and were suspended at a concentration of 10^6 cells/mL in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM Hepes and 0.1 % albumin. Initial cell viabilities assessed by Trypan blue exclusion were approximately 90%.
All incubations were performed in rotating, round-bottomed flasks at 37° C under constant flow of humidified carbogen (95% O2 and 5%

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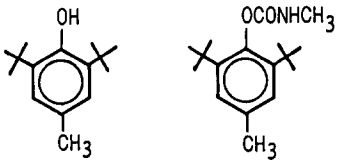

A B

Figure 1. Chemical structures of BHT (A) and Terbucarb (B)

CO_2). Reactions were initiated by the addition of BHT or Terbucarb dissolved in DMSO (final concentration less than 1%). The corresponding control groups received an equivalent volume of DMSO. Aliquots of cell suspensions were taken at intervals for the determination of cell death as well as for quantification of the concentrations of GSH and adenine nucleotides.

Liver mitochondria were isolated from Fischer-344 rats by differential centrifugation in medium containing 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4) and 1 mM EDTA. EDTA was omitted in the final wash and resuspension. The rate of oxygen consumption was measured polarographically with a Clark-type oxygen electrode (Yellow Spring Instruments Co., Model 5300) at 25°C in the presence (state 3) or exhaustion (state 4) of 100 μM ADP. Respiration buffer (3 mL, pH 7.4) contained 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 5 mM potassium phosphate and 1 μM rotenone. The respiration substrate was 5 mM succinate and the amount of mitochondria was 1 mg protein/mL. The respiration control index (RCI) was calculated as the ratio of state 3/state 4 respiration.

Adenine nucleotides in hepatocytes were measured using HPLC according to the procedure of Jones (1981). Cellular GSH levels were determined by HPLC as described by Reed et al. (1980). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The addition of BHT or Terbucarb to isolated rat hepatocytes caused a concentration (0.5, 1.0 mM)-dependent acute cell death (Figure 2). These toxicities were accompanied by the loss of cellular ATP and GSH. Based on these parameters, Terbucarb was less toxic than BHT. Table 1 shows the effects of both compounds on the levels of adenine nucleotides in hepatocytes. The rapid disappearance of cellular ATP induced by 1.0 mM BHT was accompanied by an increase in AMP level 30 min later. In addition, 1.0 mM BHT reduced the total adenine nucleotides pool to approximately 60% of control. Because BHT does not react with ATP in Krebs-Henseleit buffer without hepatocytes for 30 min (data not shown), this suggests that the depletion of ATP is due to the inhibition of adenine nucleotide synthesis and/or the activation of hydrolysis of ATP by the compounds.

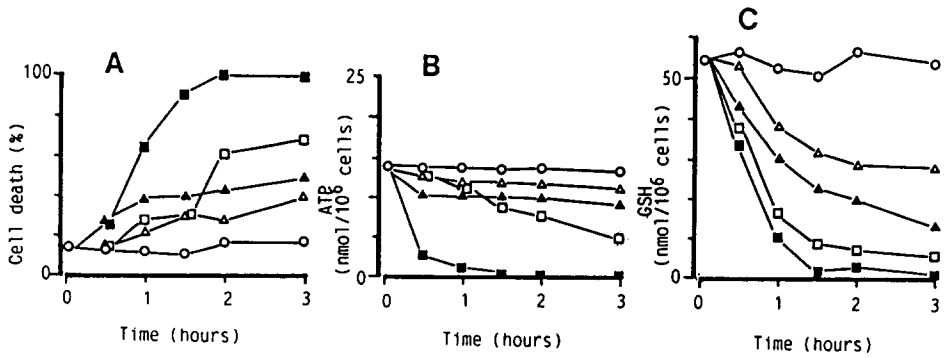

Figure 2. Effects of BHT and Terbucarb on cell death (A), levels of ATP (B) and GSH (C) of isolated hepatocytes. Hepatocytes were incubated at 10^6 cells/mL in Krebs-Henseleit buffer with no addition (○), 0.5 mM BHT (□), 1.0 mM BHT (■), 0.5 mM Terbucarb (△) and 1.0 mM Terbucarb (▲). Results of one experiment typical of three are shown.

Table 1. Effects of BHT or Terbucarb on the level of adenine nucleotides in isolated hepatocytes

Incubation Time (min)	Treatment	(mM)	Adenine nucleotides ($nmol/10^6$ cells) ATP	ADP	AMP	Total
0	None		13.7	2.57	0.22	16.49
30	None		14.1	2.59	0.22	16.91
	BHT	0.5	13.1	3.26	0.32	16.68
		1.0	2.3	2.91	4.80	10.01
	Terbucarb	0.5	13.7	3.06	0.20	16.96
		1.0	9.9	4.00	0.24	14.14

Values are the means from two separate experiments.

Thompson and Moldéus (1988) have suggested that the cytotoxicity of BHT is because of its effects on biomembranes and mitochondrial bioenergetics. The effects of BHT and Terbucarb on the oxygen consumption by isolated liver mitochondria are shown in Table 2. Addition of 0.5 mM BHT caused an increase in the rate of state 4 oxygen consumption, indicating partial uncoupling of oxidative phosphorylation in mitochondrial respiration. BHT had a greater effect than Terbucarb. Further, inhibition of the RCI, a sensitive index of mitochondrial impairment, in the 0.5 mM BHT group, was due to an inhibition of state 3 respiration and a stimulation of state 4 respiration. This result indicates that inhibition of

Table 2. Effects of BHT or Terbucarb on mitochondrial respiration

Treatment	mM	Mitochondrial respiration (ng atom O/mg protein/min) state 4	state 3	RCI
None		13.5±3.6	63.3±2.7	4.7
BHT	0.25	12.3±1.5	47.1±2.1	3.8
	0.50	42.9±7.4	38.4±2.9	0.9
Terbucarb	0.25	15.0±1.2	59.4±1.6	4.0
	0.50	15.1±1.5	51.0±2.8	3.4

Values are the means±SD from three determination.

oxidative phosphorylation is one mechanism by which BHT causes depletion of intracellular ATP levels.

We have demonstrated that BHT is metabolized to BHT-quinone methide by a cytochrome P-450-linked monooxygenase system and that the 4-methyl group of the metabolite specifically binds to the sulfhydryl groups of protein, GSH, and other sulfhydryl compounds (Nakagawa et al. 1981; Nakagawa et al. 1983). The binding between BHT intermediate(s) and sulfhydryl groups may deplete intracellular GSH level. In this study, BHT caused the loss of cellular ATP which was accompanied by a sequential increase in AMP levels and a decrease in total adenine nucleotides pool. Since BHT results in inhibition of mitochondrial respiration, the organelle may be a target for BHT and/or its metabolites(s). Phenols are effective inhibitors of a number of FAD- and NAD^+- containing oxidases and dehydrogenases via reaction mechanisms that exhibit complex kinetics (Hank and Wedding 1975; Irons and Sawahata 1985). The addition of hydroxyl group to the aromatic ring of biphenyl enhances biphenyl-induced cytotoxicity (Nakagawa et al. 1993). Therefore, the substitution with a methylcarbamate group for the hydroxyl group of BHT may reduce both the inhibition of mitochondrial respiration and the cytotoxicity caused by BHT. It is well known that the carbamates are inhibitors of esterase (Casida 1963; O'Brien 1969). However, the two tertiary butyl groups of Terbucarb may prevent carbamylation of cholinesterase, since intermediates without the methylcarbamate group were not found in hepatocyte suspension added with Terbucarb one hour later (data not shown). In the plant tissues, Terbucarb affects spindle microtubule organizing center and results in mitotic abnormality on root tips (Lehnen et al. 1990). Further examination will be necessary to determine the mechanism of Terbucarb-induced cytotoxicity and its metabolism.

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