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EFFECT OF BUTYLATED HYDROXYTOLUENE (BHT) AND ITS METABOLITE ON THE UPTAKE OF TAUROCHOLATE IN PRIMARY CULTURE OF ADULT RAT HEPATOCYTES

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ABSTRACT: *The effect of butylated hydroxytoluene (BHT) and its major metabolite, 3,5-di-tert-butyl-4-hydroxybenzoic acid (BHT-acid) on the uptake of taurocholate into hepatocytes was studied using the primary culture of rat hepatocytes. Hepatocyte were isolated by an in situ collagenase perfusion technique and maintained as a monolayer in serum-free meadia for 24 hours before use. The uptake of taurocholate was saturable with an apparent* K_m *of* 12.8 ± 2.8 *μM and* V_{max} *of* 0.18 ± 0.01 *nmol/mg/min. Both BHT and BHT-acid inhibited the hepatocellular uptake of taurocholate when they were added to the culture. BHT-acid caused more inhibition in taurocholate when they were added to the culture. BHT-acid caused more inhibition in taurocholate uptake than BHT itself and the inhibition was competitive with a* K_1 *of* 376 ± 23 *μM. The results of current study suggest that the decreased bile acid excretion in BHT-treated rats is due to an inhibition of taurocholate uptake by BHT and/or its metabolites.*

Key words: *Hepatocytes culture, Taurocholate uptake, BHT effect.*

INTRODUCTION

Butylated hydroxytoluene (BHT) is commonly added to food at level of 0.01% to 0.02% of the fat content of the food product as preservative for unsaturated lipids and the materials subject to spoilage by oxidation through donating a hydrogen to free radical. Because of the widespread use of its compound, toxicological effects of BHT have been studied extensively in recent years. BHT has toxic effects at extremely high doses, having a LD_{50} of 2.0 g/kg in most animals (Branen, 1975). When fed to experimental animals, BHT produces a variety of changes. These changes include reduced growth rate and body weight (Johnson and Hewgill, 1961), hypertrophy of the liver with the increase in smooth endoplasmic reticulum and in the mitotic activity of hepatocytes (Botham *et al.*, 1970; Brown *et al.*, 1959; Feuer *et al.*, 1965) and altera-

tions in the activity of several hepatic enzymes (Awasthi *et al.*, 1983; Halladay *et al.*, 1980). These hepatic changes appeared to be an adaptive hypertrophy and to be an increase activity rather than to be pathological alterations (Gilbert and Golberg, 1965).

In our previous study we reported that the administration of 0.25% BHT for 10 days increased bile flow significantly in rats (Choe *et al.*, 1984). The increase in bile flow was not due to an enhanced excretion of bile acids into bile because both the biliary bile acid concentration and total biliary excretion of bile acid were lower in BHT-treated rats than in control rats. It appears that the increase in bile flow produced by BHT is due to the osmotic choleresis related to the secretion of BHT and its metabolites into bile and the decreased bile acid excretion in BHT-treated rats might be due to an interference of BHT and its metabolites on bile acid transport into hepatocytes.

To prove this hypothesis, the effect of BHT and its major metabolite, BHT-acid (3,5-di-tert-butyl-4-hydroxybenzoic acid) on the uptake and secretion of taurocholate was studied in the primary culture of adult rat heaptocytes in this study.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200-250 g) were used. Rats were maintained on a powdered diet (Purina Korea) and water was given *ad libitum*. The animals were kept in 12 h light/dark cycle.

Primary Rat Hepatocytes Culture

Hepatocytes were isolated from rats by a collagenase perfusion technique and were maintained in serum-free medium on 60 mm collagen-coated plates as described previously (Yang *et al.*, 1983). Cells were initially cultured in modified Waymouth's MB 752/1 medium, previously designated $WO/BA\text{-}M_2$ (Kletzien *et al.*, 1976), containing insulin (10^{-7}M) and gentamycin (50 μg/m*l*). The medium was changed to fresh $WO/BA\text{-}M_2$ plus insulin and gentamycin 4 and 24 h after initial plating.

Uptake of Taurocholate

After 24 h incubation the culture medium was aspirated off and the plate was rinsed with warm (37°C) Hank's-Hepes salt solution (containing 8 μM glucose and 20 mM hepes buffer, pH 7.4). Then a volume of 2.0 m*l* of Hank's-Hepes salt solution containing a specific concentration of radio-active taurocholate which had been diluted with nonradioactive sodium taurocholate was added to the plate along with BHT or BHT-acid. Taurocholate uptake was measured by incubating the cells for the designated time at 37°C by placing the plate in a shallow waterbath. Incubation was terminated by aspirating off the medium and rinsing the plate 4 times with a total amount of about 12 m*l* cold Hank's-Hepes salt solution.

Cells were digested in 0.2N NaOH and aliquot of the digest were taken for protein determination and liquid scintillation counting. Protein concentration in aliquot was determined by the method of Lowry *et al.* (1951).

Statistic Analysis

Slope of lines were determined by the least sequares linear regression. Result were

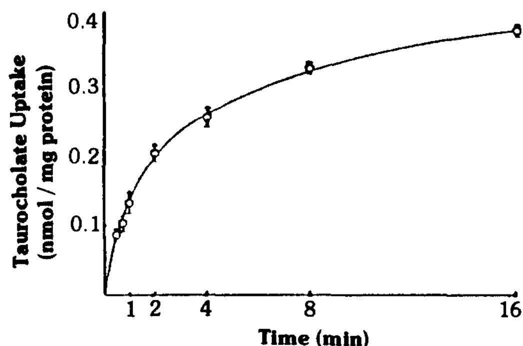

Fig. 1. Time course of taurocholate uptake by primary culture of rat hepatocytes. After 24 hr culturing, 10 μM of taurocholate was added to the plate. Each value to the mean SE of triplicate plates.

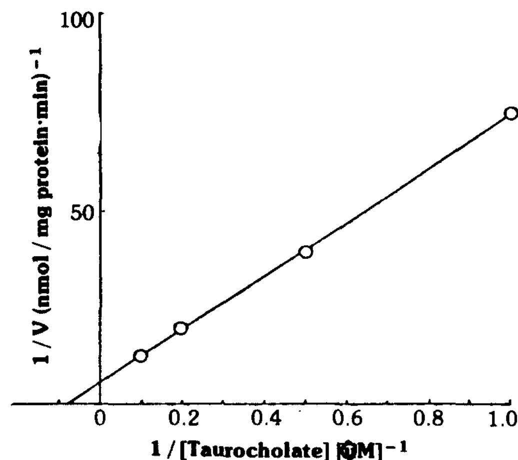

Fig. 2. Lineweaver-Burk plot of taurocholate uptake by hepatocyte monolayers. After 24 hr culturing, ^{14}C-taurocholate was added to the culture to yield final concentration of 1, 2, 5 and 10 μM. The reaction was stopped after 30, 45 and 60 sec. Uptake of taurocholate was linear for 1 min. Initial rates of uptake were calculated from the slopes of the straight lines. The data represent the average of triplicate plates.

expressed as the mean ± S.E. The significance of the difference between mean values was assessed by student's t-test ($p < 0.05$).

RESULTS

The initial experiments were designed to characterize the uptake of taurocholate into the primary culture of rat hepatocytes. As shown in Fig. 1, the process of taurocholate uptake was rapid and linear for about 1 min. It gradually decreased thereafter, and attained a plateau after about 16 min.

To determine the kinetic constant of the transport process, the initial rate of the taurocholate uptake was measured with various concentrations of taurocholate ranging from 1 to 10 μM. The reaction was stopped after 30, 45 and 60 sec. The rate of uptake was linear for 1 min. the slopes of these curves were plotted according to Lineweaver and Burk. As shown in Fig. 2, the straight line reveals an apparent K_m of 12.8 ± 2.2 μM and a maximal velocity, V_{max} of 0.18 ± 0.01 mmol/mg protein/min. Fig. 3 shows the time course of taurocholate uptake into rat hepatocytes primary cultures in the presence of BHT or BHT-acid. In this experiment 10 μM of taurocholate was added to the culture along with 300 μM of BHT or BHT-acid. The uptake of taurocholate was significantly lower in treat groups than that of the control. BHT-acid shows greater inhibition than BHT itself.

To further characterize this inhibitory effect on the uptake, the initial uptake rate at different taurocholate concentrations was measured in the presence of various concen-

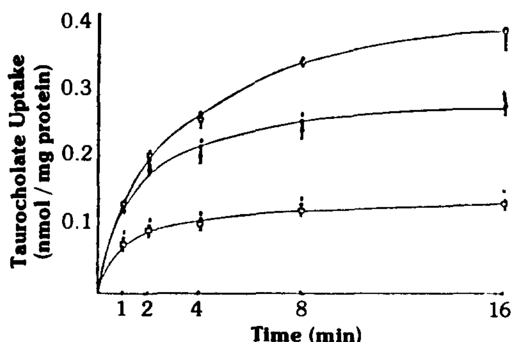

Fig. 3. Effects of BHT and BHT-acid on uptake of taurocholate in primary cultures of hepatocytes. After 24hr culturing, BHT (300 μM) and BHT-acid (300 μM) was added to the culture along with taurocholate (10 μM). The plots represent data for BHT (△), BHT acid (□) and control (○). Each value is the average of triplicate plates. An asterisk indicates values significantly different from control ($p<0.05$).

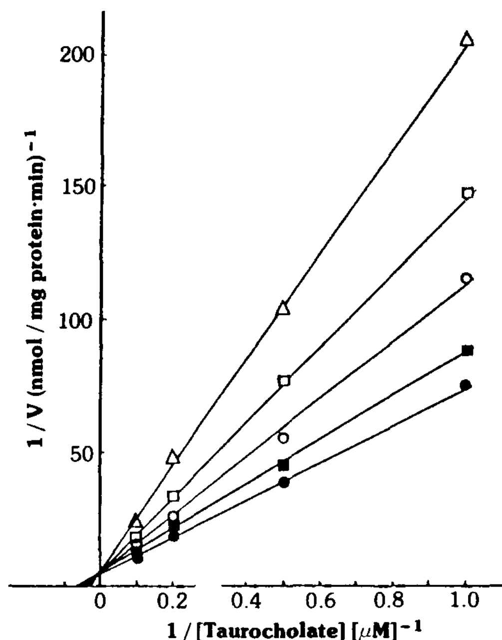

Fig. 4. Lineweaver-Burk plot for the uptake of taurocholate showing inhibition by BHT-acid. After 24 hr culturing, various concentrations of were added to the culture along with taurocholate. Initial rate of uptake was measured as described in the legend to Fig. 3. The plot represents data for various BHT-acid concentrations; without inhibitor (●), 50 μM (■), 100 μM (○), 300 μM (□), 600 μM (△).

trations of BHT-acid. The results were plotted according to Lineweaver and Burk as shown in Fig. 4. BHT-acid competitively inhibited the uptake of taurocholate as demonstrated by the common intercept on the 1/v-axia in the plot. A similar result was obtained when the experimental data was plotted according to Hanes-Woolf in Fig. 5; the family of plots were parallel indicating the competitive inhibition of taurocholate uptake by BHT-acid.

In Fig. 6, the reciprocal velocity was plotted against concentration of BHT-acid according to Dixon. The inhibitor constant, K_i, was calculated from the intercept over the base line of the plot. It amounts to a K_i of 376.8 ± 23.1 μM BHT-acid.

DISCUSSION

A major finding in this study was that BHT and its major metabolite BHT-acid competitively inhibited the uptake of taurocholate into the primary culture of rat hepatocytes. In a previous study, we reported that in treatment of rats with 0.25% BHT for 10 days, increased the bile flow but the concentration and total biliary excretion of bile acids were decreased (Choe *et al.*, 1984). We assumed that the decreased bile acid excretion in BHT-treated rats might be due to an interference of BHT and/or its metaboli-

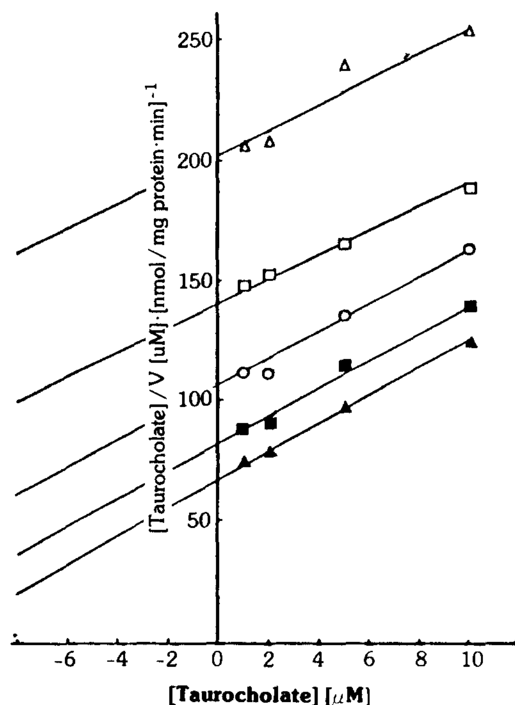

Fig. 5. Hanes-Woolf plot for the inhibition of taurocholate uptake by BHT-acid. The plot represents data for various BHT-acid concentrations: without inhibition (▲), 50μM (●), 100μM ( ), 300μM (□), 700μM (△).

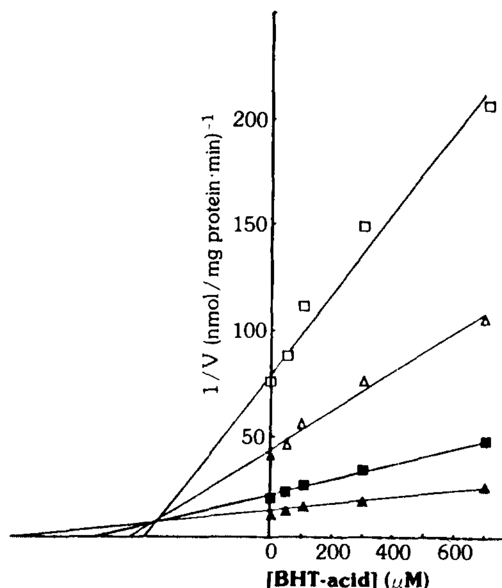

Fig. 6. Dixon plot for the inhibition of taurocholate uptake by BHT-acids. Using the experimental data of Lineweaver-Burk plot, 1/v is plotted against concentrations of BHT-acid at various taurocholate concentrations; 1 μM (□), 2 μM (△), 5 μM (▲), 10 μM (■). Projection of the intersection to the baseline gives a K_1 of 376.7 μM for BHT-acid.

tes on the transport of bile acids into hepatocytes. The results of the current study provide experimental support for this assception.

The apparent K_m of taurocholate uptake obtained in this study was 12.8 ± 2.2 μM. This value is comparable to the kinetic constant found in freshly isolated liver cells (K_m = 19 μM) by Schwarz *et al.* (1976) but much lower than the reported value in primary hepatocytes culture (K_m = 28 ± 10 μM) by Schwarz and Barth (1979). The discrepancy of K_m value might be due to the different culture condition used in each study. The inhibition constant K_i for BHT-acid was 376 μM. The high K_i value indicated lower affinity of BHT-acid to the carrier than taurocholate.

In this study, only the uptake process of taruocholate into hepatocytes was investigated for the effect of BHT and BHT-acid, since preliminary experiments suggested that the secretion process of taurocholate from hepatocytes into bile was not affected by BHT and BHT-acid.

The clinical importance of the inhibition of bile acid uptake by BHT and BHT-acid is not clear. Since bile acid synthesis is regulated by a feed back mechanism, a lowered intrahepatic bile acid concentration releases product inhibition of bile acid formation, thus increasing bile acid synthesis. However, *de novo* synthesis of bile acids may be insignificant, since it restores only a small portion of the bile flow (Salen and Shafer, 1983).

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