Cytotoxicities of Sodium Benzoate in Primary Culture of Hepatocytes from Adult Rat Liver

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OYANAGI, K., KUNIYA, Y., NAGAO, M., TSUCHIYAMA, A. and NAKAO, T. *Cytotoxicities of Sodium Benzoate in Primary Culture of Hepatocytes from Adult Rat Liver*. Tohoku J. exp. Med., 1987, **152** (1), 47-51 — The cytotoxicities of sodium benzoate was studied using primary culture of hepatocytes established from adult rat liver by a collagenase perfusion technique and maintained as a monolayer in serum-free culture medium. The activities of ornithine transcarbamylase (as a marker of mitochondria) and tyrosine aminotransferase (as a marker of cytosol) were clearly suppressed by sodium benzoate at concentration in excess of 500 $\mu\text{g/ml}$. Intracellular protein synthesis and DNA synthesis were also suppressed, and the suppression of DNA synthesis was observed even with a lower concentration of benzoate (100 $\mu\text{g/ml}$). ——— cytotoxicity; sodium benzoate; hyperammonemia; cultured hepatocytes

Since the report by Batshaw et al. (1981), sodium benzoate (BA-Na) therapy has been used on many children with congenital hyperammonemia. At present, 200-500 $\mu\text{g/ml}$ is thought to represent the clinically effective and safe concentration in blood (Green 1981). No report on functional disturbance of the liver due to BA-Na has been reported. An influence of BA-Na on mitochondrial enzymes, however, has been reported (Gatley and Sherratt 1977). In the present study, the hepatic toxicity of BA-Na was evaluated in vitro using primary culture of hepatocytes.

MATERIALS AND METHODS

The primary culture of hepatocytes from adult rats was prepared according the method of Tanaka et al. (1978). The liver of a male Wistar rat of approximately 200 g of body weight was perfused with collagenase in situ to separate the hepatocytes. The cells were seeded in a plastic culture dish at a concentration of 5×10^4 cells/cm² and cultured in Williams medium E containing 5% fetal calf serum, 10^{-9} M dexamethasone and 10^{-9} M insulin. When a monolayer of cell sheet was formed in 3-4 hr after seeding, the medium was changed to serum-free medium containing 10^{-9} M dexamethasone and 5 U/ml of aprotinin. The culture medium was changed daily. On the third day of culture, it was replaced with one of sets of medium containing BA-Na of 7 different concentrations ranging

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0 to 1×10^4 $\mu\text{g/ml}$. In order to evaluate the biochemical, ornithine transcarbamylase (OTC) as a marker of mitochondrial fraction and tyrosine aminotransferase (TAT) as a marker of cytosolic fraction were measured by Schimke's (1962) and Granner's and Tomkins (1970) methods, respectively.

Measurement of DNA synthesis. 48 hr after initiation of the culture, the medium was replaced with Williams medium E containing a range concentration of BA-Na (serum-free, with addition of 10^{-9} M insulin, 10^{-9} M dexamethasone, and 5 U/ml of aprotinin) and culture was continued for another 24 hr.

At the end of 24 hr of culture, [^3H] thymidine (2.5 $\mu\text{Ci/ml}$, 0.3 $\mu\text{Ci/mole}$) was added and the mixture was incubated for 4 hr. The cells were washed twice cold PBS and treated with 10% trichloroacetic acid (TCA) for 2 hr at 4°C , followed by digestion with 0.5 N NaOH at 37°C for 1 hr. After washing twice with 5% TCA, the sample was heated at 90°C for 15 min and the radioactivity of the TCA-soluble fraction was measured using a Beckman liquid scintillation counter.

Assay of intra- and extracellular protein synthesis. After 24 hr of incubation of the cultured liver cells in Williams E containing various concentrations of BA-Na, L-[U- ^{14}C] leucine (1 $\mu\text{Ci/ml}$, 348 $\mu\text{Ci/mole}$) was added and the incubation was continued for 3 hr. The cells were washed with cold PBS and treated with 10% TCA for 2 hr at 4°C . After digestion with 1 N NaOH at 37°C for 1 hr, the cells were washed with 5% TCA twice and dissolved in 0.1 N NaOH. An aliquot was then removed and the radioactivity of this fraction was defined as the ability of protein synthesis. The ability of extracellular (secretory) protein synthesis was measured by collecting the medium with addition of L-[U- ^{14}C] leucine and treating it with 10% TCA, followed by a centrifugation at 3,000 rpm for 15 min. The precipitate was again washed with 5% TCA and dissolved in 0.1 N NaOH. An aliquot was used for the measurement of radioactivity.

RESULTS

Time course changes of OTC and TAT activities. Figs. 1 and 2 show the time course changes of OTC and TAT activities. OTC activity tended to decrease during 3 hr of incubation even in the medium containing 200 $\mu\text{g/ml}$ of BA-Na. At 500 $\mu\text{g/ml}$ of BA-Na, a marked decrease was noted after 24 hr. While TAT activity should have been induced by dexamethasone in the culture medium, it tended to be inhibited from an early period even at low BA-Na concentrations (100–200 $\mu\text{g/ml}$). More pronounced inhibition occurred at 24 hr.

Changes of the ability of DNA synthesis. As shown in Fig. 3, the ability of DNA synthesis decreased slightly after 24 hr even without BA-Na loading. At concentrations of BA-Na higher than 100 $\mu\text{g/ml}$, DNA synthesis decreased to less than 50% of control. At concentrations higher than 2,500 $\mu\text{g/ml}$, DNA synthesis could not be detected.

Ability of intra- and extracellular protein synthesis. The ability of intracellular protein synthesis decreased significantly at 500 $\mu\text{g/ml}$ of BA-Na concentration. Regarding the ability of extracellular (secretory) protein synthesis, no significant difference from control was noted at 200 and 500 $\mu\text{g/ml}$ of BA-Na concentrations (Table 1).

DISCUSSION

BA-Na has been used safely at low dose for short period, as an addition to

Fig. 1. Effects of sodium benzoate to OTC activity in primary culture of hepatocytes. Concentrations of benzoic acid in medium: ○—○, none; ×—×, 100 $\mu\text{g/ml}$; △—△, 200 $\mu\text{g/ml}$; □—□, 500 $\mu\text{g/ml}$; ●—●, 2,500 $\mu\text{g/ml}$; ▲—▲, 5,000 $\mu\text{g/ml}$; ■—■, 10,000 $\mu\text{g/ml}$.

Fig. 2. Effects of sodium benzoate to TAT activity in primary culture of hepatocytes. Concentrations of benzoic acid in medium: ○—○, none; ×—×, 100 $\mu\text{g/ml}$; △—△, 200 $\mu\text{g/ml}$; □—□, 500 $\mu\text{g/ml}$; ●—●, 2,500 $\mu\text{g/ml}$; ▲—▲, 5,000 $\mu\text{g/ml}$; ■—■, 10,000 $\mu\text{g/ml}$.

Fig. 3. Effects of sodium benzoate to DNA synthesis in primary culture of rat hepatocytes.

TABLE 1. *Effects of sodium benzoate in protein synthesis*

Medium	Extracellular protein synthesis (dpm/mg protein per 3 hr $\times 10^4$)	Intracellular protein synthesis (dpm/mg protein per 3 hr $\times 10^4$)
WE	3.21 ± 0.24	1.10 ± 0.13
WE + BA - Na 200 $\mu\text{g/ml}$	2.84 ± 0.36	0.97 ± 0.08
WE + BA - Na 500 $\mu\text{g/ml}$	3.17 ± 0.16	0.27 ± 0.03

Values are means $\pm$ s.d. for 3 experiments. WE, Williams medium E; BA - Na, Sodium benzoate.

food and drugs for antiseptic purpose, without impairing liver functions. Even in the treatment of congenital hyperammonemia requiring a relatively large dose, BA-Na administration is reported to be safe as long as the blood concentration of BA-Na does not exceed 200–500 $\mu\text{g/ml}$ (Green 1981). BA-Na, however, is one of the uncouplers of oxidative phosphorylation in mitochondria. The authors therefore evaluated the cytotoxic action of BA-Na at currently used therapeutic concentrations, using cultured liver cells.

At a concentration of BA-Na in blood of 500 $\mu\text{g/ml}$, which is thought to represent the upper limit of the clinically safe range, enzyme activities, ability of protein synthesis, and ability of DNA synthesis were all decreased, indicating a need for caution in a long term use of high concentrations. Mitochondrial hypofunction, manifested by the decrease of OTC activity, probably interferes

with the therapeutic effect on hyperammonemia. Although no remarkable morphological or functional disturbance was noted after 24 hr, DNA synthesis was markedly inhibited even at the relatively low concentration of 100 μ g/ml. Even a low dose of BA-Na may with chronic administration suppress the liver function in patients with congenital hyperammonemia.

Since this study is an in vitro experiment using rat liver, the results cannot be directly compared with the changes of human liver in vivo. These results suggest, however, that even BA-Na concentrations currently assumed to be safe may cause some damage to the liver cells, calling for a careful reevaluation of the dose of BA-Na to be employed.

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