INDUCTION OF GLUTATHIONE S-TRANSFERASE BY PHENOBARBITAL IN RAT HEPATOCYTE CULTURE

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The in vitro effect of phenobarbital on glutathione S-transferase (GST) activity toward 1-chloro-2,4-dinitrobenzene (CDNB) in rat hepatocyte culture was investigated. Treatment of hepatocytes with a medium containing 6 or 8 mM phenobarbital for 20 h increased the specific activity of GST approx. 140% and 160% of control, respectively. When induction of GST by phenobarbital treatment was observed, the cellular level of phenobarbital determined by HPLC was approx. 10 - 15 $\mu\text{g}/\text{mg}$ of 20000 x g supernatant protein of hepatocytes. Thus, an inductive effect of phenobarbital on GST was observed in vitro as well as in rat liver. It was suggested that a certain level of cellular phenobarbital was necessary for the induction.

KEYWORDS — glutathione S-transferase; rat hepatocytes; phenobarbital; enzyme induction

INTRODUCTION

Induction of rat hepatic glutathione S-transferase (GST, EC 2.5.1.18) has been reported to be caused by the administration of such xenobiotics as barbiturates, 3-methylcholanthrene, polychlorinated biphenyls of benzo-[a]pyrene.¹⁾ Isoenzymes induced in vivo have also been studied quantitatively resulting in the variation of the level.²⁾ We³⁾ have previously reported that the rate of induction of hepatic GST by phenobarbital was greater in male rat than in female, suggesting contribution of hormone(s). Increase in the amount of mRNA^{4,5)} and transcriptional rate of GST gene⁶⁾ have been reported to account for the induction by phenobarbital. However, it is not well elucidated how phenobarbital alters the mRNA level and transcriptional rate. Therefore, more investigation is required to clarify this question. Rat hepatocytes provide a useful model to study metabolism and toxicity of xenobiotics in vitro. This system using hepatocytes is free from such in vivo factors as tissue absorption,

distribution, elimination, nutrition and hormones. In an *in vitro* study using rat hepatocytes, phenobarbital has been shown to induce microsomal cytochrome P-450,⁷⁾ ethoxycoumarin deethylase⁸⁾ and UDP-glucuronyl-transferase.⁹⁾ Recent works reported that not only microsomal enzymes but also such cytosolic enzymes as aldehyde dehydrogenase¹⁰⁾ and 2-carboxy-benzaldehyde reductase¹¹⁾ were induced by the compound. But to date, no report has appeared on the *in vitro* effect of phenobarbital on GST. In the present study, we investigated the effect of phenobarbital on GST in rat hepatocyte culture and the relationship between cellular concentration of phenobarbital and GST activity.

MATERIALS AND METHODS

Chemicals — Collagenase, 1-chloro-2,4-dinitrobenzene (CDNB) and phenobarbital sodium were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); new born calf serum from Whittaker M.A. Bioproducts Ltd. (Maryland, U.S.A.); Eagle's MEM from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); and trypsin inhibitor from Sigma Chemical Co. (St. Louis, MO., U.S.A.). The other reagents used were of analytical grade.

Animals — Male rats of Wistar strain weighing 400 - 500 g were purchased from Sankyo Laboratory Co. (Tokyo, Japan).

Preparation of Isolated Hepatocytes — Hepatocytes were isolated from male rats, under ether anesthesia, in the two-step perfusion procedure essentially according to the method of Seglen.¹²⁾ Crude hepatocytes were washed with MEM medium (pH 7.25) by repeated centrifugation (50 x g, 1 min) to purify parenchymal cells. Viability of the obtained hepatocytes was estimated by the trypan blue exclusion test (>80%). Hepatocytes were suspended in MEM medium and plated in tissue culture dishes (Falcon, ϕ 60 mm) with 10% new born calf serum (2.0×10^6 cells/dish). Eagle's MEM medium supplemented with 2×10^{-3} M L-glutamine, 10^{-9} M dexamethasone and 10^{-9} M insulin was used as an incubation medium. The dishes were incubated at 37°C for 3 h and then the medium was replaced with 4 ml of fresh medium containing phenobarbital and 10% new born calf serum. The incubation was continued at 37°C for 20 h. Phenobarbital sodium was dissolved in the medium to the final concentration of 1 - 10 mM, and it was adjusted to pH 7.25 by hydrochloric acid. After 20 h, the medium was removed, and the cell monolayers were washed twice with chilled Hanks solution (pH 7.2). Hepatocytes were collected in 4 ml of 0.1 M potassium phosphate buffer (pH 7.0) by scraping with a rubber policeman. Cell suspensions thus

obtained were sonicated for 30 s and centrifuged at 20000 x g for 30 min to obtain the supernatant. The activity of GST and concentration of phenobarbital were determined using this supernatant.

Assay of GST—The activity of GST was estimated spectrophotometrically with CDNB as a substrate according to the method of Habig *et al.*¹³⁾ Assay medium comprised 1 mM CDNB, 1 mM reduced glutathione, the enzyme source and 0.1 M potassium phosphate buffer (pH 6.5) in the final volume of 3.0 ml. One unit of GST was defined as the activity of enzyme conjugating 1 μ mole of substrate per min at 25°C.

Analysis of Phenobarbital—Phenobarbital was extracted from the 20000 x g supernatant of hepatocytes with chloroform under acidic condition. It was analyzed by HPLC equipped with Fine pak SIL C18S column (4.6 x 150 mm, Japan Spectroscopic Co., Ltd.) using a mobile phase of methanol: water (1:1).

Determination of Protein—Protein was determined according to the method of Lowry *et al.*¹⁴⁾ with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Activity of glucokinase, glucose-6-phosphatase and cytochrome P-450, and the content of glutathione in hepatocytes have been reported to be rapidly reduced after the start of culture.^{15,16)} Therefore, alteration of GST activity during the incubation period was checked by control dishes. CDNB was used as a substrate to determine overall activity of GST because all of the transferases are active toward it. As shown in Fig. 1, the activity of GST was kept almost constant for at least 20 h after the medium was renewed 3 h after plating. Grant and Hawksworth also reported the maintenance of

Fig.1. Activity of GST in Rat Hepatocytes during Incubation. Hepatocytes were incubated for various periods in control medium without phenobarbital after the medium was renewed 3 h after plating. Each value represents mean $\pm$ S.E. from 3 or 4 dishes.

Fig.2. Effect of Phenobarbital on GST Activity in Rat Hepatocytes. GST activity was determined in 20000 x g supernatant of hepatocytes after treatment with phenobarbital for 20 h. Each value represents mean + S.E. of 4 dishes. Statistical significance as examined by Student's t-test: a), $p < 0.01$; b), $p < 0.001$.

GST activity from 24 h to 72 h in cultured hepatocytes.¹⁷⁾

As shown in Fig. 2, treatment of hepatocytes with a medium containing 6 or 8 mM phenobarbital increased specific activity of GST up to 140% and 160%, respectively. Thus, it was shown that isolated hepatocytes also responded to phenobarbital resulting in an elevation of GST activity as well as in the case of *in vivo* experiments. Treatment of hepatocytes with 10 mM phenobarbital, however, resulted in a decrease in the activity. This concentration seemed to damage hepatocytes, because depression of flattening

Fig.3. Content of Phenobarbital in Rat Hepatocytes.

Content of phenobarbital was determined in 20000 x g supernatant of hepatocytes after treatment with phenobarbital for 20 h. Each value represents mean + S.E. of 4 dishes.

and aggregation of hepatocytes were observed.

Figure 3 shows the content of phenobarbital in 20000 x g supernatant of hepatocytes. The amounts of phenobarbital in hepatocytes linearly increased with the concentration of phenobarbital added to the medium. Phenobarbital was at a level of 10 - 15 μ g/mg protein in hepatocytes treated with 6 to 8 mM phenobarbital, where increase in the GST activity was observed. At least a few consecutive days of phenobarbital administration is necessary to increase GST activity.¹⁸⁾ This delay in the induction was explained as a requirement for a certain amount of the compound in the liver in vivo. It was suggested from Fig. 3 that the induction was related to cellular intake of phenobarbital and a certain amount of phenobarbital was necessary for the induction of GST. A contribution of cytosolic binding protein (Ah receptor) to the induction of aryl hydrocarbon hydroxylase by polycyclic aromatic hydrocarbons has been reported.¹⁹⁾ Such an interaction between hepatocytes and phenobarbital is not yet clear. The isolated hepatocytes may offer a great advantage in the investigation of the mechanism which regulates an induction of GST by xenobiotics. Further studies on qualitative and quantitative characterization of GST isoenzymes induced in vitro and the relationship between cellular concentration of phenobarbital and GST induction are in progress.

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(Received September 13, 1989)