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Effect of phenobarbital on the expression of glutathione *S*-transferase isoenzymes in cultured rat hepatocytes

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Cultured adult rat hepatocytes were treated daily with 3.2 mM phenobarbital (PB) in order to study its effect on the expression of cytosolic glutathione *S*-transferase isoenzymes. Glutathione *S*-transferase (GST) activities, using 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates, were increased when PB was present in the culture medium. After purification and separation of GST on glutathione Sepharose 6 B and reversed-phase HPLC, respectively, it was observed in vitro that PB caused an increase in the relative amounts of subunits 1, 3 and 7 compared to subunits 2 and 4. Using Northern blot technique, elevated levels of GST subunit 1/2 and 7 mRNA were measured, after addition of PB to the cultures.

Glutathione *S*-transferase; Phenobarbital; (Rat hepatocyte)

1. INTRODUCTION

During the last few years, cultured adult rat hepatocytes have been increasingly used as an alternative model for in vivo pharmacotoxicological studies. Especially the maintenance of phase I biotransformation reactions has been widely investigated in hepatocyte cultures, while less attention has been paid to phase II biotransformation enzymes. Although glutathione *S*-transferases (EC 2.5.1.18) play an important role in intracellular detoxification [1,2]. Glutathione *S*-transferases (GST) are a family of multifunctional isoenzymes which catalyze the conjugation of a wide variety of hydrophobic electrophiles with glutathione [3,4]. In addition, they also have the capacity to bind lipophilic non-substrate ligands and to catalyze some key steps in the biosynthesis of prostaglandins and leukotriene C_4 [2]. Up to now, at least eight GST subunits have been identified and characterized. On the basis of their primary structure, they belong to at least 3 gene families, namely the α family (subunits 1, 2 and 8), the μ family (subunits 3, 4 and 6) and the π family (subunit 7); subunit 5 has not yet been classified [5].

Each rat tissue has its own characteristic isoenzyme composition. Adult rat liver is the tissue in which GST isoenzymes are the most abundant. The major subunits expressed are 1, 2, 3 and 4. The isoenzyme profile changes during development and may be modulated by endogenous hormones and exposure to compounds such as enzyme inducers and carcinogens [6]. In vivo, the effect of PB on GST has been studied in detail [7-9]: this barbiturate is an inducer of GST with a subunit specific effect. The transcription rate of especially subunits 1 and 3 is increased, whereas subunit 2 mRNA level is barely affected [10]. These changes on transcription by PB treatment in vivo result in an increase in the relative concentrations of sub-

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units 1 and 3 when compared with those of subunits 2 and 4 [7].

In previous works [11–13], it has been shown that changes occur in the GST enzymic activity, GST subunit composition and corresponding mRNA levels, including 'de novo' expression of subunit 7, when rat hepatocytes are seeded in culture. Subunit 7 is known to be absent in normal adult rat hepatocytes, but is a part of the GST mRNA of fetal rat hepatocytes [13,21]. However, the effect of PB on the GST subunit composition of cultured rat hepatocytes is unknown in the existing literature. It is also not known if its effect in vitro is subunit specific as observed in vivo. Therefore, finding the answers to these questions was the aim of this study.

After PB treatment (3.2 mM) of conventionally and co-cultured rat hepatocytes, GST activities, using 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates, have been measured. Northern blot technique and reversed-phase HPLC have been used in order to study the effect of PB on the GST mRNA levels of subunits 1/2 and 7 and the GST subunit composition, respectively.

2. MATERIALS AND METHODS

2.1. *Materials*

Culture media and fetal calf serum were obtained from Gibco, Scotland. Collagenase, bovine albumin, bovine insulin and epoxy activated-Sepharose 6B were products from Sigma, USA. The nick-translation kit, nitrocellulose hybond C, [α-^{32}P]dCTP and [^{35}S]methionine were obtained from Amersham, England. All other compounds were readily available commercial products and used without further purification.

2.2. *Cell isolation and culture*

Adult rat hepatocytes were isolated from 2-month-old Sprague-Dawley rats as described by Guguen et al. [17]. They were seeded in culture and co-culture with rat liver epithelial cells, as described previously [14]. The culture medium consisted of 75% minimal essential medium and 25% medium 199, containing 200 μg/ml bovine serum albumin, 10 μg/ml bovine insulin and 10% fetal calf serum (standard medium, St). Four hours after cell seeding, the medium was renewed and every 24 h thereafter. The medium was supplemented with 7×10^{-5} M hydrocortisone hemisuccinate for conventional culture or with 7×10^{-6} M for co-culture respectively. PB (3.2 mM) was added to the culture medium (St + PB) 4 hours after cell seeding for conventional culture and in co-culture when confluency was reached between parenchymal and epithelial cells (day 1). Thereafter it was added every day by renewing the medium. At the end of the culture period, cells were stored at -80°C until analysis.

2.3. *GST activity measurements*

GST activity was measured as previously described [11] using CDNB and DCNB as substrates. Enzyme activities were calculated as units per milligram of protein. A unit activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of product/min at 30°C [15]. Protein concentrations were measured using the Bio-Rad assay, with bovine serum albumin as a standard.

2.4. *Purification of GST isoenzymes and HPLC analysis*

GST isoenzymes were purified as described previously [12]. Separation of GST subunits was carried out by HPLC using a 10×0.8 cm μBondapak C-18 reversed-phase column in a Z module (Millipore Waters, Milford MA, USA). The solvents

Table 1

Effect of phenobarbital on the glutathione *S*-transferase activity of conventional cultured rat hepatocytes

Days of culture	CDNB[a]		DCNB[a]	
	St	St + PB	St	St + PB
2	0.87 ± 0.18	1.06 ± 0.06	0.019 ± 0.007	0.035 ± 0.005[b]
4	0.65 ± 0.05	1.48 ± 0.08[b]	0.049 ± 0.018	0.062 ± 0.014
6	0.56 ± 0.19	1.54 ± 0.29[b]	0.034 ± 0.012	0.077 ± 0.019[b]

[a] 1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were used as substrates

[b] Indicates values that are significantly ($p < 0.001$, Student's *t*-test) higher than those from non-phenobarbital-treated cells

Results are expressed in U/mg cell homogenate proteins. GST activities of freshly isolated hepatocytes: 0.90 ± 0.13 and 0.036 ± 0.013 U/mg protein towards CDNB and DCNB, respectively. Each value represents the mean ± SE of three independent experiments in triplicate

Table 2

Effect of phenobarbital on the glutathione *S*-transferase activity of co-cultured rat hepatocytes

Days of culture	CDNB[a]		DCNB[a]	
	St	St + PB	St	St + PB
4	0.66 ± 0.05	1.16 ± 0.14[b]	0.021 ± 0.003	0.034 ± 0.005[b]
8	0.86 ± 0.08	1.57 ± 0.10[b]	0.043 ± 0.009	0.079 ± 0.011[b]
16	0.94 ± 0.10	1.67 ± 0.13[b]	0.059 ± 0.008	0.069 ± 0.010[c]

[a] 1-Chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) were used as substrates

[b] Indicates values that are significantly ($p < 0.001$, Student's *t*-test) higher than those from non-phenobarbital-treated cells

[c] Indicates values that are significantly ($p < 0.05$, Student's *t*-test) higher than those from non-phenobarbital-treated cells

Results are expressed in U/mg cell homogenate proteins. GST activities of freshly isolated hepatocytes: 0.90 ± 0.13 and 0.036 ± 0.013 U/mg protein towards CDNB and DCNB, respectively. Each value represents the mean ± SE of three independent experiments in triplicate

were water (A) and acetonitrile (B) each containing 0.06% trifluoroacetic acid. The samples were injected at 35% B. During a run, a linear gradient was used from 35% to 53% B over 60 min with a flow rate of 1.5 ml/min. Detection was carried out at 214 nm. GST subunits, separated by HPLC, were identified by comparing their retention times with those from purified GST. Quantitative data were obtained by the recoveries of the respective subunits from HPLC and their molar extinction coefficients at 214 nm, as given by Ostlund-Farrants et al. [16].

2.5. *Northern blot analysis of GST subunits 1/2 and 7*

Total RNA was isolated by the procedure of Chirgwin et al. [18]. 20 μg of RNA isolated from the hepatocytes was subjected to electrophoresis in a denaturing formaldehyde-agarose gel and transferred onto nitrocellulose sheets. These sheets were prehybridized and hybridized with nick-translated probes (400–600 10^6 cpm/μg DNA). pGSTr 155 [19] is complementary to mRNA encoding subunit 1, but also cross-hybridizes to subunit 2 mRNA and pGSTr 7 [20] is complementary to subunit 7 mRNA. After hybridization, filters were washed, dried and autoradiographed at −80°C.

3. RESULTS

3.1. *Effects of PB on GST activities*

GST activities of conventionally and co-cultured adult rat hepatocytes are summarized in tables 1 and 2, respectively. In conventional culture, PB addition increased GST activity toward CDNB 2- to 3-fold after 4 and 6 days, respectively. Using DCNB as substrate, a maximal 2-fold increase was noticed after 6 days. In co-culture, PB addition to the cells increased GST activity toward CDNB at an average of 80% over a 16 day culture period. Toward DCNB, a maximal increase of 83% on day

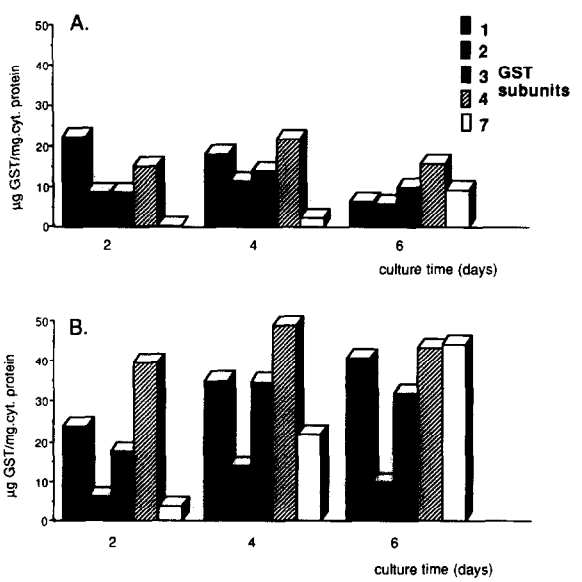

Fig.1. GST subunit composition of control (A) and PB-treated (B) conventionally cultured rat hepatocytes. GST subunits were purified and separated by HPLC technique. Cells were harvested on day 2, 4 and 6 of culture. Results are expressed in μg GST subunit/mg cytosolic protein and the GST subunit profile on each culture time is representative for a mixture of 6 petri dishes containing each 10×10^6 hepatocytes.

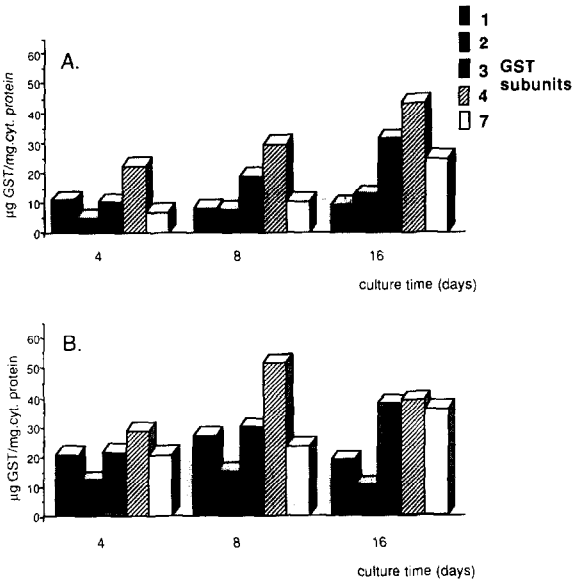

Fig.2. GST subunit composition of control (A) and PB-treated (B) co-cultured rat hepatocytes. GST subunits were purified and separated by HPLC technique. Cells were harvested on day 4, 8 and 16 of culture. Results are expressed in μg GST subunit/mg cytosolic protein and the GST subunit profile on each culture time is representative for a mixture of 6 petri dishes containing each 10×10^6 hepatocytes.

8 was observed. GST activity of rat liver epithelial cells (0.17 U/mg protein toward CDNB) was not affected by PB treatment.

3.2. *Effects of PB on the GST subunit composition*

GST isoenzymes were purified by use of affinity chromatography. The purified transferases were then separated into subunits by reversed-phase HPLC. Freshly isolated rat hepatocytes, in which subunit 7 is absent, contain 39.9 μg, 14.8 μg, 13.5 μg and 38.8 μg of subunits 1, 2, 3 and 4, respectively, per mg cytosolic protein. In conventionally cultured non-PB-treated rat hepatocytes (fig.1), a constant decrease of subunit 1 and an increase of subunit 7 were observed during the 6 days of culture. When treated daily with PB, an increase was noticed for subunits 1, 3 and 7, while the amounts of subunits 2 and 4 remained relatively stable over the total culture period. In co-culture (fig.2), PB treatment had a less pronounced increasing effect on subunits 1, 3 and 7 compared to its effect in conventional culture. On days 4 and 8, but not on day 16, the amounts of subunits 2 and

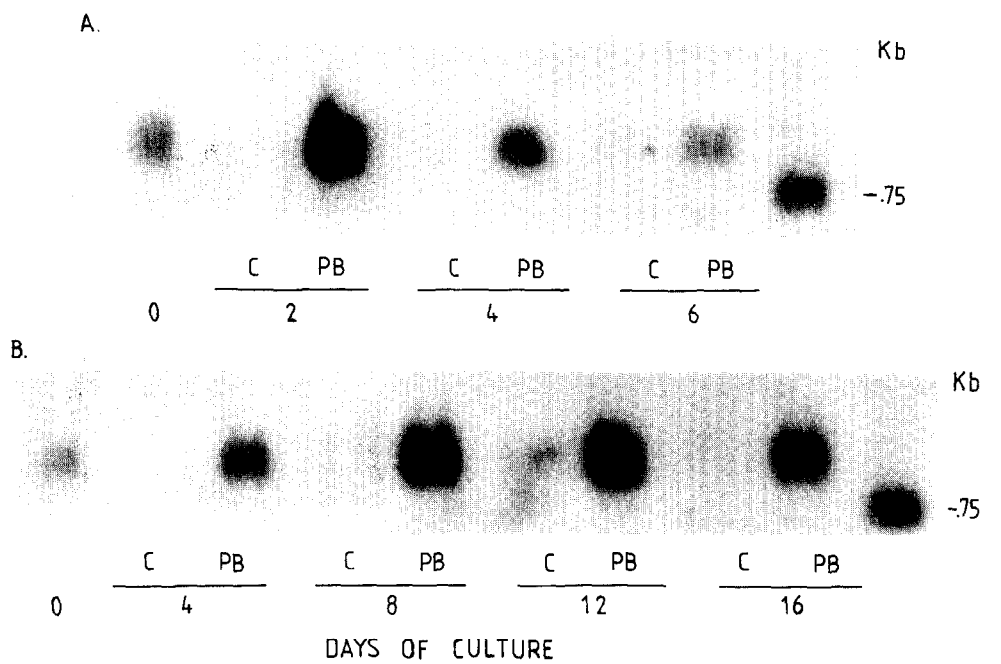

Fig.3. Effect of PB on the GST 1 and 2 mRNA expression in conventionally (A) and co-cultured (B) rat hepatocytes. In conventional culture RNA was isolated from standard medium cultured hepatocytes (control: C) and PB-treated cells (PB) after 2, 4 and 6 days. In co-culture, RNA was isolated after 4, 8, 12 and 16 days of culture, control (C) and PB-treated (PB). 0 days, freshly isolated hepatocytes.

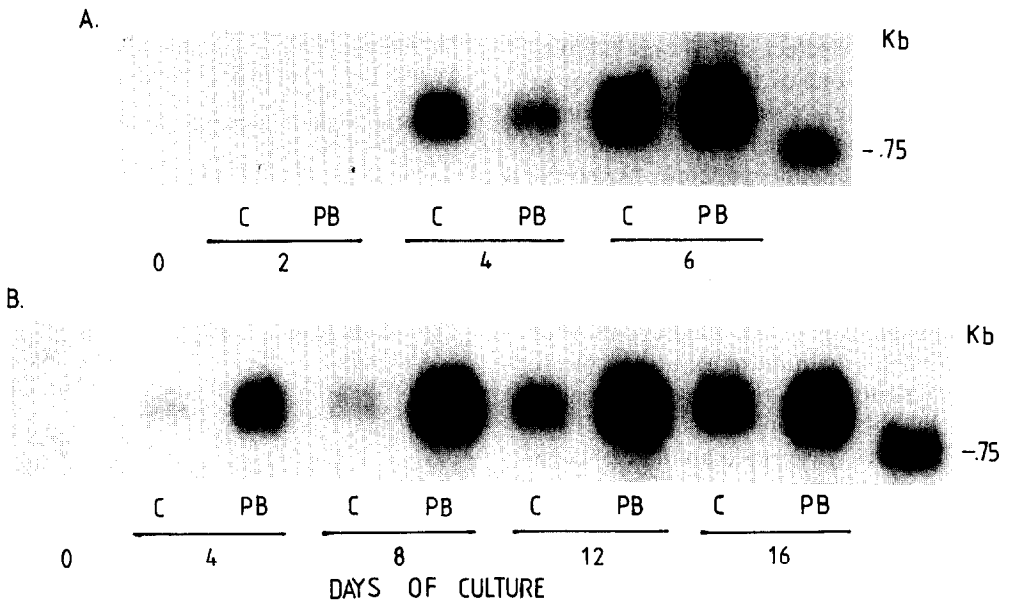

Fig.4. Effect of PB on the GST 7 mRNA expression in conventionally (A) and co-cultured (B) rat hepatocytes. In conventional culture RNA was isolated from standard medium cultured hepatocytes (control: C) and PB treated cells (PB) after 2, 4 and 6 days. In co-culture, RNA was isolated after 4, 8, 12 and 16 days of culture, control (C) and PB-treated (PB). 0 days, freshly isolated hepatocytes.

4 were greater in PB-treated hepatocytes than in St medium cultured cells (control).

3.3. *Effect of PB on the GST mRNA levels 1/2 and 7*

Northern blots reflect changes on the mRNA levels encoding GST subunits 1 and 2 (fig.3) and GST subunit 7 (fig.4). It is noted that in both culture systems the GST 1/2 mRNA level is not maintained for control cultured hepatocytes. PB treatment in conventional and in co-culture increased and maintained the 1/2 mRNA amount at a level comparable or higher than observed for freshly isolated hepatocytes. In co-culture, but not in conventional culture, PB addition to the cells increased subunit 7 mRNA level over the 16 day culture period. The GST mRNA level of rat liver epithelial cells, which contain subunit 7 but no 1/2 mRNA, was not affected by PB addition (results not shown).

4. DISCUSSION

The results of this present study show that treatment of cultured adult rat hepatocytes with 3.2 mM PB increases the level of hepatic cytosolic glutathione *S*-transferases. The increase of GST activity by PB treatment, toward CDNB and DCNB depends on the culture conditions used, resulting in a more important increase of GST activity in conventional culture than in co-culture, when the daily basal values of both culture systems are considered. In earlier studies [11,13], it has also been observed that the effect of additional medium factors, such as dimethylsulphoxide, is less pronounced when rat liver epithelial cells are added to the hepatocyte cultures. This could probably be explained by the fact that co-cultured hepatocytes maintain their differentiated state better than the conventional cultured cells and thus retain higher daily basal values for GST activities than conventional cultures do. Indeed, compared to the values of freshly isolated hepatocytes, the induction by PB in co-culture is as important as in conventional culture toward both substrates. Since only limited information concerning the in vitro effect of phenobarbital on the GST subunit pattern can be obtained by measuring GST activities toward various substrates, more specific techniques have been chosen to study the effect of PB on the expression of GST subunits in cultured rat hepatocytes.

Using a HPLC separation technique, it was shown that in vitro the effect of PB on GST proteins is not specific to one of the three, α, μ or π, classes, but varies for each subunit. This strongly suggests that, according to the in vivo situation [7], each subunit is regulated independently. When the HPLC results are expressed as percent of the total amount of GST protein, it is observed that especially the amounts of subunits 1 and 7, and slightly of subunit 3 are increased, while those of subunits 2 and 4 are decreased. Incorporation of [^{35}S]methionine in a methionine omitted culture medium and separation of the purified labelled GST proteins by gel electrophoresis showed that the increase of subunit 1 is due to an increased 'de novo' synthesis of this subunit by PB treatment (unpublished results). The mRNA levels coding for the GST proteins 1 and 2 are strongly elevated in both culture systems: up to 10- and 7-times higher after 2 days of conventional and 12 days of co-culture, respectively. The increased GST 1/2 mRNA level, by PB treatment in both culture systems, is not completely translated since the increased protein amounts of subunits 1 and 2 never exceed the increases in mRNA encoding this subunit. In contrast, it is shown that in the case of subunit 7, the increase in protein amount exceeds the elevated corresponding mRNA level, when PB-treated cells are compared to control ones. This could mean that differences in translational efficiency and turnover of protein and mRNA may exist in vitro for each GST subunit, indicating the importance of studying mRNA as well as protein levels for the evaluation of any induction of glutathione *S*-transferase isoenzymes.

From this in vitro study, it is clear that as in the in vivo situation [7], PB exerts a subunit specific effect. The total effect of this barbiturate in vitro on the GST proteins, results in a GST profile which resembles more the one found in vivo or in freshly isolated hepatocytes, than that observed for hepatocytes cultured in the absence of PB. One exception is the appearance and inducibility of subunit 7. Further studies are needed to investigate if the increased GST 1,2 and 7 mRNA levels obtained after PB treatment were caused by an elevation of the transcription rate or by a stabilisation of mRNA.

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