Constitutive and inducible profile of glutathione S-transferase subunits in biliary epithelial cells and hepatocytes isolated from rat liver

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The constitutive and inducible cytosolic glutathione S-transferase (EC 2.5.1.18) subunit compositions of parenchymal cells (hepatocytes) and biliary epithelial cells (BEC) from rat liver have been quantitatively analysed using reverse-phase h.p.l.c. Hepatocytes, analysed in the absence of non-parenchymal cells, expressed constitutively the following subunits, in order of their concentration: 3, 4, 2, 1a, 1b, 8, 6 and 10. BEC express constitutively only four of the GST subunits expressed by hepatocytes and these are, in order of their concentration: subunits 2, 7, 4 and 3. Notable differences from hepatocytes are that BEC completely lack the Alpha-class subunits 1a and 1b that are major subunits in hepatocytes, Mu-class subunits make up a very low proportion of the total, and the Pi-class subunit 7 is a major subunit in BEC, whereas it is essentially absent from hepatocytes. For the first

time, the effects of the inducing agents phenobarbitone (PB), β -naphthoflavone (β -NF) and ethoxyquin (EQ) have been characterized in a comprehensive and quantitative manner in both cell types. PB, β -NF and EQ increased total GST protein in hepatocytes by approx. 2-fold, 3-fold and 4-fold respectively. Subunits significantly induced in hepatocytes were (in order of fold-induction): by PB, 1b > 8 > 3 > 2 > 4; by β -NF, 1b > 8 > 2 > 3 > 4; and by EQ, 7 > 1b > 10 > 8 > 3 > 2 > 1a > 4. In BEC, neither PB nor β -NF had significant effects on the total amount of GST protein, although PB did significantly induce subunit 3 at the expense of other subunits. EQ increased total GST protein nearly 5-fold in BEC, subunits 7 and 3 being induced dramatically above constitutive levels.

INTRODUCTION

Mammalian liver is known to play a major role in the metabolism and disposition of xenobiotics as well as of endogenous compounds. The liver consists of a considerable number of different cell types including hepatocytes (parenchymal cells), endothelial cells, Kupffer cells, fat-storing cells and biliary epithelial cells (BEC), all of which could potentially contribute to the overall pattern of metabolism. Several investigators, by taking advantage of immunohistochemical techniques and especially by using isolation procedures for the single cell types, have begun a direct analysis of the cellular distribution of the phase I and phase II drug-metabolizing enzymes and the response of the different cell types to compounds that induce the activities of these enzymes in whole liver. The first results obtained by these approaches in recent years indicate that metabolism of xenobiotics is certainly not restricted only to parenchymal liver cells (Redick et al., 1982; Hiley et al., 1988; Rich and Lodola, 1989; Steinberg et al., 1987, 1989, 1990).

BEC are small cells of epithelial origin that line intrahepatic bile ductules and ducts in mammalian liver and represent only a minority of the total cell population of the organ (Schaffner and Popper, 1961). However, they have attracted the attention of several investigators because of their unique phenotypic profile (Baptista et al., 1983; Desmet, 1987; Tavoloni, 1987; Sirica et al., 1990; Parola et al. 1991). They have attracted attention particularly because of the phenomenon of ductular proliferation during certain examples of toxic liver injury and during hepatocarcinogenesis. The latter phenomenon and the similarities in phenotype have led to the hypothesis that BEC are directly

related to, and possibly the precursors of, the so-called 'oval cells' that appear during hepatocarcinogenesis; this contentious issue is reviewed fully by Sirica et al. (1990). A specific similarity between the two cell types is a resistance to certain xenobiotics that are toxic to hepatocytes. Previous studies have provided evidence that both normal BEC (Parola et al., 1988, 1989, 1990a; Schrenk et al., 1991) and also hyperplastic BEC (Mathis et al., 1989; Sirica et al., 1990) lack cytochrome P-450 and related phase I xenobiotic-metabolizing activities. This characteristic may be relevant in explaining BEC resistance to some toxic compounds, such as carbon tetrachloride and ethoxycoumarin, that need to be metabolized in order to damage and kill cells (Parola et al., 1989, 1990a, 1990b, 1991; Sirica et al., 1990). Very little information is at present available concerning enzymes related to phase II metabolism and, in particular, to glutathione S-transferases (GSTs) in these cells.

Cytosolic GSTs (EC 2.5.1.18) are a family of multifunctional enzymes that catalyse the nucleophilic attack of GSH on a wide variety of electrophilic compounds, and may function as binding proteins for hydrophobic molecules such as bilirubin, haem and polycyclic hydrocarbons (Mannervik et al., 1985, 1990; Ketterer and Coles, 1991). The cytosolic GSTs are homo- or heterodimers of 12 different subunits with molecular masses between 23000 Da and 30000 Da and have been classified into four gene families Alpha, Mu, Pi and Theta (Mannervik et al., 1985; Meyer et al., 1991). In adult male rat liver cytosol the major Alpha-class subunits are 1a, 1b, and 2 and the minor Alpha-class subunits are 8 and 10, the latter of which appears to be closely related to subunit 2 (discussed later). The major Mu-class subunits are 3 and 4 and the minor Mu-class subunits are 6 and

Abbreviations used: BEC, biliary epithelial cells; PB, phenobarbitone; β -NF, β -naphthoflavone; EQ, ethoxyquin; GST, glutathione S-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 3,4-dichloronitrobenzene; GGT, γ -glutamyltransferase.

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|| Sadly, Professor Trevor Slater died during the course of these studies (15 April 1992).

11, the latter of which is relatively labile (Kispert et al., 1989) and not considered in this study. The only Pi-class isoenzyme, a homodimer of subunit 7, is not found in adult rat hepatocytes but is expressed in foetal liver, in hepatomas and, as shown by immunohistochemical techniques, in BEC of the adult liver. Until recently, only immunohistochemical studies had addressed the question of which GSTs are expressed in BEC under either normal or pathological conditions (Redick et al., 1982; Hayes et al., 1987; Hiley et al., 1988). In recent years, preliminary studies performed on BEC isolated from normal liver (Parola et al., 1990a) or on hyperplastic BEC isolated from the liver of bile-duct-ligated animals (Mathis et al., 1989) have shown the existence of detectable GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) and 3,4-dichloronitrobenzene (DCNB) but have given no details of the particular GST subunits involved. The principle aim of the present study was to examine in detail both the constitutive and inducible isoenzyme profiles of GSTs in preparations of rat liver BEC, and to compare them with the profiles exhibited by the parenchymal cells.

A secondary aim was to produce up-to-date quantitative information on the expression of GST subunits in the hepatocytes after treatment with certain inducing agents. Many of the published studies of induction were carried out before the techniques to measure the full range of GST subunits had been developed. Early studies described only changes in activity and some later ones described changes in isoenzyme expression only in terms of class and/or in semiquantitative terms. As the individual subunits of the dimeric isoenzymes possess independent activities, and since no truly specific substrates exist, activity measurements do not provide information on the subunit composition of cells. Moreover, the diversity of species, strains and inducers used previously presents a confusing and incomplete picture. We therefore aimed to obtain a more complete picture of the effects of selected inducing agents [phenobarbitone (PB), β -naphthoflavone (β -NF) and ethoxyquin (EQ)] on the expression of a full range of GST subunits in a quantitative fashion in a single strain of rat (Wistar). We have analysed the cytosol of isolated hepatocytes, rather than whole liver, to obviate possible distortion of the results by GST subunits present in the various non-parenchymal cell types and to allow a direct comparison with the BEC for the first time. Moreover, we have quantified the various GST subunits using h.p.l.c., an approach that is more informative as regards GST-subunit expression than either activity measurements or immunohistochemistry.

MATERIALS AND METHODS

Animals and pretreatments

Male Wistar rats (200–250 g body wt.) were purchased from Nossan (Correnzana, Italy). The animals were housed one per cage and allowed free access to water and standard pelleted food (diet no. 48, Fratelli Piccioni, Brescia, Italy). PB (100 mg/kg body wt.) and β -NF (75 mg/kg body wt.) in corn oil were administered intraperitoneally for three consecutive days before the animals were used. EQ (6-ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline) was incorporated into the diet at a final concentration of 0.5% (w/w) and was stored at 5 °C. Animals were acclimatized to the control diet for a week and then fed on the EQ-supplemented diet for 10 days before cell isolation.

Isolation of hepatocytes and BEC and preparation of cytosolic fractions

Hepatocytes were isolated using the *in situ* collagenase perfusion technique described by Poli et al. (1981), followed by

centrifugation on a Percoll gradient (Albano et al., 1991) producing a cell suspension free of non-parenchymal cells within 1 h. The normal yield of cells at this stage is $2\text{--}3 \times 10^8$ cells having a viability of greater than 90%.

BEC were isolated from the liver of control, as well as induced, animals according to the procedure described in detail previously (Parola et al., 1988, 1990a, 1991). By means of this procedure we obtained a final cell suspension of $5\text{--}15 \times 10^6$ cells from each rat liver within 5–6 h. The cell viability was always in the range of 90–95% (as evaluated by means of the Trypan Blue exclusion test). BEC were routinely identified by means of the histochemical staining for γ -glutamyltransferase (GGT) to which 80–90% of cells of the final cell suspension were usually positive. GTT-negative cells were identified mainly as Kupffer cells and endothelial cells by morphological criteria; other cells, like fat-storing cells and lymphocytes, were only occasionally seen as very minor contaminants (no more than 1% of the final cell suspension). Contamination by liver parenchymal cells was always absent due to the specifically designed Pronase step in the procedure which kills hepatocytes (Parola et al., 1990a, 1991).

In order to obtain cytosolic fractions the final cell suspensions were washed once in 10 mM potassium phosphate buffer, pH 7.0, made to 0.15 M with respect to KCl (KCl/phosphate buffer), then resuspended in a known volume of the same buffer and sonicated with an MSE sonicator as already described (Parola et al., 1990a). The cell homogenate obtained in this way was then centrifuged at 100 000 g for 60 min by using a Beckman 50.3 rotor (r_{av} , 6.42 cm) that was operated at 4 °C in a Beckman model L8-55 ultracentrifuge. Cytosolic fractions were immediately frozen in liquid nitrogen and then stored at -80 °C until GST analysis.

Analysis of GST subunits

GST subunits were quantified by GSH-agarose affinity chromatography plus reverse-phase h.p.l.c. as described previously (Meyer et al., 1989) with the following modifications. For improved recovery of GST subunits bound to GSH-agarose, the elution buffer used was 5 mM S-hexyl GSH/5 mM GSH/0.1 M Tris/NaOH, pH 9.6; and for improved resolution during reverse-phase h.p.l.c. a column of 5 μ m particle size was used at a flow rate of 1 ml/min.

RESULTS

The GST subunits described below refer only to those retained by the GSH-agarose column. Previous experience has shown us that for control hepatocytes and BEC the proportion of total cytosolic GST activity (towards CDNB) failing to bind to GSH-agarose is in the range 0–12%, and in liver samples induced with PB is in the range 10–20%. The bulk of the extra unbound activity in the induced liver is due to subunits 1b and 2 (D. J. Meyer, unpublished work).

Tables 1 and 2 show the constitutive and inducible GST-subunit composition of cytosolic fractions obtained from BEC preparations isolated from the liver of control rats and from PB-, β -NF- and EQ-pretreated animals. In Table 1 results are shown as the means of cytosolic levels of individual GST subunits; in Table 2 the results are expressed as percentages of each subunit relative to total levels of GST and the subtotals of each class of subunit (Alpha, Mu and Pi) are shown. Table 3 shows corresponding results for isolated hepatocytes from control and treated rats and again the class distribution is displayed in Table 2.

Table 1 GST subunit composition of BEC isolated from untreated, PB-treated, β -NF-treated and EQ-treated rats

Results are expressed as means ($\mu\text{g}/\text{mg}$ of protein) $\pm$ S.D., with the fold increase relative to control levels indicated. Abbreviation: n.d., not detectable. Values in parentheses indicate number of experiments. Significantly different from control levels (Student's *t*-test): ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

Subunit	Control	PB-treated cells		β -NF-treated cells		EQ-treated cells	
	Content ($\mu\text{g}/\text{mg}$ of protein)	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase
3	0.36 $\pm$ 0.45 (6)	1.35 $\pm$ 0.67* (5)	3.8	0.96 $\pm$ 0.52 (4)	2.7	9.17 $\pm$ 4.65** (4)	25.5
4	0.72 $\pm$ 0.51 (6)	0.78 $\pm$ 0.33 (5)	1.1	0.95 $\pm$ 0.70 (4)	1.8	0.78 $\pm$ 0.90 (4)	1.1
7	2.25 $\pm$ 0.48 (6)	1.42 $\pm$ 0.31** (5)	0.6	2.23 $\pm$ 0.88 (4)	1.0	17.83 $\pm$ 3.32*** (4)	7.9
2	3.90 $\pm$ 0.58 (6)	2.14 $\pm$ 1.04** (5)	0.6	0.99 $\pm$ 0.80 (3)	0.3	8.65 $\pm$ 4.85 (4)	2.2
10	n.d. (3)	0.49 $\pm$ 0.17 (5)		0.18 $\pm$ 0.13 (4)		n.d.	
6	n.d. (5)	0.26 $\pm$ 0.22 (4)		0.04 $\pm$ 0.07 (4)		n.d.	
1a	n.d. (5)	0.10 $\pm$ 0.22 (4)		0.12 $\pm$ 0.13 (4)		n.d.	
1b	n.d. (5)	0.26 $\pm$ 0.35 (4)		0.13 $\pm$ 0.15 (4)		n.d.	
8	n.d. (5)	0.80 $\pm$ 0.51 (4)		0.61 $\pm$ 0.24 (4)		n.d.	
Total	7.23	7.65	1.1	6.21	0.8	36.43	5.0

Table 2 Effect of inducing agents on the class distribution of GST subunits in bile-duct cells (BEC) and hepatocytes (HEP)

Results are expressed as percentages of total GST protein. Alternative subunit nomenclature in parentheses for reference.

Subunit	Total GST protein (%)							
	Control		PB-treated		β -NF-treated		EQ-treated	
	HEP	BEC	HEP	BEC	HEP	BEC	HEP	BEC
Alpha-class								
1a (Y _{a1})	10.0	0	6.4	1.3	6.1	1.6	7.5	0
1b (Y ₂)	5.9	0	13.2	3.9	13.6	1.6	17.6	0
2 (Y _{C1})	21.3	53.9	19.1	28.0	21.9	15.9	15.9	23.7
8 (Y _k)	2.5	0	3.5	10.3	4.1	9.8	3.1	0
10 (Y _{C2})	1.8	0	2.0	6.6	1.8	3.3	2.7	0
Alpha subtotal	41.5	53.9	44.2	50.0	47.5	32.7	46.8	23.6
Mu-class								
3 (Y _{b1})	29.2	5.0	32.1	17.7	26.6	15.5	32.4	25.2
4 (Y _{b2})	27.2	10.0	22.1	10.2	24.0	15.3	16.3	2.1
6 (Y _{n1})	1.9	0	1.3	3.9	1.7	0	0.8	0
Mu subtotal	58.3	15.0	55.5	31.6	52.3	31.2	49.5	28.0
Pi-class								
7 (Y _f)	0.2	31.1	0.3	18.6	0.3	35.9	3.8	48.9

Table 3 GST subunit composition of hepatocytes isolated from untreated, PB-treated, β -NF-treated and EQ-treated rats

Results are expressed as means ($\mu\text{g}/\text{mg}$ of protein) $\pm$ S.D., with the fold increase of induced levels relative to control levels indicated. Values in parentheses indicate number of experiments. Significantly different from control levels (Student's *t*-test): ****P* < 0.001; ***P* < 0.01; **P* < 0.05.

Subunit	Control	PB-treated cells		β -NF-treated cells		EQ-treated cells	
	Content ($\mu\text{g}/\text{mg}$ of protein)	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase	Content ($\mu\text{g}/\text{mg}$ of protein)	Fold increase
3	3.62 $\pm$ 1.29 (6)	7.80 $\pm$ 2.26** (5)	2.2	9.73 $\pm$ 3.01** (4)	2.7	14.7 $\pm$ 1.8*** (4)	4.06
4	3.37 $\pm$ 0.28 (6)	5.37 $\pm$ 1.16** (5)	1.6	8.76 $\pm$ 3.32* (4)	2.6	7.4 $\pm$ 1.2*** (4)	2.19
7	0.02 $\pm$ 0.03 (6)	0.06 $\pm$ 0.06 (5)	3.0	0.12 $\pm$ 0.11 (4)	6.0	1.7 $\pm$ 1.2* (4)	85.0
2	2.64 $\pm$ 0.88 (6)	4.65 $\pm$ 1.29* (5)	1.8	8.01 $\pm$ 3.36* (4)	3.0	7.2 $\pm$ 1.0*** (4)	2.7
10	0.22 $\pm$ 0.12 (4)	0.48 $\pm$ 0.38 (5)	2.2	0.64 $\pm$ 0.49 (4)	2.9	1.2 $\pm$ 0.1*** (4)	5.5
6	0.23 $\pm$ 0.18 (7)	0.31 $\pm$ 0.11 (5)	1.4	0.61 $\pm$ 0.32 (4)	2.7	0.35 $\pm$ 0.08 (4)	1.5
1a	1.24 $\pm$ 0.37 (6)	1.56 $\pm$ 0.66 (5)	1.3	2.23 $\pm$ 0.99 (4)	1.8	3.4 $\pm$ 0.6*** (4)	2.7
1b	0.73 $\pm$ 0.31 (6)	3.20 $\pm$ 1.11*** (5)	4.4	4.98 $\pm$ 2.01** (4)	6.8	8.0 $\pm$ 1.0*** (4)	11.0
8	0.31 $\pm$ 0.12 (6)	0.86 $\pm$ 0.28** (5)	2.8	1.49 $\pm$ 0.88* (4)	4.8	1.4 $\pm$ 0.4*** (4)	4.5
Total	12.38	24.29	2.0	36.57	3.0	45.35	3.7

Constitutive levels of GST subunits in BEC and hepatocytes

BEC express constitutively only four of the several GST subunits that are known to be expressed by the whole liver. Without treatment, the major subunit by far is the Alpha-class subunit 2, which accounts for approx. 50% of the total BEC content. No other Alpha-class subunit is expressed. Interestingly, BEC also express constitutively very high levels of subunit 7 (Pi class), which represents approx. 30% of the total GST protein in these cells. The constitutive pattern of GST in BEC is completed by relatively low amounts of subunits 3 and 4, the two major hepatic Mu-class isoenzymes, which together account for 15% of the total GST protein. In some BEC preparations from untreated rats subunit 3 was actually undetectable. In comparison with the parenchymal cells of the liver, it is striking that BEC completely lack subunits 1a and 1b that are important in hepatocytes and also subunits 8 and 10. The Mu-class GST proteins make up a relatively low proportion of the total compared with their dominant position in hepatocytes. On the other hand, hepatocytes from untreated animals displayed only a trace of subunit 7 and subunits 1(a and b), 2, 3 and 4 make up over 90% of the total GST protein. In terms of class, the Pi-class GST is insignificant and hepatocytes from untreated rats can be seen to contain slightly more Mu-class GSTs (58%) than Alpha-class (42%).

Effects of inducing agents on GST-subunit composition of BEC

PB did not significantly increase the total amount of GST protein in BEC, despite a significant increase in the level of subunit 3, because subunits 7 and 2 were significantly decreased (Table 1). The overall effect was to change the subunit-class distribution in these cells, decreasing the Pi-class in favour of the Mu-class with no change in the representation of the Alpha-class (Table 2). These gross patterns do not reveal the changes in the individual subunits, however; and the subunits that were at undetectable levels in BEC from untreated rats, subunits 10, 6, 1a, 1b and 8, were sufficiently elevated by PB treatment to be measurable.

β -NF treatment did not increase the amount of total GST protein in BEC, in fact, there was a 20% decrease. This largely resulted in a significant fall in the level of subunit 2, normally the major subunit of these cells. Subunit 3 was again induced, apparently, though this was not statistically significant and, as previously noted for PB the subunits normally undetectable in BEC were induced to low but measurable levels. The overall effect on subunit-class distribution was to increase the representation of the Mu class, but in this case at the expense of the Alpha class.

EQ treatment had the most striking effects on BEC. The amount of total GST protein was elevated some 5-fold; subunits 7 and 3 were the most strongly affected, being elevated 8-fold and 25-fold respectively. The subunits not detectable in BEC from untreated rats were not induced by EQ treatment. Subunit 7 was therefore the most dominant GST subunit in these cells (nearly 50% of total GST) and the overall subunit-class distribution altered relative to controls such that both Mu and Pi classes were elevated at the expense of the Alpha class.

Effects of inducing agents on GST-subunit composition of hepatocytes

After treatment with PB the overall amount of cytosolic GST protein in hepatocytes was doubled. The inductive effect was not specific, with significant increases seen in all the major subunits

except 1a. Not all subunits are increased equally. The subunit whose concentration increased most was 1b, the protein concentration of which rose, on average, 4-fold above control levels. This represents a change from around 6% of total GST protein in controls to around 13% in PB-treated rats. Subunits 3, 4, 8 and, to a lesser degree of significance, 2 were also elevated above control levels. The overall class composition was only slightly altered at 56% Mu and 44% Alpha.

After β -NF treatment the overall average content of cytosolic GST protein was tripled. Again, all the major subunits were induced significantly except 1a, and subunit 1b was again the most affected, being elevated some 7-fold. Subunit 1b thus makes up some 14% of total GST protein in hepatocytes of β -NF-treated rats, though the most dominant subunits, in terms of protein concentration, remain subunits 3, 4 and 2. Notably, β -NF treatment caused a nearly 5-fold increase in the average content of minor subunit 8. A substantial average increase in subunit 7 was seen but was too variable to be statistically significant and, in any case, did not increase its representation above 0.3% of total GST. The balance of GST class shifted to 52% Mu, 48% Alpha.

EQ treatment was the most effective in increasing the overall average content of GST protein: an average increase of nearly 4-fold was recorded. As with PB and β -NF, all of the major subunits of GST were induced by EQ and, of these, subunit 1b to the greatest extent. The average concentration of subunit 1b increased 11-fold and as a proportion of the total GST protein this represented 18%, as compared with 6% in controls. EQ also induced statistically significant increases in all of the minor subunits of GST, with the exception of subunit 6. On average subunits 10 and 8 were induced 5.5- and 4.5-fold respectively, albeit that the final protein concentrations remained low compared with the major subunits. Alone among the inducers, EQ elicited a statistically significant increase in the concentration of the Pi-class subunit 7. The fold-increase is dramatic, since it is based on such a low constitutive figure, but the induced level is still low compared with the major subunits. The overall class composition after EQ treatment was 49% Mu, 47% Alpha and 4% Pi.

DISCUSSION

Constitutive expression of GST subunits in BEC

In recent years it has been increasingly stressed that phase I and phase II drug-metabolizing enzymes in the liver are not restricted to the parenchymal cells, and that the activities of such enzymes in non-parenchymal cells may be of great importance in protecting the liver from toxic compounds. For example, Steinberg and colleagues (1987; 1989; 1990) have made important contributions towards our understanding of metabolism of xenobiotics in the non-parenchymal cells, demonstrating the constitutive expression and inducibility of phase II enzymes in endothelial and Kupffer cells. The GSTs are very important components of phase II metabolism and in this paper we present the first complete analysis of the constitutive pattern of GST subunits in BEC.

The GST profile of BEC is completely different from that expressed by other cell types in the liver. BEC do not express detectable levels of subunits 1a, 1b, 6, 8, and 10 which are usually observed in whole liver (Ostlund-Farrants et al., 1987; Meyer et al., 1993) and isolated hepatocytes (this paper). Subunit 1 (more precisely, subunits 1a and 1b) accounts for approx. 16% of the total GST protein in hepatocytes and more than one third of Alpha-class subunits. BEC also lack subunit 8 that has a particularly high activity with the lipid-peroxidation product

4-hydroxynonenal (Ålin et al., 1985). Like the parenchymal cells, Kupffer cells and liver endothelial cells also express subunits 1 and 8 (Steinberg et al., 1989), so that lack of these subunits in our preparations from BEC can be taken as an indication of their purity. Almost all of the previously published results concerning GST-isoenzyme expression in BEC were obtained by immunohistochemical studies (Redick et al., 1982; Hayes et al., 1987; Hiley et al., 1988) for only some of the subunits now known to exist, or with polyclonal antibodies. Other studies have used non-specific substrates such as CDNB and DCNB to measure GST activities in BEC isolated from normal liver (Parola et al., 1990a), from bile-duct-ligated animals (Mathis et al., 1989) and BEC in culture (Schrenk et al., 1991). From the relative activities towards these two substrates the presence in BEC of GST isoenzymes 3-3 and 3-4 was proposed (Mathis et al., 1989; Parola et al., 1990a) and this hypothesis is fully supported by the present analysis.

Our study clearly illustrates that in BEC, in strong contrast with hepatocytes, subunit 7 is a major form of GST. The quantification of subunit 7 in BEC and hepatocytes confirms and extends previous observations based on immunohistochemical techniques that have reported strong staining of bile-duct epithelium in the liver of rats (Hayes et al., 1987) and man (Hiley et al., 1988). Neal et al. (1988) describe localization of GST 7-7 in bile-duct epithelium and 'a few isolated hepatocytes' in sections of control rat liver. BEC make up a small percentage of the total mass of the liver and subunit 7 is almost undetectable in cytosolic preparations of whole normal adult liver (Kitahara et al., 1984; Meyer et al., 1985). Using both immunohistochemical and h.p.l.c. analytical techniques, Tee et al. (1992) demonstrated that GST subunit 7 is strongly expressed in both bile-duct cells and hepatocytes of rat foetal liver during late gestation, but in the final few days before birth its expression is specifically down-regulated in the parenchymal cells, such that it is almost absent after birth. These results are in general agreement with those of Strange et al. (1990) for the human foetal liver. The normal physiological substrates of GST 7-7 and its exact physiological role are, in common with those of other GST isoenzymes, unknown. GST 7-7 is the major isoenzyme found in bile (Howie et al., 1989) and the group of Hayes has proposed that GST 7-7 acts as a carrier protein for non-substrate toxic ligands in order to remove unidentified substances from BEC and prevent their reabsorption. However, since the biliary concentrations of GST subunits are simply proportional to their concentrations in the BEC, their presence in the bile fluid may merely reflect general leakage from BEC rather than specific secretion. Other observations concerning Pi-class GST and detoxification concern its expression in cells transformed *in vitro* by *v-ras* or *v-raf*, co-expression of MDR-1 and association with the phenomenon of multi-drug resistance (Burt et al., 1988). Finally, GST 7-7 is strongly expressed in hepatoma cells (Meyer et al., 1985; Pemble et al., 1986; Tahir et al., 1989) and has been suggested to be a sensitive and specific marker of preneoplasia in rat liver (Kitahara et al., 1984; Jensson et al., 1985; Neal et al., 1988; Tatamatsu et al., 1988a,b; Rushmore et al., 1988; Sato, 1989).

Induction of GST subunits by PB, β -NF and EQ in BEC and hepatocytes

It has been known for many years that several classes of compound can induce an increase in activity and a change in isoenzyme patterns of GST. The great significance of the induction of GST activity by various exogenous compounds lies in the protective effect this generally affords against toxic and carcinogenic chemicals (see Hayes et al., 1990a). This

'chemoprotective' effect is not specific for GST activity but is associated with induction of other phase II drug-metabolizing enzymes such as NAD(P)H: (quinone-acceptor) oxidoreductase (EC 1.6.99.2) and UDP-glucuronyltransferase (Talalay and Spencer, 1990). Previous studies on the effects of GST-inducing agents utilized a wide variety of species, strains, inducers and methods of measurement and, moreover, were largely conducted when our knowledge of the range of GST subunits present in animal tissue was meagre. Reviewing the literature therefore yields a confusing and incomplete picture. One aim of this study was to use current chromatographic techniques to provide a quantitative and comprehensive characterization of the effects of three well-known inducers in a single strain of rat. This approach provides important information on the mechanisms of their induction, e.g. it can indicate whether different subunits are expressed independently or co-ordinately.

As mentioned earlier, we do have indications that PB induction results in a decreased retention of subunits 1b and 2 by the GSH-agarose affinity column and so the possibility that the results reported here represent underestimates of the levels of those subunits has to be considered. We do not believe that such effects are of sufficient magnitude to alter the general conclusions reached here. The results obtained here for induction of GST subunits in hepatocytes generally correlate well with published data, where comparisons are appropriate. At the fundamental level, for example, the GST subunit composition in hepatocytes of our untreated Wistar rats is largely identical to that in the whole liver of Sprague-Dawley rats used in another study; moreover, the subunit composition after PB treatment is also almost identical in the two strains (Meyer et al., 1993).

The overall magnitude of the effect of each of the inducers, studied in terms of total amount of GST protein, is in accord with reports of the increases in GST activity induced respectively, by EQ (Benson et al., 1978; Kensler et al., 1985, 1986), by PB (Igarishi et al., 1987; Vos et al., 1988; Di Simplicio et al., 1989) and by β -NF (McLellan et al., 1991), notwithstanding the range of different experimental conditions represented in those papers.

Those reports that have specifically quantified the effect of inducers on individual subunits are few and mostly incomplete in terms of the range of subunits measured, but all indicate a general trend for an increase in levels of subunits 1 and 3. For example, Kensler et al. (1986) reported that EQ (in Fischer 344 rats) induced isoforms 1-1, 1-2 and 3-3 to the greatest extent; isoforms 2-2, 3-4 and 4-4 less so. Vos et al. (1988) reported increases in isoenzymes 1-1 and 3-3 after PB induction in Wistar rats and Rogiers et al. (1991) noted that subunit 1 was that most increased by PB treatment. Our results support these conclusions but go further. Vos et al. (1988) and Rogiers et al. (1991) were unable to distinguish between subunits 1a and 1b. Our results make clear the great difference in responsiveness to inducers of these two variants: 1b is much more responsive to all three inducers. The group of Hayes et al., which distinguishes between subunits 1a and 1b, and terms them Y_{a1} and Y_{a2} respectively, have also reported the difference in response of these two subunits to aflatoxin B₁ (Hayes et al., 1990b) and to EQ in Fischer 344 rats (Hayes et al., 1991). In fact, Hayes et al. (1990b) have demonstrated that subunits 1a (Y_{a1}) and 1b (Y_{a2}) are distinct gene products corresponding respectively to the cDNAs pGTR (Lai et al., 1984) and pGTB (Pickett et al., 1984). Induction of subunit 1b is likely to be controlled by the so-called 'anti-oxidant-responsive element' (previously referred to as the β -NF-responsive element) regulating the gene for this protein (Paulsen et al., 1990; Rushmore et al., 1991).

With regard to the chemoprotective effect of induction of GST activity, the role of subunit 10 in conferring resistance to aflatoxin

may be significant, despite the fact that it is normally considered a 'minor' subunit. Hayes et al. (1991b) have suggested that induction by EQ of a subunit they term Yc_2 is the major mechanism by which EQ induces resistance to aflatoxin. They report that EQ has most effect (in terms of fold-induction) on subunit Yc_2 and that this subunit is very active towards aflatoxin B_1 -8,9-epoxide. Although not noted by Hayes et al., this subunit, Yc_2 , is closely related or identical to subunit 10, as is clear from the amino-acid sequence (Meyer et al., 1991). If the equivalence of Yc_2 and subunit 10 is accepted, then the results of Hayes et al. (1991b) and our reported results are in agreement: EQ induces all of the GST subunits measured and most strongly induces 1b (Ya_2), 10 (Yc_2) and 3 (Yb_1). However, the magnitude of the EQ effect on subunit 10 in our study is less dramatic than that reported by Hayes et al., possibly due to the different strains of rat used. The latter group also posed the question as to whether other compounds including PB and β -NF also induced ' Yc_2 ' (subunit 10). Our results indicate that the effect of both these compounds on subunit 10 was too weak to be statistically significant. This does not appear to support a role for the 'antioxidant-responsive element' in mediating the effect of EQ on the regulation of the gene for this protein. Another GST-inducing agent that protects against aflatoxin-induced hepatotoxicity, 1,2-dithiole-3-thione, was investigated in a separate study and found to induce strongly expression of subunit 10, and it was also shown that this subunit exhibited by far the highest activity towards aflatoxin B_1 -8,9-epoxide (Meyer et al., 1992).

Hayes et al. (1991b) did not report data for the effect of EQ on subunit 7, which we found to be significantly induced by EQ. Manson et al. (1987) reported 'modest' induction by EQ of GST π in periportal rat hepatocytes, as judged by immunohistochemistry, only after 23 weeks of treatment. Subunit 7 is normally not present, or present only at exceedingly low levels, in adult rat hepatocytes and induction has otherwise only been reported previously with lead nitrate (Di Ilio et al., 1989), *trans*-stilbene oxide (Tahir et al., 1989) and, very recently, with dithiolthione (Meyer et al., 1993). This is the most complete quantitative analysis of the subunits induced by EQ and also the first report of quantitative induction by EQ of subunit 7 *per se*. As we used isolated hepatocytes rather than whole liver we believe we have avoided artefactual measurements arising from BEC that express subunit 7 constitutively, and do not believe that this result is due to contamination. From our knowledge of the level of subunit 7 in BEC after EQ treatment, discussed above, we can calculate that the contamination of the hepatocyte preparation that would be required to account for this finding is unfeasibly high.

We have also arranged the results to indicate the effects of the inducers on GST expression in hepatocytes in terms of GST class (Table 2) since some of the literature also tends to discuss the phenomenon in these terms and since the different classes represent different gene families. However, this approach is much less informative than discussion of the individual subunits. The overall tendency in our study is for the inducing agents to increase expression of subunits in both of the major classes, Alpha and Mu, but with the Alpha-class tending to be expressed slightly more than the Mu-class proteins. This does not result in any gross distortion of the normal balance: even at the extreme condition, after EQ induction, the ratio of Alpha:Mu has only shifted from 42:58 in controls to 47:50 in treated livers. This finding is in contrast with that reported by Di Simpicio et al. (1989) who, using mice, reported Mu-class proteins to be most responsive to inducing agents.

As BEC express a different pattern of GST subunits to hepatocytes, it is probably not surprising that they also respond

quite differently to inducing agents. In fact, PB and β -NF are unable to elicit an increase in total GST protein expression in BEC, but they do nevertheless alter the pattern of subunits. Subunit 3 concentration is elevated by PB treatment, as it is in hepatocytes, but subunits 4 and 2, also induced in hepatocytes, are not induced in BEC; in fact subunit 2 levels decrease. Subunit 2 also decreased in BEC from β -NF-treated rats, in contrast with its rise in hepatocytes of the same animals. The apparent average rise in levels of subunits 3 and 4 of BEC after β -NF treatment approximates to that occurring in hepatocytes from the same animals, but is not statistically significant due to the large variance in the values obtained. With both PB and β -NF it is remarkable that subunits not expressed in BEC of control rats become detectable, albeit at low individual levels. For this to be due to contamination by other non-parenchymal cells would require that the level of purity in these BEC preparations was lower than that in control BEC preparations.

In terms of its effects on BEC, EQ is the most interesting of the three inducers studied. It is the only inducer to increase the total level of GST protein (which it does substantially) in these cells. Its effects are limited to subunits that BEC express constitutively (subunits 2, 3 and 7) but one of the constitutive subunits, subunit 4, is not responsive, despite it being induced in hepatocytes of the same animals. Following EQ treatment, the concentrations of GST subunits in BEC are remarkably high. For example, the concentration of subunit 3 in BEC from EQ-treated rats exceeds that of hepatocytes from control rats and approaches the concentrations of this subunit in hepatocytes of EQ-treated rats. Subunit 7, of course, is at a very high concentration in these cells, such that Pi is the dominant class. Given that BEC represent a very small number of cells in the liver and that they are small cells compared with hepatocytes, it can be estimated that on a total liver basis, the amount of subunit 7 protein in parenchymal cells actually far exceeds the amount of subunit 7 in BEC, after EQ treatment. Nevertheless, specific GST 7-7 activity will be at its highest in BEC where that isoenzyme is most concentrated.

As mentioned above EQ is very well known to protect against aflatoxin hepatocarcinogenicity and this has been attributed to its induction of GST activity. In the past, BEC have been envisaged as target cells for carcinogens because the so-called 'oval cells' seen during hepatocarcinogenesis have been postulated as being derived from BEC, a concept that is the source of much debate and is reviewed fully by Sirica et al. (1990). If BEC are indeed targets for chemical carcinogens then the potent activity of the anti-carcinogen EQ in inducing GST in these cells must be of interest and merits mention here. Of the GST subunits active towards aflatoxin B_1 -8,9-epoxide, only subunit 3 is expressed in BEC, and the others (1b and 10, the latter of which is the most active) are not, even after EQ treatment. Moreover, since we have shown that BEC lack cytochrome *P*-450 activity (Parola et al., 1990a) it seems most unlikely that they are capable of activating aflatoxin to the carcinogenic epoxide and for this species to be an effective carcinogen in these cells it would have to migrate there from hepatocytes. In summary, the potent GST-inducing effects of EQ in BEC seem unlikely to be related to its anti-carcinogenic properties, whereas its effects in hepatocytes seem far more relevant.

Finally, it should be stressed that the effects of inducing agents that we have described in BEC have been investigated at a single time point. It is possible that BEC are slow to respond to inducing agents and that where lack of induction has been described here, a positive effect might be seen with extended treatment. Such a phenomenon was described by Steinberg et al. (1989) for the effects of Arochlor-1254 on Kupffer and liver

endothelial cells. The effects of extended treatment regimes and of other inducing agents can only be discovered by further studies.

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