Hormonal regulation of glutathione S-transferase expression in co-cultured adult rat hepatocytes

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Abstract

Glutathione S-transferases (GSTs) are subject to regulation by thyroid and sex hormones and by GH. We have used an *in vitro* experimental system comprising adult rat hepatocytes co-cultured with rat liver epithelial cells of primitive biliary origin, to distinguish between direct and indirect effects of various hormones on GSTs; to identify the GST subunits affected by individual hormones; and to investigate the level at which the hormones act. Tri-iodothyronine (T₃), thyroxine (T₄) and 17 β -oestradiol (OE₂) reduced GST activities, whereas testosterone, dihydrotestosterone, and human growth hormone (hGH) had little effect on total GST activity. HPLC separation of

the various GST subunits revealed that T₃ and T₄ reduced total GST content, in particular the abundance of subunits M1 and M2. The amount of the Pi-class subunit P1 was reduced by OE₂. Treatment of the co-cultured cells with this hormone altered the GST subunit profile to one that is more similar to that observed in freshly isolated hepatocytes. Analysis of mRNAs demonstrated that some of the hormones act at a pre-translational level, whereas others act at a translational or post-translational level to regulate the expression of various GST subunits.

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Introduction

Cytosolic glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of dimeric proteins consisting of two identical or closely related subunits belonging to the same class. A species-independent scheme of classifying these subunits into Alpha, Mu, Pi, Theta and Sigma classes has been widely accepted (Mannervik *et al.* 1985, Tsuchida & Sato 1992, Hayes & Pulford 1995). GSTs play a role in the detoxification of electrophilic xenobiotics or electrophilic metabolites of xenobiotics produced by mixed-function oxygenases (Mannervik & Danielson 1988, Pickett & Lu 1989, Hayes & Pulford 1995). In addition, GST proteins control the uptake and transport of numerous hydrophobic endogenous compounds, including bilirubin, glucocorticoids, steroids and thyroid hormones, via non-covalent glutathione (GSH)-dependent binding to GST (Listowsky *et al.* 1988, Ishigaki *et al.* 1989, Wilce & Parker 1994).

Two types of GST inhibitors are known. Irreversible inhibitors, such as halogenated quinones, covalently modify a cysteine residue near, or in, the active site of the GST enzyme (Vos & Van Bladeren 1990). Reversible inhibitors, on the other hand, act through non-covalent binding and include, among others, thyroid hormones, as

mentioned above (Boyer *et al.* 1984, Listowsky *et al.* 1988).

Transcriptional activation by exogenous substances has been best documented for the GSTA1/2 subunit gene of the rat. Several enhancer elements, including the xenobiotic-responsive element (XRE), the antioxidant-responsive element (ARE), the glucocorticoid-responsive element (GRE) and the barbiturate-responsive element (Barbie box) have been identified in the 5'-flanking regulatory region of this gene and are responsible for its induction by polycyclic aromatic hydrocarbons including 3-methylcholanthrene and β -naphthoflavone, products of oxidative stress, dexamethasone and phenobarbital respectively (Rushmore *et al.* 1991, Hayes & Pulford 1995). With respect to the endogenous regulation of GST expression, it is known that adrenocorticotrophic (Mankowitz *et al.* 1990), growth (Staffas *et al.* 1992), thyroid (Kelley & Bjeldanes 1995, Beckett *et al.* 1988) and steroid hormones (Hatayama *et al.* 1986) are involved. At the regulatory level, it has been shown that insulin induces the transcription of the human GSTP1 gene via one of the SP-1 sites (GC/box) in the 5'-flanking regulatory region, whilst retinoic acid suppresses hGSTP1 expression through the AP-1 site (Xia *et al.* 1993, 1996). However, compared

with xenobiotic-mediated GST regulation, the endogenous hormonal control of GST expression has received much less attention.

Several aspects of both endogenous and exogenous hepatic GST regulation have been mainly studied *in vivo* (McLellan & Hayes 1987, Scott & Kirsh 1987, Li & Tu 1989, Meyer *et al.* 1991, 1993, Singhal *et al.* 1992 *a,b*, Hatayama *et al.* 1993). It is, however, generally agreed that it is difficult to make a distinction between the direct and indirect effects of hormones and drugs administered *in vivo*. Such a discrimination is more feasible *in vitro* (Gebhardt *et al.* 1990, Vind *et al.* 1992). At present, no ideal hepatocyte culture system exists. However, previous work by our group has shown that hepatocytes co-cultured with rat-liver epithelial cells of primitive biliary origin maintain the expression of many differentiated liver functions and may provide a useful model for studying enzyme regulation (Vandenbergh *et al.* 1988a, 1991, Rogiers *et al.* 1990, Akrawi *et al.* 1993, Coecke *et al.* 1993, Rogiers 1993, Rogiers & Vercruysse 1993). In this paper, we report the use of adult rat hepatocytes co-cultured with rat-liver epithelial cells of biliary origin, to distinguish between direct and indirect effects of various hormones on GSTs, to identify the GST subunits affected by individual hormones, and to investigate the level at which each hormone acts.

Materials and Methods

Materials

Crude collagenase type I, bovine serum albumin (bsa) (fraction V), bovine insulin, 1-chloro-2,4-dinitrobenzene (CDNB), hGH (2 IU/mg), GSH and epoxy-activated Sepharose 6B were obtained from the Sigma Chemical Co. (St Louis, MO, USA). All culture media, foetal calf serum and trypsin-EDTA solution were obtained from Gibco (Brussels, Belgium). 5 α -dihydrotestosterone (5 α -DHT) was purchased from Janssen Chimica (Geel, Belgium). OE₂, T₃ and T₄ were obtained from Fluka Chemie (Bornem, Belgium) and testosterone was purchased from K&K Rare and Fine Chemicals (Asse, Belgium). 1,2-Dichloro-4-nitrobenzene (DCNB) was from Merck-Belgolabo (Darmstadt, Germany). RNazol B was purchased from A.M.S. Biotechnology (Witney, UK). The Klenow fragment of *Escherichia coli* DNA polymerase was from Pharmacia Biotech Ltd (Milton Keynes, UK). All other chemicals were of reagent grade and were purchased from general commercial sources.

cDNA probes

Full-length cDNA probes complementary to GST A1/2 mRNA (cross-hybridization to GST A3 mRNA occurs), GST M1 mRNA (cross-hybridization to GST M2

mRNA occurs) and GST P1 were the kind gifts of Dr S Pemble (University College London, London, UK) (Pemble *et al.* 1986).

Preparation of hepatocyte cultures and cytosolic fractions

Hepatocytes were isolated from outbred adult male Sprague-Dawley rats (specific pathogen-free Carworth Farms Elias, 15 weeks old, 350 g), obtained from Iffa Credo (Brussels, Belgium), as described by De Smet *et al.* (1998). Cell integrity was tested by trypan blue exclusion and ranged from 85 to 95%. Rat-liver epithelial cells were obtained by trypsinization of the livers of 10-day-old Sprague-Dawley rats (Williams & Gunn 1974) and were used between their 20th and 30th passages, as described in detail by Vanhaecke *et al.* (1998). Twenty-four hours after cell plating, the cultures were incubated with serum-free medium containing testosterone, 5 α -DHT, OE₂, T₃ or T₄ at a final concentration of 10 μ M. Such high concentrations are required because of the high degradation rate of these hormones in co-cultures (Coecke *et al.* 1998). The cultures also contained the hormone vehicle ethanol, at a concentration of 0.1% (v/v). Only in the case of T₄ addition was a concentration of 0.4% (v/v) used. For treatment of cells with hGH, the hormone was dissolved in phosphate-buffered saline (PBS) solution and added to the cells to a final concentration of 0.1 μ g/ml. In this case, the vehicle was present at a final concentration of 0.1%. Appropriate control cultures were run and contained 0.1% ethanol, 0.4% ethanol or 0.1% PBS.

Cytosolic fractions from freshly isolated hepatocytes (T0) and co-cultures were prepared as described by Johnson *et al.* (1992). Storage at -80°C for three months did not modify GST activity (Vandenbergh *et al.* 1988a, and our own observations).

Enzyme and protein assays

Total and Mu-class M1-1 and M1-2 GST activities towards CDNB and DCNB respectively, were assayed according to Habig *et al.* (1974). The protein content of the cytosolic fractions was determined by the method of Bradford (1976), using a protein assay kit (Bio-Rad, Brussels, Belgium) with bovine serum as a standard.

Isolation and separation of GST subunits

GSTs were isolated from cytosolic fractions by affinity chromatography as previously described (Vandenbergh *et al.* 1988b). The separation and quantification of GST subunits was carried out according to Vandenbergh *et al.* (1990), using an LC10 system (Shimadzu, Berelux, 's-Hertogen-bosch, Netherlands) with a Prolinea 4/33 HPLC-dedicated integrator (Compaq, Houston, TX, USA). The GST subunits were identified by comparison

of the retention time obtained for the individual peaks with those of purified GST subunits and by comparison of the patterns generated with characteristic extrahepatic GST profiles and previously published GST HPLC profiles (Hiratsuka *et al.* 1990, Vandenberghe *et al.* 1990, Meyer *et al.* 1991, Johnson *et al.* 1990, 1992, 1993, Kispert *et al.* 1989, Meyer *et al.* 1989). GST subunits were quantified using peak areas of the respective subunits from HPLC, together with molecular masses (Mannervik & Danielson 1988) and molar extinction coefficients at 214 nm, as given by Johnson *et al.* (1992). The concentrations of the individual subunits were expressed as μg per mg cytosolic protein.

A peak with a retention time of 11 min was found to be due to the TRIS–GSH buffer used to elute the column.

RNA isolation and Northern blots

RNA was isolated from freshly isolated and co-cultured hepatocytes using RNazol B and the procedure described by Chomczynski & Sacchi (1987). Equal amounts of total RNA (15 μg per lane) were size-fractionated by electrophoresis in a denaturing formaldehyde agarose gel and transferred to a nylon membrane (Hybond- N^+ , Amersham, Buckinghamshire, UK) (Fourney *et al.* 1988). Filters were prehybridized and hybridized with full-length [$\alpha^{32}\text{P}$]dCTP-labelled GST cDNA probes according to Feinberg & Vogelstein (1983). After hybridization, filters were washed, dried, and autoradiographed at -80°C using RX-NIF X-ray films (Fuji Film, USA Inc). Hybridization signals were quantified by scanning densitometry using a Bio-Rad densitometer (Model GS670). To correct for any unequal loading of the RNA, signals were normalized with respect to the 28S rRNA content.

Statistical analysis

Statistical evaluation of the differences obtained was achieved by Student's *t*-tests, using the Software Package of the Social Sciences, SPSS/PC+ (Norusis 1986). $P < 0.05$ was the set level of significance.

Results

Ability of freshly isolated hepatocytes to express GST activities

During the process of isolation, hepatocytes lose some of the differentiated functions associated with the intact liver *in vivo* (Mertens *et al.* 1993a). It was thus necessary to ensure that isolated hepatocytes for use in the co-culture system maintained levels of GST activities similar to those observed *in vivo* (Crocchi & Williams 1985, Vandenberghe *et al.* 1988a). Therefore, suspensions of freshly isolated hepatocytes were used for co-cultures only if they contained GST activities with respect to CDNB and DCNB

Figure 1 GST activity towards CDNB and DCNB in co-cultures of adult rat hepatocytes maintained in serum-free medium. Each point plotted represents the average of values obtained from three independent co-cultures of hepatocytes and is expressed as a percentage of the GST activities towards CDNB and DCNB obtained for freshly isolated hepatocytes (broken line; 1.263 ± 0.246 and 0.037 ± 0.006 U per mg cytosolic protein respectively). The bars represent standard deviation. *Significantly different from freshly isolated hepatocytes according to a paired Student's *t*-test ($P < 0.05$).

of at least $1.2 \mu\text{mol}/\text{min}/\text{mg}$ cytosolic protein and $35 \text{ nmol}/\text{min}/\text{mg}$ cytosolic protein respectively. Cells that met these criteria maintained constant levels of GST activities after 7–14 days in culture, thus providing a suitable system for studying hormonal regulation of GST expression (Fig. 1).

Effect of hormones on GST activities

Administration of either OE_2 , T_3 or T_4 for 10 days in co-culture reduced GST activity towards CDNB and DCNB by approximately 30 and 40% respectively ($P < 0.05$). Addition of hGH to the co-cultured hepatocytes reduced the activity of isoenzyme M1–1/M1–2 ($P < 0.05$), but had no significant effects on total GST activity. Neither testosterone nor $5\alpha\text{-DHT}$ had a significant effect on either total GST or isoenzyme M1–1/M1–2 activity. As ethanol, used as a vehicle for all the hormones tested except GH, is known to induce the activities of certain enzymes involved in the metabolism of xenobiotics (Skett & Paterson 1985, Okey 1990), its effect on GST activities in co-cultured hepatocytes was investigated. Ethanol at a final concentration of 0.1% (equivalent to that present in the medium during treatment of cells with testosterone, $5\alpha\text{-DHT}$, OE_2 or T_3) had no significant effects on either total or Mu-class M1–1/M1–2 GST activities. In contrast, 0.4% ethanol, equivalent to the final concentration present during treatment of cells with T_4 , increased GST activity with respect to CDNB by 35% ($P < 0.05$). The small increase in GST activities obtained with DCNB as a substrate was not significant. In order to exclude any misleading conclusions because of possible interference of the vehicle with GST results,

Figure 2 Effect of T₃ and T₄ delivery in HPBC on GST activity towards CDNB and DCNB in co-cultures of adult rat hepatocytes. The hormonal concentration was 10 µM T₃ and T₄ were taken up in 0.02 and 0.10% (w/v) HPBC respectively. Each value represents the average $\pm$ standard deviation of values obtained from three independent co-cultures of hepatocytes. The results are expressed as a percentage of the GST activities towards CDNB and DCNB obtained for 10-day-old, untreated, control co-cultures (0.397 ± 0.089 and 0.017 ± 0.004 U per mg cytosolic protein respectively). * $P < 0.05$ or ** $P < 0.01$ for significant difference from 10-day-old, untreated, co-cultures according to a paired Student's *t*-test.

hydroxypropyl- β -cyclodextrin (HPBC) was also used to deliver T₃ and T₄. When 0.1 and 0.4% (v/v) ethanol were replaced by 0.02 and 0.1% (w/v) HPBC to deliver T₃ and T₄ to the cultured cells respectively, no statistically significant effects on GST activities towards CDNB and DCNB could be observed (Fig. 2). Moreover, T₃ and T₄ administered via HPBC reduced total and Mu-class GST activities by approximately 30–40%, which corresponds well with our observations on GST activities with ethanol as a solvent. These findings indicate that co-cultured unexposed cells that are treated with appropriate concentrations of ethanol represent the correct controls. Consequently, the corresponding results of hormone-exposed and non-exposed cells used to calculate hormonal effects on co-cultured cells led to relevant conclusions.

Effect of hormones on GST subunit profiles

The GST subunit compositions of cytosolic fractions obtained from freshly isolated rat hepatocytes (T0) and from co-cultured hepatocytes in the presence or absence of various hormones are given in Table 1. The total content of GST protein present in freshly isolated hepatocytes was 76.3 ± 6.3 µg per mg cytosolic protein. This comprised similar amounts of Alpha- and Mu-class proteins. After hepatocytes had been co-cultured for 10 days in a serum-free medium, the total content of GST protein had

decreased to 60.3 ± 4.0 µg per mg cytosolic protein. Although the concentration of each of the Mu-class subunits was maintained during culture, the concentration of almost all Alpha-class subunits decreased substantially during culture, whereas that of the Pi-class subunit, which is not detectable in freshly isolated hepatocytes, increased. Thus, the complement of GST subunits present in hepatocytes is altered during culture.

Treatment of co-cultured hepatocytes with testosterone or with 5 α -DHT had no significant effects on the total content of either the Alpha, Mu or Pi classes of GST subunits (Table 1), which corresponds with the results obtained from activity measurements. However, the amounts of subunits A1 and A2 were increased almost twofold in response to testosterone. Subunit A2 was also increased (about threefold) by OE₂. This hormone had no significant effects on any other subunit, except for subunit P1, which was reduced by 50%. Treatment of cells with hGH had no significant effects on the amounts of any of the cytosolic GST subunits.

Compared with their appropriate control co-culture, T₃ and T₄ reduced the total GST protein content by 60 and 70% respectively. Analysis of subunits showed that T₃ reduced the total content of Alpha-class subunits, whereas T₄ reduced the total content of Mu-class subunits (Table 1). The latter was due to 80% reductions in the amounts of subunits M1 and M2.

Table 1 Effect of hormones on the GST subunit content of freshly isolated hepatocytes (T0) and co-cultured hepatocytes. The concentrations of individual GST subunits were determined by HPLC. The values presented are the mean $\pm$ S.D. of four separate isolation and culture experiments

Subunit concentration ($\mu\text{g}/\text{mg}$ cytosolic protein)										
Subunit	T0	Control	Control + 0.1% EtOH	Control + 0.4% EtOH	Testosterone	5 α -DHT	OE ₂	hGH	T ₃	T ₄
Alpha class										
A1	18.5 $\pm$ 3.8*	2.3 $\pm$ 0.7	2.7 $\pm$ 2.3	6.4 $\pm$ 5.6	6.9 $\pm$ 4.6	3.1 $\pm$ 2.4	2.5 $\pm$ 1.5	3.2 $\pm$ 2.1	1.1 $\pm$ 1.2	3.8 $\pm$ 1.8
A2	4.8 $\pm$ 4.5	2.3 $\pm$ 1.4	3.9 $\pm$ 2.6	6.8 $\pm$ 9.1	8.5 $\pm$ 7.8	3.6 $\pm$ 1.9	13.9 $\pm$ 9.8	2.1 $\pm$ 0.4	0.6 $\pm$ 0.6	2.7 $\pm$ 1.5
A3	12.6 $\pm$ 2.5*	2.8 $\pm$ 0.7	12.5 $\pm$ 13.4	5.1 $\pm$ 3.2	7.0 $\pm$ 3.7	8.3 $\pm$ 7.2	7.0 $\pm$ 3.1	5.1 $\pm$ 4.8	2.7 $\pm$ 0.7	4.2 $\pm$ 4.6
A4a	2.5 $\pm$ 2.1	0.8 $\pm$ 0.2	0.8 $\pm$ 0.6	1.4 $\pm$ 1.3	1.3 $\pm$ 1.4	1.0 $\pm$ 0.3	1.1 $\pm$ 0.7	1.4 $\pm$ 1.1	1.1 $\pm$ 1.2	1.0 $\pm$ 1.1
A4b	2.6 $\pm$ 0.2*	0.7 $\pm$ 0.4	0.8 $\pm$ 0.6	1.9 $\pm$ 2.3	0.6 $\pm$ 0.7	1.1 $\pm$ 0.4	3.1 $\pm$ 2.6	1.2 $\pm$ 1.2	1.0 $\pm$ 1.2	0.5 $\pm$ 0.5
A5	0.0 $\pm$ 0.0	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	0.4 $\pm$ 0.7	0.6 $\pm$ 0.8	0.4 $\pm$ 0.5	0.2 $\pm$ 0.3	0.3 $\pm$ 0.3	1.0 $\pm$ 1.2	0.5 $\pm$ 0.5
Subtotal	40.9 $\pm$ 3.5*	9.5 $\pm$ 3.5	20.7 $\pm$ 8.4	21.9 $\pm$ 21.0	24.9 $\pm$ 14.6	17.4 $\pm$ 11.8	27.8 $\pm$ 13.4	13.2 $\pm$ 6.6	7.6 $\pm$ 3.7*	13.4 $\pm$ 4.8
Mu class										
M1	12.9 $\pm$ 1.1	11.4 $\pm$ 4.7	22.0 $\pm$ 11.5	33.6 $\pm$ 8.6*	22.2 $\pm$ 10.7	23.7 $\pm$ 11.9	18.3 $\pm$ 9.1	18.6 $\pm$ 9.6	9.7 $\pm$ 3.1	6.4 $\pm$ 3.0*
M2	21.6 $\pm$ 2.1	22.1 $\pm$ 4.0	36.4 $\pm$ 16.3	52.0 $\pm$ 7.8*	36.1 $\pm$ 11.1	46.4 $\pm$ 16.0	28.0 $\pm$ 9.8	32.8 $\pm$ 12.7	13.2 $\pm$ 1.4	11.8 $\pm$ 5.9*
M3	0.7 $\pm$ 0.2	0.8 $\pm$ 0.7	0.8 $\pm$ 1.0	1.1 $\pm$ 1.1	1.2 $\pm$ 1.4	0.7 $\pm$ 0.3	0.7 $\pm$ 0.7	1.3 $\pm$ 1.2	0.2 $\pm$ 0.2	0.3 $\pm$ 0.2
Subtotal	35.2 $\pm$ 3.1	34.2 $\pm$ 8.8	59.1 $\pm$ 27.4	86.8 $\pm$ 14.9*	59.4 $\pm$ 21.1	70.9 $\pm$ 27.0	47.0 $\pm$ 18.5	52.8 $\pm$ 21.4	23.1 $\pm$ 2.8	18.5 $\pm$ 8.9*
Pi class										
P1	0.0 $\pm$ 0.0*	15.4 $\pm$ 6.3	18.3 $\pm$ 5.4	14.6 $\pm$ 2.3	15.3 $\pm$ 1.5	16.5 $\pm$ 5.1	9.7 $\pm$ 1.6*	18.1 $\pm$ 3.5	8.3 $\pm$ 6.6	8.0 $\pm$ 7.7
T 16.5 min	0.2 $\pm$ 0.1	1.2 $\pm$ 0.9	0.4 $\pm$ 0.7	3.7 $\pm$ 5.0	3.3 $\pm$ 3.3	6.4 $\pm$ 9.7	8.2 $\pm$ 9.4	2.0 $\pm$ 1.1	0.7 $\pm$ 0.8	0.9 $\pm$ 0.7

*Significantly different from the corresponding control value according to non-paired (T0) and paired Student's *t*-tests ($P < 0.05$).

Figure 3 Northern blot analysis of mRNAs encoding GST subunits. Total RNA was isolated from freshly isolated hepatocytes (lanes 1) or from untreated co-cultured cells (lanes 2) or from co-cultured cells treated with 0.1% (v/v) ethanol (lanes 3), 0.4% (v/v) ethanol (lanes 4), testosterone (lanes 5), 5 α -DHT (lanes 6), T₃ (lanes 7), hGH (lanes 8), T₄ (lanes 9) or OE₂ (lanes 10). Each lane contained 15 μ g total RNA. Northern blots were hybridized with cloned cDNAs that hybridized to mRNAs encoding GST subunits A(1/2) and A3 (A), M1 and M2 (B) or P1 (C). In each case, a photograph of the ethidium bromide-stained gel in the region of the 28S rRNA is shown.

Effects of hormones on the amounts of GST mRNAs

To gain some insight into the levels at which the various hormones act to exert their effects on GSTs, the mRNAs encoding the GST subunits were investigated. Total RNA from freshly isolated hepatocytes and from co-cultured hepatocytes was analysed by Northern blot hybridization with cloned cDNA probes encoding GST A1/2 (which cross-hybridizes with subunit A3), GST M1 (which cross-hybridizes with subunit M2) and GST P1. Both GST (A1/2)/A3 mRNAs (Fig. 3A, lane 1) and GST M1/M2 mRNAs (Fig. 3B, lane 1) were present in freshly isolated hepatocytes, whereas GST P1 mRNA was not detectable in these cells (Fig. 3C, lane 1). Quantification of GST mRNAs showed that, in comparison with its control (Fig. 3B, lane 4), T₄ (Fig. 3B, lane 9) reduced GST M1/M2 mRNA amounts by 75%. This is in accord with the effects of the hormone on the amounts and activities of the corresponding GST subunits. None of the other hormones affected the amounts of mRNAs encoding the various GST subunits.

Discussion

Co-cultured adult male rat hepatocytes have been proved to be a useful model to study hormonal regulation of phase I flavin-containing mono-oxygenase activity (Coecke *et al.* 1998). In the present study, we used this *in vitro* experimental system to investigate the direct effects of various

hormones on the activities and amounts of GSTs and on the amounts of the corresponding mRNAs.

Addition of OE₂ to the co-cultured cells inhibited both total and Mu-class-specific GST activities, but had no effect on the abundance of Mu-class protein or mRNA. Johnson and co-workers have reported that GST M1-1 and M1-2 activities are negatively regulated post-translationally by methylation (catalysed by a specific GST methyltransferase) and that Mu-class subunits M1 and M2 are the preferred substrates for this enzyme in the liver (Johnson *et al.* 1992). Under normal physiological conditions, tissue concentrations of GSH, an inhibitor of GST methylation (Neal *et al.* 1988), may be sufficient to suppress GST methylation *in vivo* (Johnson *et al.* 1992). Methylation of GSTs may therefore take place only when GSH is depleted. However, previous studies from our group have demonstrated that the intracellular GSH concentration is, after an initial increase immediately after hepatocyte isolation, quickly stabilized to normal levels in co-cultures (Mertens *et al.* 1991, 1993b). It has, however, been shown that OE₂, at physiological doses, reduces GSH concentrations in isolated hepatocytes (Ruiz-Larrea *et al.* 1993). It is therefore possible that the decrease in GST activities in response to treatment with OE₂ (in the absence of any effect on the proteins or their mRNAs) may be mediated via post-translational methylation of GSTs. The decrease in the amount of GST subunit P1 in response to OE₂ cannot account for the observed gender differences in GST amounts as this subunit is not normally present in the livers of adult rats.

In vivo studies have already demonstrated a sexual dimorphism in hepatic GSTs. In rat, total and Mu-class GST activities (Hales & Neims 1976, Hatayama *et al.* 1986, Coecke *et al.* 1990), and the amounts of GST proteins, in particular M1 and M2 (Coecke *et al.* 1991, Igarashi *et al.* 1987), and of the corresponding mRNAs (Igarashi *et al.* 1987, Pickett *et al.* 1987, Coecke *et al.* 1991, Rogiers *et al.* 1991) are higher in male than in female liver. However, here we found no effect of the male sex hormones testosterone and 5 α -DHT on the activities or amounts of GST proteins in co-cultured male rat hepatocytes. GSTs are regulated *in vivo* by androgenic programming through neonatal imprinting (Lamartiniere 1981, Skett 1987), which may explain the finding that addition of androgens *in vitro* cannot upregulate GST activity (the male GST activity has already been imprinted at birth).

The pituitary gland appears to be important in regulating endogenous GST expression in rats. In particular, gender differences in GH secretion patterns are involved in the regulation of GSTs *in vivo* (Staffas *et al.* 1992). Continuous administration of GH yields a female isoenzyme pattern, whereas discontinuous administration of GH yields a male pattern (Staffas *et al.* 1992). A similar observation has been made for members of the cytochrome P450 family (Mode *et al.* 1989, Vind *et al.* 1992).

However, our results indicate that in co-cultured rat hepatocytes hGH has no effect on the GST activities or amounts of GST proteins or on the amounts of the corresponding mRNAs.

Both of the thyroid hormones, T_3 and T_4 , reduced the activities of GSTs towards CDNB and DCNB and reduced the total GST protein content. These *in vitro* results correspond well with earlier *in vivo* observations that prolonged oral administration of T_3 or T_4 causes a decline in GST activity in rat liver, which can be related to a decrease in the amounts of Alpha- and Mu-class subunits (Beckett *et al.* 1988). Often, effects exerted by T_4 are comparable to those of T_3 since 80% of T_3 is derived from conversion of T_4 in peripheral tissue, particularly the liver (Visser 1988). We found that in the case of T_3 the decrease in total GST content was due to a decrease in Alpha-class subunits, whereas T_4 reduced the Mu-class subunits. These results were confirmed at the mRNA level. As proper controls for both T_3 and T_4 have been used in our experiments, these apparently conflicting observations are not due to different concentrations of the vehicle used. However, it is known that part of T_4 is deactivated through extensive metabolism (Visser 1996) and since our investigation of the hormonal effects at day 10 of culture with daily changes of the medium, we believe that, because of a decrease in T_4 metabolism as a function of culture time, the strong, suppressive effect of T_4 on the amounts of Mu-class subunits results from a direct effect of T_4 rather than from its conversion to T_3 .

In conclusion, our results with respect to the activity, subunit composition and abundance of steady-state mRNAs for the various GSTs indicate that GST activity is regulated by hormones both transcriptionally and post-translationally. Such hormone-related differences in GST expression may provide a molecular basis for tissue, gender, age and interindividual differences in the toxicity of certain xenobiotics (Lock *et al.* 1984, Coecke *et al.* 1990, 1991, Singhal *et al.* 1992 *a,b*). The effects of the different hormones under investigation are most pronounced for the Mu-class GSTs. In humans, Mu-class GSTs have attracted particular interest because 45% of the European population fails to express GSTM1, because of the presence of two null alleles (Zhong *et al.* 1993). GSTM1-containing enzymes are very effective in deactivating mutagenic and carcinogenic epoxides (Hayes & Pulford 1995). Moreover, GSTM1 polymorphism has been linked with the development of cancers (Harris *et al.* 1998, Whalen & Boyer 1998). Therefore, it is of general importance to increase our understanding of the molecular regulation of Mu-class GSTs and, more specifically, the effect of Mu-class polymorphism on endogenous GST regulation. In this respect, human hepatocyte cultures could represent a good *in vitro* model. However, the limited availability of human liver tissue is still a major problem (Rogiers 1993).

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