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Hydroxypropyl-β-cyclodextrin as delivery system for thyroid hormones, regulating glutathione *S*-transferase expression in rat hepatocyte co-cultures

Tamara Vanhaecke[a]*, Marie-Paule Derde[b], Antoine Vercruysse[a], Vera Rogiers[a]

[a]*Department of Toxicology, Free University Brussels, Laarbeeklaan 103, B-1090 Brussels, Belgium*
[b]*Department of Statistics, Free University Brussels, Laarbeeklaan 103, B-1090 Brussels, Belgium*

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Abstract

Thyroid hormones play a role in the regulation of glutathione S-transferase (GST) expression. Here, co-cultures of rat hepatocytes with bile duct epithelial cells have been used to study the direct effects of both triiodothyronine (T3) and thyroxine (T4) on GST activities and proteins. Because T3 and T4 are poorly water soluble and organic solvents used to dissolve them often interfere with biotransformation pathways, an alternative delivery system namely hydroxypropyl-β-cyclodextrin (HPBC) has been applied. Appropriate control cultures contained either 0.02 or 0.10% (w/v) HPBC, the concentrations necessary to supply T3 and T4 (10^{-9} to 10^{-5} M) to the cells, respectively. No effect of the vehicle HPBC on the different GST isoenzyme activities and proteins could be observed. On the contrary, after 10 days of co-culture, T3 and T4 decreased GST protein concentrations as well as GST activities measured by 1-chloro-2,4-dinitrobenzene (broad spectrum), 1,2-dichloro-4-nitrobenzene (Mu class M1/M2-specific) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Alpha class A1/2-specific) in a concentration-dependent manner. The Alpha class subunits A1/2 and A3, and the Mu class subunit M2 were mostly affected. No effect was observed on the Pi class enzyme. These findings indicate that a combination of co-cultured hepatocytes with an HPBC-based delivery system for hydrophobic compounds represents a powerful *in vitro* tool in drug development.

Keywords: Glutathione S-transferase; Hydroxypropyl-β-cyclodextrin; Inhibition; Rat hepatocyte co-cultures; Triiodothyronine; Thyroxine

1. Introduction

During the pre-clinical phase of drug development, a number of questions with respect to metabolism, pharmacokinetics and possible interactions of the new drug with exogenous and endogenous molecules have to be answered. Efforts have been focused on the development and practical use of various culture models of hepatocytes. Co-cultures of adult rat hepatocytes with primitive biliary epithelial cells have proven to be useful *in vitro* models since they retain several key phase I and phase II metabolic pathways [1–4], including the expression for at least 10 days of the key phase II GST (EC 2.5.1.18), a group of dimeric multifunctional isoenzymes which catalyses the conjugation of glutathione to a wide variety of hydrophobic electrophiles [5,6]. GST expression is subject to regulation by different hormones, including the thyroid hormones T3 and T4 [7–9]. However, it is difficult to discriminate direct from indirect effects when animal models are used [10,11]. Such a distinction is easier to make *in vitro*. A problem, however, remains the fact that T3 and T4, like many other exogenous and endogenous substances, are lipophilic and thus poorly solve in hydrophilic culture media. Usually, their solubilization is achieved by means of organic solvents. However, interference between organic solvents and drug metabolism pathways often occurs [12,13]. In order to overcome this problem, we tried to use an alternative delivery system, namely cyclodextrin.

Cyclodextrins are cyclic oligosaccharides obtained from the enzymatic conversion of starch. Related to their three-

* Corresponding author. Tel.: +32-2-477-4518; fax: +32-2-477-4582.
E-mail address: tamaravh@vub.vub.ac.be (T. Vanhaecke).

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; GST, glutathione S-transferase; HPBC, hydroxypropyl-β-cyclodextrin; LDH, lactate dehydrogenase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; T3, 3,3′,5-triiodo-L-thyronine; T4, L-thyroxine.

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dimensional structure, cyclodextrins show a relatively hydrophobic cavity and hydrophilic exterior, which enable them to form inclusion complexes with lipophilic components. These non-covalent inclusion complexes can improve the aqueous solubility, chemical and physical stability and thus the bio-availability of the sequestered molecule [14–17]. In this study, a derivative of the naturally occurring β-cyclodextrin, namely HPBC, has been used. First, the conditions for using HPBC in rat hepatocyte co-cultures have been optimised. Subsequently, HPBC has been applied to deliver physiological concentrations of T3 and T4 in order to investigate their effect on the expression of the individual GST isoenzymes at the activity and protein levels.

2. Materials and methods

2.1. Materials

Crude collagenase type I, bovine serum albumin fraction V, insulin, EA, glutathione, and epoxy-activated Sepharose 6B were purchased from the Sigma Chemical Co. Fetal bovine serum and trypsin-EDTA solution were from Gibco BRL. Hydrocortisone hemisuccinate came from Upjohn s.a. and the fungicide amphotericin B from Bristol-Myers-Squibb Belgium n.v. CDNB and DCNB were purchased from Merck Belgolabo. NBD-Cl and HPBC (Encapsin™ HPB, 30.221.54) came from Janssen Chimica. T3 and T4 sodium salts were obtained from Fluka Chemie. All other chemicals were readily available commercial products and were used without further purification.

2.2. Isolation and co-culture of rat hepatocytes

Hepatocytes were isolated from outbred adult male Sprague–Dawley rats weighing 200–250 g (Iffa Credo) by a two-step perfusion of the liver with a collagenase solution [18]. After testing the cell integrity by trypan blue exclusion, the hepatocytes were co-cultured with rat liver epithelial cells as described elsewhere [19]. The medium was changed daily thereafter with serum-free medium supplemented with T3 or T4 (from 10^{-9} to 10^{-5} M). For T3 and T4, final concentrations of 0.02 and 0.10% HPBC (w/v) in PBS were used, respectively. Appropriate control cultures, containing either 0.02 or 0.10% HPBC (w/v), were included in this study.

2.3. LDH leakage test

Cytotoxicity of HPBC in co-cultures was assessed by measuring LDH leakage into the culture medium (Merck Belgolabo) and was expressed as the percentage of the ratio of the LDH release in the medium versus the total LDH present in the medium and cells.

2.4. Enzyme assays

GST isoenzyme activities were measured spectrophotometrically in cytosolic fractions. Four different substrates were used, namely CDNB as broad spectrum substrate [20], DCNB as Mu class M1/M2-specific substrate [20], NBD-Cl as Alpha class A1/2-specific substrate [21] and EA as Pi class P1 substrate [22].

2.5. Isolation and HPLC analysis of GST subunits

GST isoenzymes were isolated from cytosolic fractions by affinity chromatography using a glutathione affinity column (1.6×1.2 cm) packed with epoxy activated Sepharose 6B that had been reacted with glutathione [23]. The different GST subunits were separated and quantified as described previously [3,24], originally based on the method of Ostlund Farrants *et al*. [25].

2.6. Measurement of cytosolic protein content

The cytosolic protein content was determined using the Bio-Rad protein assay based on the principle of Bradford [26] (Bio-Rad protein Assay kit, Bio-Rad Laboratories) with bovine serum albumin as a standard.

2.7. Determination of hormonal concentrations

The concentrations of T3 and T4 in freshly prepared solutions and media taken from co-cultures 24 hours after media renewal were determined by a radioimmunoassay using Corning-Reagent kits (Corning Medical no 474207-09). Hormonal concentrations in freshly prepared media did not differ more than 5% from the theoretical concentrations. However, 24 hr after medium renewal (daily change), the T3 concentration in the medium was decreased by approximately 85 and 50% during the first 2 and 4 days of culture, respectively, whilst for T4 the medium concentration was reduced by 45 and 30%, respectively. From 7 days on, a decline of about 17% was observed for both T3 and T4 concentrations. Physiological serum concentrations of T3 and T4 in adult male rats have been determined previously and were 7.2 ± 4.1 nM for T3 and 0.22 ± 0.09 μM for T4 [27].

3. Results

3.1. HPBC in co-cultured rat hepatocytes

In order to detect eventual cytotoxic effects of HPBC on co-cultured rat hepatocytes, the LDH leakage index was measured as a function of culture time and HPBC concentration (Fig. 1). It was found that in non-HPBC-treated control cultures, the LDH index remained constant at $11 \pm 3\%$ throughout the whole culture period.

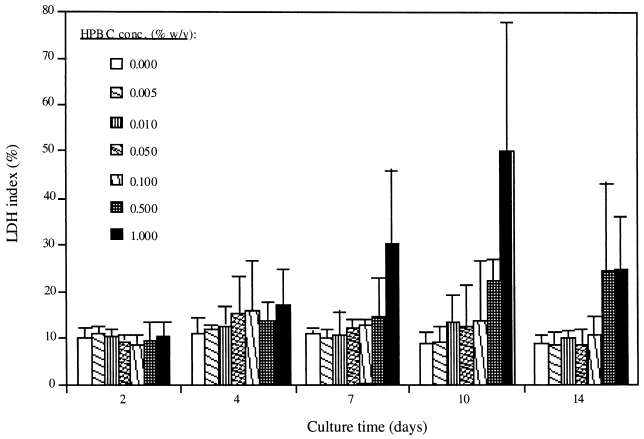

Fig. 1. LDH index (%) in co-cultured rat hepatocytes, measured as a function of culture time and HPBC concentration in w/v% (N = 3). The results are shown as mean ± SD. Each determination is performed in triplicate.

However, increased membrane permeability was observed in cells exposed for 7 and 10 days to concentrations ≥1.00% and 0.50% HPBC, respectively. Based on these results, the maximum non-cytotoxic concentration of HPBC in rat hepatocytes, co-cultured for 14 days, was assumed to be 0.10%. This concentration has never been exceeded in further experiments. With respect to the effect of HPBC on GST expression, it was found that the final concentrations of 0.02 and 0.10% HPBC, used to dissolve T3 and T4, respectively, exhibited no statistically significant inducing nor inhibiting effects on any particular GST activity (Fig. 2). A significant increase of the P1 content was, however, observed in cultures ex-

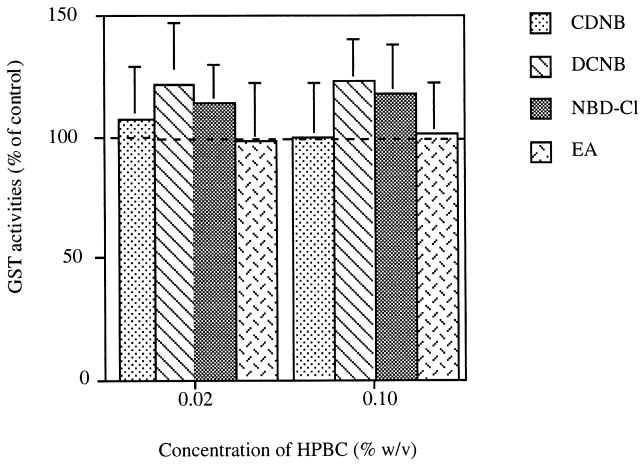

Fig. 2. GST activities measured toward CDNB, DCNB, NBD-Cl, and EA in 10-day-old co-cultured adult rat hepatocytes, untreated or exposed to 0.02 and 0.10% (w/v) HPBC. The results are shown as the mean ± SD and are expressed as a percentage of the values obtained in control cultures (dashed line) i.e. 0.397 ± 0.089, 0.017 ± 0.004, 0.070 ± 0.018, and 0.027 ± 0.008 U/mg cytosolic proteins for the CDNB, DCNB, NBD-Cl, and EA activity, respectively. No statistical significant differences could be detected between the values obtained for the HPBC-exposed and non-exposed cultures by using a paired Student's *t*-test (N = 8).

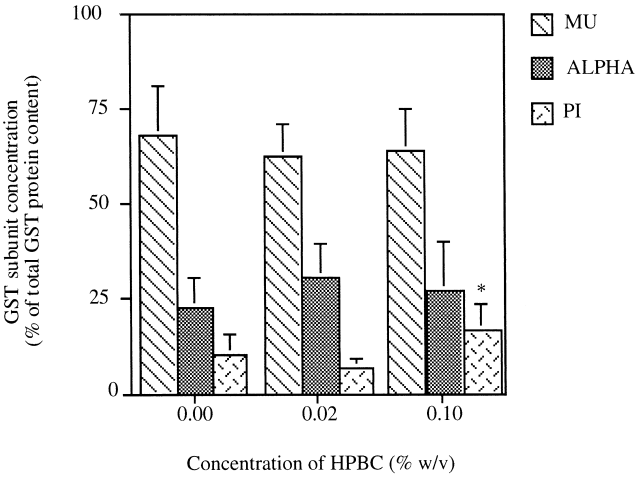

Fig. 3. Mu, Alpha and Pi class GST protein contents of adult rat hepatocyte co-cultures, either non-exposed or exposed for 10 days to different concentrations of HPBC (% w/v). The mean total GST protein content found after 10 days of co-culture without HPBC was 168.0 ± 41.3 μg/mg cytosolic proteins. *Denotes a statistically significant difference between the values obtained in HPBC-treated and untreated cultures with $P < 0.05$ according to a paired Student's *t*-test (N = 8).

posed to 0.10% HPBC, but no concentration-dependent effects of HPBC on Mu, Alpha or Pi class subunit contents could be detected (Fig. 3). These results indicate that whenever hepatocytes in culture have to be exposed to lipophilic substances, including thyroid hormones, to investigate their effects on GST expression, HPBC is a convenient delivery system.

3.2. Effect on GST of T3 delivered by HPBC

After continuous exposure of co-cultured rat hepatocytes for 10 days to T3 (concentrations from 10^{-8} to 10^{-5} M), the GST activity measured toward CDNB, DCNB and NBD-Cl significantly decreased by 20 to 40%, when compared to control values (Fig. 4). 10^{-9} M T3 did not have any significant effect on the GST activities measured. The GST activity toward EA was also not affected after 10 days exposure to the various T3 concentrations. Similar to the activity results, a reduction of the total GST protein content by 15 to 40% was observed in co-cultures exposed to T3 (concentrations from 10^{-7} to 10^{-5} M), related to a reduction in the amounts of Alpha class subunits A1/2 and A3, and Mu class subunits M1 and M2 (Table 1). An increase of P1 content, inversely proportional to the T3 concentration was also observed, indicating that T3 probably had an inhibitory effect on the *de novo* synthesis of P1 in the co-cultures. In general, a significant concentration-dependent effect of T3 was found on GST activities and proteins measured.

3.3. Effect on GST of T4 delivered by HPBC

When compared to the corresponding control values (0.10% HPBC), exposure for 10 days to concentrations of

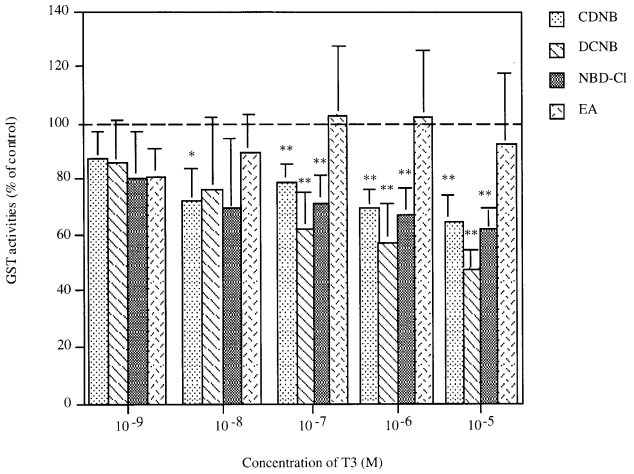

Fig. 4. GST activities measured toward CDNB, DCNB, NBD-Cl and EA in co-cultured adult rat hepatocytes after a 10-day exposure to different T3 concentrations. Enzyme activities are expressed as a percentage of the values obtained in controls exposed to 0.02% HPBC (w/v) (dashed line). In the latter case, mean specific activities toward CDNB, DCNB, NBD-Cl, and EA were 0.421 ± 0.093, 0.022 ± 0.006, 0.079 ± 0.021, and 0.026 ± 0.008 U/mg cytosolic proteins, respectively. Statistical significant differences between the values obtained in T3-treated and control cultures were determined by a paired Student's *t*-test (N = 3 for 10^{-8} and 10^{-9} M; N = 5 for 10^{-5}, 10^{-6}, and 10^{-7} M) (*$P < 0.05$; **$P < 0.01$).

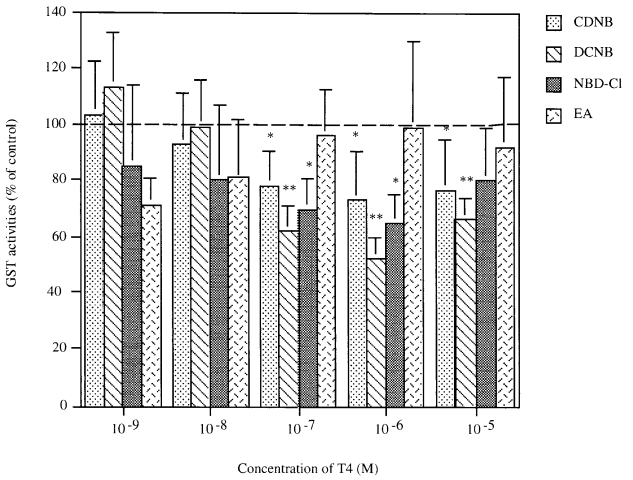

Fig. 5. GST activities measured toward CDNB, DCNB, NBD-Cl, and EA in co-cultures of adult rat hepatocytes following a 10-day exposure to different T4 concentrations. Enzyme activities are expressed as a percentage of the values obtained in controls exposed to 0.10% HPBC (w/v) (dashed line) i.e. 0.395 ± 0.096, 0.021 ± 0.003, 0.082 ± 0.024, and 0.027 ± 0.007 U/mg cytosolic proteins for the CDNB, DCNB, NBD-Cl, and EA activity, respectively. Statistical significant differences between the values obtained in T4-treated and control cultures were determined by a paired Student's *t*-test (N = 3 for 10^{-8} and 10^{-9} M; N = 5 for 10^{-5}, 10^{-6}, and 10^{-7} M) (*$P < 0.05$; **$P < 0.01$).

10^{-5}, 10^{-6} and 10^{-7} M T4 significantly decreased the GST activities of the co-cultured hepatocytes measured toward CDNB, DCNB and NBD-Cl (Fig. 5). No effect of T4 on the EA activity could be detected. Exposure to the lowest concentrations 10^{-8} and 10^{-9} M T4 for 10 days, did not significantly affect the activity of the different GSTs under study.

HPLC analysis showed that, compared to control values, the total GST protein concentration decreased by 35% when the cultures were exposed to 10^{-6} and 10^{-7} M T4, whereas the abundance of GST proteins was only 20% reduced upon exposure to the highest concentration of 10^{-5} M T4 (Table 2). Also here, a general concentration-dependent decrease of the GST activities, due to a decrease of the GST subunit contents, was found after T4 exposure. However, it was less significant than the effects elicited by T3.

4. Discussion

Although *in vivo* studies in rats clearly indicate that thyroid hormones inhibit GST expression [7], no direct

Table 1
GST Subunit profile of co-cultured rat hepatocytes exposed for 10 days to different concentrations of T3

Subunit	GST subunit concentration (μg/mg cytosolic protein)					
	Control 0.02%	10^{-9} M T3	10^{-8} M T3	10^{-7} M T3	10^{-6} M T3	10^{-5} M T3
Alpha class						
A1/2	5.9 ± 3.4	5.4 ± 1.0	5.0 ± 2.4	2.8 ± 2.4	1.6 ± 0.6	1.9 ± 0.2
A3	5.7 ± 3.4	2.7 ± 0.1	2.9 ± 1.0	2.8 ± 2.7	1.4 ± 0.8	3.5 ± 1.7
A4	0.6 ± 0.5	1.1 ± 0.4	0.8 ± 0.3	0.7 ± 0.2	0.6 ± 0.3	0.4 ± 0.3
Subtotal	12.2 ± 5.2	9.2 ± 1.2	8.8 ± 3.3	6.4 ± 5.1*	3.6 ± 1.2*	5.7 ± 1.8*
Mu class						
M1	12.1 ± 2.2	21.8 ± 6.9	17.3 ± 4.6	11.8 ± 8.1	7.9 ± 2.4	7.2 ± 3.8*
M2	12.0 ± 3.9	18.2 ± 2.7**	16.0 ± 3.9	8.1 ± 4.5	5.8 ± 3.2	3.9 ± 0.7*
M3	0.3 ± 0.1	1.2 ± 0.2**	1.8 ± 0.6	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Subtotal	24.4 ± 4.3	41.3 ± 9.4	35.6 ± 9.1	20.1 ± 12.6	13.8 ± 5.4	11.3 ± 4.4*
Pi class						
P1	2.3 ± 0.7	22.8 ± 4.7*	20.6 ± 2.3*	7.0 ± 2.2*	6.4 ± 1.9*	6.2 ± 2.2
Total	39.4 ± 7.8	73.4 ± 8.9	65.0 ± 9.5	33.5 ± 17.7	23.8 ± 4.6	22.0 ± 5.4

Values are mean ± SD ($N = 3$ for 10^{-8} and 10^{-9} M; N = 5 for 10^{-5}, 10^{-6}, and 10^{-7} M). Statistical significant difference between values obtained in T3-treated and control cultures are given by *$P < 0.05$ or **$P < 0.01$, according to a paired Student's *t*-test.

Table 2
GST subunit profile of co-cultured rat hepatocytes exposed for 10 days to different concentrations of T4

Subunit	GST subunit concentration (μg/mg cytosolic protein)					
	Control 0.10%	10^{-9} M T4	10^{-8} M T4	10^{-7} M T4	10^{-6} M T4	10^{-5} M T4
Alpha class						
A1/2	6.9 ± 3.5	7.7 ± 1.5	5.0 ± 2.9	2.4 ± 0.7	2.0 ± 0.4*	2.3 ± 1.7
A3	4.3 ± 2.1	5.5 ± 0.4	4.7 ± 1.6	3.9 ± 1.4	3.3 ± 2.4	4.6 ± 3.4
A4	1.4 ± 0.9	1.0 ± 0.7	1.0 ± 0.3	0.9 ± 0.6	0.6 ± 0.4	1.2 ± 1.1
Subtotal	12.7 ± 5.8	14.2 ± 2.6	10.7 ± 2.8	7.2 ± 1.3	5.9 ± 2.8	8.1 ± 4.6
Mu class						
M1	16.7 ± 5.8	26.4 ± 4.7	23.4 ± 5.0	11.4 ± 3.7	10.7 ± 4.3	13.4 ± 5.2
M2	15.2 ± 8.3	26.6 ± 0.8	25.3 ± 6.0	6.0 ± 2.5*	6.3 ± 1.3	8.9 ± 3.8
M3	0.5 ± 0.2	1.9 ± 0.5	3.5 ± 1.8	0.1 ± 0.1*	0.5 ± 0.3	0.3 ± 0.2
Subtotal	32.4 ± 13.6	54.9 ± 6.0	52.2 ± 11.2	17.5 ± 6.0	17.4 ± 4.8	22.5 ± 7.5
Pi class						
P1	7.5 ± 2.1	21.8 ± 10.8	26.0 ± 8.2	7.9 ± 1.7	8.3 ± 1.9	13.3 ± 7.9
Total	49.9 ± 14.5	90.9 ± 7.4	88.9 ± 2.6	32.6 ± 7.8	31.7 ± 7.3	44.0 ± 13.7

Values are mean ± SD ($N = 3$ for 10^{-8} and 10^{-9} M; N = 5 for 10^{-5}, 10^{-6}, and 10^{-7} M). Statistical significant difference between values obtained in T4-treated and control cultures are given by $*P < 0.05$, according to a paired Student's *t*-test.

effect of T3 on total GST activity [10] nor on the cytosolic T3-binding protein [28] could be observed when monolayer cultures of rat hepatocytes were used as *in vitro* models. Several possible explanations could be forwarded: (i) monolayer cultures of rat hepatocytes are not suitable as *in vitro* models because of the rapid loss of metabolic integrity [1], (ii) organic solvents used to dissolve the hormones interfere with several metabolic pathways present within cultured hepatocytes [12,13], or (iii) a combination of both events occurs.

In previous work, we observed that exposure of more sophisticated culture models, namely co-cultures of rat hepatocytes with primitive rat biliary epithelial cells, to T3 and T4 solved in ethanol, also decreased GST expression [24]. The results, however, remained questionable because of significant inducing effects of ethanol on GST expression in control cells [13]. These results were only measurable when unphysiologically high concentrations of hormones were used, namely 10^{-5} M for both T3 and T4 [24]. In this study, co-cultures of rat hepatocytes were again exposed to T3 and T4, but the hormones were included into HPBC. Using this alternative delivery system, a direct concentration-dependent decrease of T3 (10^{-5}, 10^{-6} and 10^{-7} M) on CDNB, DCNB and NBD-Cl activities could be observed. No effect, however, could be detected on the Pi class GST activity measured toward EA, which is in accordance with earlier data of Tsuchida and Sato [29].

To become bioactive, T4 needs to be converted into T3 via outer deiodination [30,31]. However, 20% of available T4 is also metabolised through glucuronidation or sulfation of its phenolic hydroxyl group, leading to either excretion into the bile or irreversible degradation, respectively [32]. Here we found that the effects observed for T3 and T4 were nearly identical, suggesting that co-cultivated rat hepatocytes were capable of converting T4 into T3. However, as the highest concentration of T4 (10^{-5} M) exerted a smaller inhibition on GST activities and proteins than 10^{-6} and 10^{-7} M T4, only a limited amount of T4 seemed to be metabolised into active T3.

The effects observed on GST of T3 and T4 delivered by HPBC do, however, not exactly match those found when ethanol was used as a solvent [24]. In the latter study, it was shown that T3 and T4 decreased mainly the levels of Alpha and Mu class proteins, respectively, whereas here similar effects of T3 and T4 on GST expression have been found. A possible explanation therefore might be that ethanol, in contrast to HPBC, inhibits the enzyme 5′-deiodinase that converts T4 into T3. If such is the case, a 'net effect' of T4 on GST expression is detected in the presence of ethanol, while in the presence of HPBC T4 is well converted into T3 and thus T4 and T3 exert comparative effects. This hypothesis needs to be further elucidated. However, since 5′-deiodinases require selenium for their activity [33–35] and it has been shown that ethanol diminishes selenium blood levels after chronic alcohol consumption [36,37], it is well possible that the activity of 5′-deiodinase is reduced by withdrawal of its cofactor.

To our best knowledge, the use of HPBC in hepatocyte cultures has not yet been reported. Findings in this study do, however, indicate that HPBC at a maximum concentration of 0.10% (w/v) is a good alternative for organic solvents to supply hydrophobic molecules such as T3 and T4 to hydrophilic culture media. We further show that, on exposure to more physiological concentrations of both hormones by their inclusion into HPBC, T3 and T4 concentration-dependently decreased the levels of GST expression. Therefore, the co-culture model combined with the HPBC delivery system could represent a powerful *in vitro* tool in drug development.

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