

Suppression of bile acid synthesis by thyroid hormone in primary human hepatocytes

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Supported by the Swedish Research Council, the Karolinska Institute and the Swedish Society for Medical Research

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Received: 2005-10-04 Accepted: 2005-11-10

Key words: Hepatocytes; Human; Bile acids; CYP7A1; CYP8B1; CYP27A1

Ellis ECS. Suppression of bile acid synthesis by thyroid hormone in primary human hepatocytes. *World J Gastroenterol* 2006; 12(29): 4640-4645

<http://www.wjgnet.com/1007-9327/12/4640.asp>

Abstract

AIM: It is known that thyroid hormones alter the bile acid metabolism in humans, however the effect on individual enzymes has been difficult to elucidate. This is mainly due to the lack of human liver cell lines producing bile acids. We used cultures of primary human hepatocytes to study the effects of triiodothyronine (T_3) on bile acid synthesis.

METHODS: Primary hepatocytes were isolated from liver tissue obtained from three different patients undergoing liver resection due to underlying malignancy. The hepatocytes were cultured under serum-free conditions and treated from d 1 to d 5 with culture containing 0.1 - 1000 nmol/L of T_3 . Bile acid formation and mRNA levels of key enzymes were analysed.

RESULTS: The lowest concentration of T_3 decreased cholic acid (CA) formation to 43%-53% of controls and chenodeoxycholic acid (CDCA) to 52%-75% of controls on d 5. The highest dose further decreased CA formation to 16%-48% of controls while CDCA formation remained at 50%-117% of controls. Expression of mRNA levels of cholesterol 7α -hydroxylase (CYP7A1) and sterol 12α -hydroxylase (CYP8B1) dose-dependently decreased. Sterol 27-hydroxylase (CYP27A1) levels also decreased, but not to the same extent.

CONCLUSION: T_3 dose-dependently decreased total bile acid formation in parallel with decreased expression of CYP7A1 and CYP8B1. CA formation is inhibited to a higher degree than CDCA, resulting in a marked decrease in the CA /CDCA ratio.

INTRODUCTION

The effect of thyroid hormone on human bile acid synthesis is still unclear. Several *in vivo* observations have suggested that thyroid hormone influences the bile acid synthesis^[1-3]. However, due to the lack of human *in vitro* tools such as cell lines synthesizing normal bile acids, investigators have not been able to study this at a cellular level. We have previously shown that primary human hepatocytes cultured under serum-free conditions in the presence of matrixgel (EHS) synthesize and conjugate normal bile acids^[4,5]. We also showed that under these conditions they synthesize bile acids with the same capacity and composition as under similar *in vivo* conditions.

In the conversion of cholesterol into bile acids two main pathways are recognized^[6-8]. The neutral pathway is initiated by the 7α -hydroxylation of cholesterol, catalyzed by the microsomal rate-limiting enzyme cholesterol 7α -hydroxylase (CYP7A1). The acidic pathway is initiated by the 27 -hydroxylation of cholesterol, catalyzed by the mitochondrial enzyme sterol 27 -hydroxylase (CYP27A1). In the acidic pathway the microsomal oxysterol 7α -hydroxylase (CYP7B1) mediates the 7α -hydroxylation. Humans synthesize two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA). CA has a hydroxyl group at the 12^{th} carbon distinguishing it from CDCA. The hydroxyl group is introduced by the microsomal enzyme sterol 12α -hydroxylase (CYP8B1). Therefore, the total amount of intermediates (from either pathway) that undergoes 12α -hydroxylation determines the ratio of CA to CDCA. Both pathways can synthesize CA and CDCA, however the acidic pathway is believed to produce mainly CDCA. The most important mechanism for regulation of bile acid synthesis is the portal flux of bile acids returning to the liver. The loss of bile acids in the intestine (5%-10%) is compensated by *de novo* synthesis.

In this study we examined the effect of different doses

of triiodothyronine (T₃) on bile acid formation and on mRNA levels of *CYP7A1*, *CYP8B1* and *CYP27A1* in cultures of primary human hepatocytes.

MATERIALS AND METHODS

Materials

RPA III™ kit and pTRI-cyclophilin-Human antisense control template were purchased from Ambion Inc. (Huntingdon, UK). ³⁵S-UTP was purchased from Hartmann Analytic GmbH (Braunschweig, Germany). William's medium E with glutamax, gentamycin, penicillin-streptomycin, Dulbecco's phosphate buffered saline, Trizol reagent and Superscript II RT were purchased from Invitrogen (Täby, Sweden). Actrapid (insulin) 100 IU/mL was from Novo Nordisk Pharma (Malmö, Sweden). Collagenase XI-S (Cat. No. C7557), methylthazolyl-diphenyl-tetrazolium bromide (Cat. No. M5655) and triiodothyronine (Cat. No. T6397) were purchased from Sigma-Aldrich (Sweden). Random hexamers (Cat. No. C1181), recombinant RNasin ribonuclease inhibitor (Cat. No. N2511) and RQ DNase I (Cat. No. M6101) were purchased from Promega (Southampton, UK). Taqman universal master mix (Cat. No. 4304437) and human cyclophilin pre-developed assay (now discontinued and replaced by Human PPIA endogenous control (Cat. No. 4310883E) were purchased from Applied Biosystems (Stockholm, Sweden). Dithiothreitol (Cat. No. 708984) was purchased from Roche (Bromma, Sweden).

Isolation of primary human hepatocytes

Human liver tissue was obtained from three different patients undergoing reduction hepatectomy for metastatic cancer to the liver where normal liver tissue was resected due to the localization of the tumor. Patient A was a 62 year old female, patient B a 47 year old male and patient C a 49 year old female.

Approval to use parts of resected human liver specimens for research was given by the ethics committee at Huddinge University Hospital and the patient's informed consent was obtained.

Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as described in detail by Li *et al.*⁹¹. In brief, liver tissue weighing between 20-150 g was cannulated in 1-2 vessels depending on the size and appearance. The tissue was then perfused with EGTA buffer for about 15 min at a flow rate of 20 mL/min, followed by perfusion with CO₂ (5%) buffered solution containing collagenase XI-S (200 mg /L) at 40°C for approximately 75 min.

Culture conditions

Hepatocytes (3.5×10^6) were cultured under standard conditions in 60 mm culture dishes containing 200 µL EHS matrigel and 3 mL William's E medium with glutamax, supplemented with insulin (2 IU/L), penicillin G sodium (100 U/mL), streptomycin sulphate (100 µg/mL) and gentamycin (85 µg/mL). Cell culture media were collected and renewed everyday. Three 60 millimeter culture dishes each containing 3.5×10^6 hepatocytes were used for each treat-

ment. The cells and media from three dishes were pooled when harvested. The cells were subjected to different concentrations of T₃ (0.1, 1, 10, 100 and 1000 nmol/L) during four days, from d 1 to d 5. On d 5 cell culture media were collected for bile acid analysis and the cells were harvested for RNA isolation and subsequent quantification of specific mRNAs.

Analysis of bile acids in cell culture media

Bile acids in cell culture media were analysed as previously described¹⁴¹. Briefly, 2 mL of the harvested culture medium, together with deuterium labelled cholic acid (D₅) and chenodeoxycholic acid (D₄), was subjected to basic hydrolyzation to remove conjugates and the bile acids were extracted using acidic ether. Bile acids were analysed by gas chromatography-mass spectrometry, as described previously^{11,101}.

Isolation of total RNA

Total RNAs were isolated using Trizol reagent as described by the manufacturer. RNA concentration was determined by spectrophotometry at 260 nm. The purity of the RNA was determined by spectrophotometry at 280 nm and the integrity checked by agarose gel electrophoresis stained with ethidium bromide.

Quantification of CYP7A1 by real time PCR

mRNA levels of CYP7A1 were determined using quantitative single-plex real time PCR. From each group, 40 µg of total RNA was incubated with 5 U of RQ DNase, 5 µL 10 × RQ buffer in a total volume of 50 µL at 37°C for 20 min, at 20°C for 15 min and finally at 70°C for 10 min. From this, 4 µg of DNase treated RNA was used for cDNA synthesis by mixing together with 2 µL random hexamer primers (100 ng/µL), 1 µL dNTP (10 mmol/L of each) in a total volume of 12 µL. After incubation at 65°C for 5 min, 25°C for 10 min and 42°C for 2 min, 2 µL of DTT (0.1 mol/L), 1 µL of RNasin (40 U/µL), 4 µL 5 × first strand buffer and 1 µL superscript II (200 U/µL) were added. The incubation was continued at 42°C for 50 min and at 70°C for 10 min. For real time PCR 3 µL undiluted cDNA/sample was used in triplicates.

Sequences for CYP7A1 primers and probe were kindly provided by Dr. Hermansson, AstraZeneca, Mölndal, Sweden. Reversed primer is: 5'AGA GCA CAG CCC AGG TAT G 3'; forward primer, 5' TGA TTT GGG GGA TTG CTA TA 3' (300 mmol/L); and probe 5' TGG TTC ACC CGT TTG CCT TCT CCT 3' (200 mmol/L). The taqman probe was labelled with FAM and TAMRA. The assays were performed according to ABI user bulletin #2 protocol in a total volume of 25 µL. VIC labelled human cyclophilin pre-developed taqman assay from Applied Biosystems was used for internal control. The assay was performed and analysed using ABI Prism 7700 sequence detector system.

Quantification of CYP8B1 and CYP27A1 by RNase protection assay

Abundance of specific mRNA was quantified using RPA III™. cRNA probes were synthesized using T₃- or T₇-polymerase and labelled with ³⁵S-UTP. Cyclophilin was

used as an internal standard and gave a protected fragment size of 103 bp.

The protected fragment size of the probe for CYP27A1 was 297 bp and for CYP8B1 442 bp. The assay was performed according to the manufacturer's protocol. In brief, 10-15 µg of total RNA was co-precipitated in 2.5 volumes of ethanol and 0.5 mol/L NH₄OAc with about 80 000 cpm of each probe at -20°C for 15 min. Following centrifugation at 15 000 rpm for 15 min the pellets were dissolved in 10 µL hybridisation buffer, heated to 95°C for 3 min and incubated at 46°C overnight. The hybridised RNA samples were then treated with RNase A and RNase A/T1 diluted 1:50 in RNase solution at 37°C and inactivated after 30 min by adding inactivation/precipitation solution. Following precipitation at -20°C for 15 min the samples were centrifuged at 15 000 r/min for 15 min and the pellets were air dried and dissolved in 10 µL of loading buffer.

The protected fragments were separated on a denaturing 5% polyacrylamide urea gel and detected and quantified using a Fuji BAS 1800 Phospho-imager.

Statistical analysis

The significance of differences between groups for the mRNA analysis was tested by a one-way ANOVA followed by a post-hoc test. To stabilize the variances, data were logarithmically transformed. Due to variances between patients, statistical analysis of the bile acid formation could not be performed.

RESULTS

Purity of hepatocyte preparations

We have analyzed the purity of the preparation of hepatocytes that we plated. Following the final low speed centrifugation step the cell pellet was resuspended in plating media and samples of the cell preparation were spotted onto microscopic glass slides, air dried and fixed in formalin. Preparations from 2 separate human cases were analyzed. Duplicate slides were exposed to antibodies for low molecular weight cytokeratins (AE1/AE3), albumin or hepatocyte specific antibody HEPR. The results showed that 93% of the cells in the preparation reacted with antibodies to cytokeratin, albumin and HEPR, indicating that 93% of the cells plated on culture dishes were epithelial (cytokeratin positive) and parenchymal hepatocytes (HEPR and albumin positive). Most of the remaining cells were small and appeared to be hematopoietic. Hematopoietic cells would not be expected to attach to culture dishes and would be removed by media changes.

Patient A

The cells were treated for 4 d (from d 1 to d 5) with different concentrations of T₃. The cell culture media were changed daily and collected on d 5 of culture after 24 h incubation. In control cells the total bile acid formation was 1515 ng/mL of culture media (920 ng CA and 595 ng CDCA per mL) and the CA/CDCA ratio 1.5. Treatment with 0.1 nmol/L of T₃ decreased total formation by

Table 1 Formation of CA and CDCA in primary human hepatocytes obtained from 3 patients

Patient	Addition of T ₃	CA	CDCA	Ratio
		ng/mL	Medium/24 h	CA/CDCA
A	Control	920	595	1.5
	0.1 nmol/L	400	310	1.3
	1 nmol/L	375	375	1.0
	10 nmol/L	340	390	0.9
	100 nmol/L	250	285	0.9
	1 µmol/L	245	295	0.8
B	Control	280	60	4.7
	0.1 nmol/L	135	45	3.0
	1 nmol/L	95	70	1.4
	10 nmol/L	55	60	0.9
	100 nmol/L	45	65	0.7
	1 µmol/L	45	70	0.6
C	Control	805	502	1.6
	0.1 nmol/L	430	345	1.2
	1 nmol/L	477	392	1.2
	10 nmol/L	395	425	0.9
	100 nmol/L	372	405	0.9
	1 µmol/L	382	420	0.9

The cells were cultured in a serum-free medium and treated with different concentrations of T₃ for 5 d. Bile acids were measured in the cell-free medium. CA: Cholic acid; CDCA: Chenodeoxycholic acid.

53% to 710 ng/mL and lowered the CA/CDCA ratio to 1.3 (Table 1). Higher doses of T₃ decreased bile acid formation even further in a dose dependent manner, down to 36% of control with a CA/CDCA ratio of 0.8 for the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 4.45 to 0.54 (arbitrary value) (12% of control), 10 nmol/L to 0.44 and 1000 nmol/L further down to 0.34 (8% of control). Two of the measure points were lost due to human error (Figure 1).

Similarly, expression of CYP8B1 mRNA decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 from 0.226 to 0.056 (arbitrary value) (12% of control), 10 nmol/L to 0.028 and 1000 nmol/L to 0.017 (8% of control) (Figure 1).

Also CYP27A1 mRNA levels decreased with T₃ treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 1.050 to 0.408 (arbitrary value) (39% of control), 10 nmol/L to 0.373 and 1000 nmol/L to 0.334 (32% of control) (Figure 1).

Patient B

The bile acid formation in hepatocytes from patient B was much lower than in cells from patients A and C. In control cells the total bile acid formation was 340 ng/mL of culture media (280 ng CA and 60 ng CDCA per mL) and the CA/CDCA ratio was 4.7. Treatment with 0.1 nmol/L of T₃ decreased total formation by 47% to 180 ng/mL, and lowered CA/CDCA ratio to 3.0 (Table 1). Higher doses of T₃ decreased bile acid formation even further down to 34% of control and CA/CDCA ratio to 0.6 for

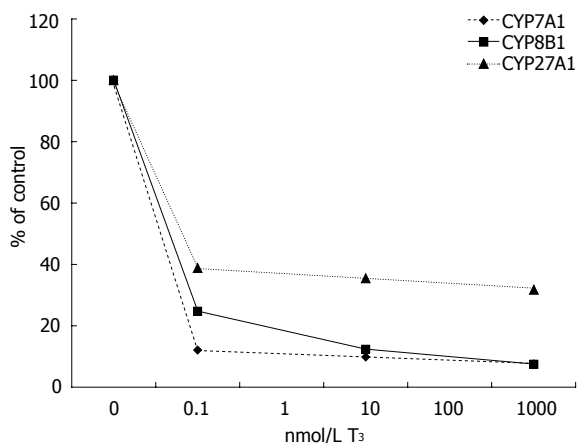


Figure 1 Patient A, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

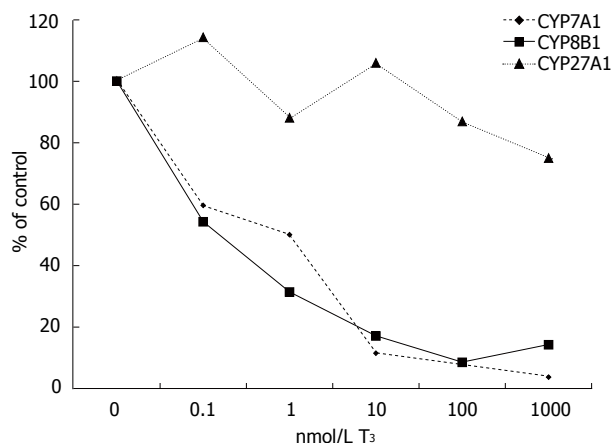


Figure 2 Patient B, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested but not to the same extent as in cells from patient A. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 0.745 to 0.444 (arbitrary value) (60% of control), 1 nmol/L to 0.373, 10 nmol/L to 0.086, 100 nmol/L to 0.063 and 1000 nmol/L further down to 0.028 (4% of control) (Figure 2).

Similarly, expression of CYP8B1 decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 mRNA levels from 0.175 to 0.095 (arbitrary value) (54% of control), 1 nmol/L to 0.055, 10 nmol/L to 0.030, 100 nmol/L to 0.015 and 1000 nmol/L further down to 0.025 (14% of control) (Figure 2).

T₃ had a modest effect on the expression of CYP27A1 in patient B. Addition of 0.1 nmol/L of T₃ changed CYP27A1 mRNA levels from 0.420 to 0.480 (arbitrary value) (114% of control), 1 nmol/L to 0.370, 10 nmol/L to 0.445, 100 nmol/L to 0.365 and 1000 nmol/L down to 0.315 (75% of control) (Figure 2).

Patient C

In control cells total bile acid formation was 1308 ng/mL of culture media (805 ng CA and 502 ng CDCA per mL) and the CA/CDCA ratio was 1.6. Treatment with 0.1 nmol/L of T₃ decreased the total formation by 41% to 775 ng/mL and lowered the CA/CDCA ratio to 1.2 (Table 1). Higher doses of T₃ decreased bile acid formation to 61% of control and lowered the CA/CDCA further down to 0.9 for the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested, but not as strongly as in patients A and B. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 3.15 to 1.64 (arbitrary value) (52% of control), 1 nmol/L to 2.06, 10 nmol/L to 1.06, 100 nmol/L to 0.81 and 1000 nmol/L further down to 0.65 (21% of control) (Figure 3).

Similarly, expression of CYP8B1 mRNA decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 mRNA levels from 0.093 to 0.070 (arbitrary value) (75% of control), 1 nmol/L to 0.040, 10 nmol/L to 0.050, 100 nmol/L and 1000 nmol/L both to

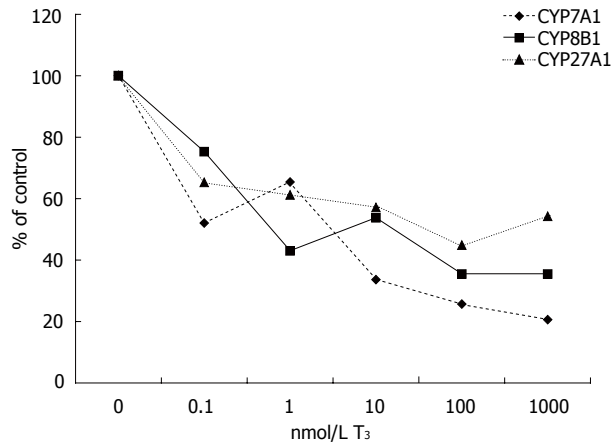


Figure 3 Patient C, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

0.033 (35% of control) (Figure 3).

Also CYP27A1 mRNA levels decreased with T₃ treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 0.245 to 0.160 (arbitrary value) (65% of control), 1 nmol/L to 0.150, 10 nmol/L to 0.140, 100 nmol/L to 0.110 and 1000 nmol/L to 0.133 (54% of control) (Figure 3).

Following logarithmical transformation, statistical analysis of mRNA levels from all three patients showed that the highest dose (1000 nmol/L) of T₃ significantly decreased CYP7A1 mRNA levels ($P < 0.05$). CYP8B1 mRNA levels was significantly decreased by 10 and 100 nmol/L of T₃. Treatment with T₃ did not significantly decrease CYP27A1 mRNA levels.

DISCUSSION

In accordance with our previous studies^[4,5,11,12], CA and CDCA, constituted more than 95% of total sterols and the ratio between the two bile acids averaged 2.6.

T₃ dose-dependently decreased formation of bile acids to 44% of controls. The reduction of CA formation was greater than the decline in CDCA formation and

resulted in a marked decrease in the CA/CDCA ratio. These results are in reasonable agreement with previous *in vivo* studies. Angelin *et al*^[13] studied bile acid metabolism in hypothyroid patients before and during replacement therapy to euthyroid state and found that CDCA synthesis was stimulated by approximately 40%, whereas CA did not change with hormone treatment. However, the relative concentration of deoxycholic acid (DCA) in bile decreased from 30% to 19% and CDCA concomitantly increased with hormone therapy. This was confirmed by Kosuge *et al*^[2] who concluded that the characteristic effect of thyroid hormone on serum bile acid composition in man is the shift from the "family" of cholic to that of CDCA by showing that DCA is the most prominent bile acid in hypothyroid patients and that CDCA is the most prominent bile acid in hyperthyroid patients. Paulezki *et al*^[3] studied bile acid metabolism in hyperthyroid patients before and during treatment to euthyroid state and showed that thyroid hormone excess caused a 34% reduction in CA synthesis with no apparent change of CDCA synthesis and a 20% decrease in total bile acid synthesis. The authors suggested that thyroid hormone inhibited hepatic 12 α -hydroxylation.

In contrast to this, Sauter *et al* failed to show any effect of T₃ treatment on the serum levels of the bile acid intermediate 7 α -hydroxy-4-cholestene-3-one (C4) (believed to reflect CYP7A1 activity *in vivo*) in hypo- and hyperthyroidism before and after treatment to euthyroid state^[14]. A recent study by Galman *et al* showed that there are marked diurnal changes of C4 levels during the day^[15]. The authors could also see a weak but significant correlation of C4 with cholesterol and therefore corrected C4 levels for cholesterol. These findings make it difficult to evaluate the results of the C4 levels of the mentioned study. Also, the fact that C4 is the main substrate for CYP8B1 and that one study suggested that CYP8B1 might be inhibited by thyroid hormone needs to be considered.

The mRNA levels of the rate-limiting enzyme CYP7A1 in the neutral pathway were also dose-dependently decreased by T₃. Our data support the human promoter studies by Wang *et al* who found that thyroid hormone repressed CYP7A1 activity^[16]. Furthermore, Drover *et al* found a distinct thyroid hormone response element that mediates T₃ repression in the human CYP7A1 promoter^[17]. This is in contrast to the study in rats where Crestani *et al* failed to demonstrate an effect of thyroid hormone on the *Cyp7a1* promoter activity^[18]. However addition of 1 μ mol/L of T₄ to primary rat hepatocytes increased the transcriptional activity of *Cyp7a1* by 3.7 fold but did not alter steady state mRNA levels^[19].

In the present study we did not measure the enzyme activity of CYP7A1 because of lack of material, but since the effects on the mRNA levels were in agreement with the effects on the bile acid formation we believed that it would be unlikely that T₃ would have an opposite effect on the enzyme activity.

Similar to CYP7A1, the CYP8B1 mRNA levels were also dose-dependently decreased by T₃. Although previous *in vivo* data pointed towards an effect on CYP8B1, this is to our knowledge the first report of the effects of T₃ on human CYP8B1 mRNA levels. In rats however, T₃ has

been shown to suppress both Cyp8b1 activity and mRNA levels^[20].

Treatment with T₃ also decreased CYP27A1 levels, but to a much lower degree than CYP7A1 and CYP8B1. This finding is in line with the contention that CYP27A1 is regulated to a lower degree than CYP7A1 in humans, as has been shown in connection with bile acid mediated feedback regulation^[21]. According to a previous study by Stravitz *et al*^[22] *Cyp27a1* is not regulated by thyroid hormone at all in rat.

In a previous study^[4] we investigated the effect of the combination of T₃ and dexamethasone on bile acid synthesis in rat *vs* human hepatocytes. Since it was previously shown that T₃ and dexamethasone had a synergistic effect on *Cyp7a1* mRNA levels in primary rat hepatocytes^[19] and since neither T₃ nor dexamethasone alone had any effect in rat hepatocytes, we did not focus on the effect of T₃ or dexamethasone alone in primary human hepatocytes. However, we could see that T₃ and dexamethasone alone tended to decrease bile acid formation but had no consistent effect on CYP7A1 mRNA levels (data not shown). The results of the present study and the fact that combination of T₃ and dexamethasone did not change human bile acid formation or CYP7A1 mRNA levels in our prior study, suggest that dexamethasone might have an effect on human bile acid synthesis. This is very interesting and needs to be elucidated in future studies.

The concentrations of T₃ used in this study ranged from 0.1 nmol/L to 1000 nmol/L. The highest dose was included because this is a dose commonly used in combination with dexamethasone in cultures of primary rat hepatocytes. Reduction of methylthiazolyldiphenylterazolium bromide (MTT) showed no signs of cytotoxicity in the dose range used here (data not shown). It should be emphasized that the most dramatic effects were seen with the lowest T₃ concentration, supporting that the effects observed are physiological.

In conclusion, it has been shown that T₃ dose-dependently decreases bile acid formation and expression levels of CYP7A1 and CYP8B1 mRNAs in primary human hepatocytes. CYP27A1 is also decreased, but to a lower degree. CA formation is inhibited to a higher degree than CDCA formation, resulting in a marked decrease in the CA/CDCA ratio.

ACKNOWLEDGMENTS

The author would like to thank Professor Curt Einarsson and Professor Ingemar Björkhem for critical discussions regarding the manuscript. The skilful technical assistance of Mrs. Anita Lövgren-Sandblom regarding the GC/MS analysis is gratefully acknowledged.

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