9-*cis*-Retinoic Acid Regulation of Four UGT Isoforms in Hepatocytes from Rats with Various Thyroid States

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Purpose. To investigate the influence of thyroid hormone status on the regulation of UGTs expression by 9-*cis*-retinoic acid in cultured rat primary hepatocytes.

Methods. Hepatocytes from rats with various thyroid states were isolated and treated with 9-*cis* retinoic acid ($1 \cdot 10^{-6}$ M). mRNA was amplified by reverse transcription and polymerase chain reaction (RT-PCR) and quantified by UV light densitometry. Variations in the expression levels of four different UGT isoforms (UGT1A1, 1A2, 1A5, and 1A6) that are involved in the glucuronication of bilirubin and phenols were determined by comparison with those of an internal standard, β-actin, which is known to be insensitive to nutritional and hormonal conditions.

Results. Primary hepatocyte cultures from rats with various thyroid states present similar metabolite characteristics to those from hypo- or hyperthyroid animals. The treatment of hepatocytes from hypothyroid rats with 9-*cis*-retinoic acid ($1 \cdot 10^{-6}$ M) did not significantly modify bilirubin and phenol-UGT isoform expression. In contrast, in hepatocytes from normal and specially hyperthyroid rats treated with 9-*cis*-retinoic acid, UGT mRNA levels were modified. This suggests that the effect of retinoic acid on UGT mRNA expression requires the presence of thyroid hormone. This was confirmed by the treatment of cultured hepatocytes from hypothyroid rats with both retinoic acid and L-T_3.

Conclusions. This study demonstrates that in cultured hepatocytes, the thyroid status can differentially modulate the expression of four UGT isoforms, and the regulation of their expression can be affected by 9-*cis*-retinoic acid.

KEY WORDS: UDP-glucuronosyltransferase; 9-*cis*-retinoic acid; thyroid hormone; hepatocytes; rats.

INTRODUCTION

UDP-glucuronosyltransferases (UGT, E.C. 2.4.1.17), a superfamily of drug-metabolizing enzymes located in the endoplasmic reticulum of hepatocytes (1), catalyze the conjugation of glucuronic acid from UDP-glucuronic acid on both endogenous and exogenous compounds. They are actively involved in the metabolism of neurotoxic bilirubin, bile acids, or steroids and thyroid hormones. They have been classified on the basis of amino acid sequence homology into two major families: UGT1 and UGT2. The UGT1 family is involved in the glucuronidation of bilirubin, phenols, and quinones. The UGT2 family includes enzymes that are capable of glucuronidating steroid hormones, bile acids, and biogenic amines. Each UGT1-specific isoform results from the splicing of one of the nine promoters/first exons with common exons 2 through 5. There are five bilirubin first exons (A1–A5) and four phenol UGT1 exons (A6–A9). UGT1A1 and UGT1A6 are the major bilirubin- and phenol-glucuronidating forms in rat liver.

UGTs have been investigated from the perspectives of toxicology, oncology, and endocrinology, and some studies have examined their regulation. Evidence from different studies demonstrates that UGTs are regulated by hormone levels, including sex hormones (2), glucocorticoides (3), and thyroid hormones. Thus, *in vivo* and *in vitro* studies carried out in our laboratory have shown that in adult rat liver, treatment by thyroid hormones stimulates phenol-UGT gene (UGT1A6) expression and inhibits major bilirubin UGT gene (UGT1A1) expression, whereas the opposite situation was observed in thyroidectomized rats (4). Recently, we demonstrated that the expression of the phenols-UGT gene is regulated at the transcription level by thyroid hormone in rat livers and requires *de novo* protein synthesis (5,6).

In a recent *in vivo* study (7), we showed that vitamin A status differentially affects the activity and expression of two major hepatic isoforms, UGT1A1 and UGT1A6. Moreover, we observed that dietary vitamin A influences the regulation of the expression of three isoforms because of L-triiodothyronine in rat liver. Thus, the presence of high amounts of vitamin A could negatively modulate the effects of thyroid hormone on UGT activity and mRNA level, whereas the specific effect of L-T_3 on UGT1A1 and UGT1A6 is not observed in animals fed a vitamin A–deficient diet.

Our working hypothesis was to consider the existence of a strong relationship between retinoic acid (the active metabolite of vitamin A) and thyroid hormone on the regulation of the gene encoding UGTs. This study was designed to specify if the vitamin A effect on UGT expression observed *in vivo* corresponds to the effect of metabolites of vitamin A, specifically 9-*cis*-retinoic acid. Therefore, we decided to test the effect of 9-*cis*-retinoic acid on hepatocyte primary cultures, which present the advantages of direct cell exposure, and this allows the study of specific effects on gene expression. We also postulated that hepatocytes from rats in various thyroid states present a characteristic glucuronidation capacity dependent on hormonal states, which may modulate the expression of UGTs and have toxicologic consequences. Then, we investigated the influence of the thyroid status on the modulation by retinoic acid of the expression of four rat liver UGT isoforms implicated in the glucuronidation of bilirubin (major UGT1A1; minors UGT1A2 and UGT1A5) and UGT1A6.

MATERIALS AND METHODS

Animals and Diets

Official French regulations for the care and use of laboratory animals were followed. Male Wistar rats weighing 40 g were obtained from Iffa-credo (L'arbresle, France) and were housed in a cage in an air-conditioned room of mean temperature 21°C and a photoperiod of 12 h light/day. The animals were provided *ad libitum* with food and drinking water. After 1 week of adaptation, the rats were randomly divided into four groups of four rats: one normothyroid group (group

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1); two hypothyroid groups (groups 2 and 3), the rats underwent surgical thyroidectomy four weeks before the culture of hepatocytes; and one hyperthyroid group, the animals were injected intraperitoneally for 10 consecutive days with 50 μg of triiodo-L-thyronine (sodium salt, Sigma, T2752)/kg body weight per day before hepatocyte isolation.

Hepatocyte Primary Cultures

Rat hepatocytes were isolated as previously described (6) by *in situ* collagenase (Gibco BRL 17103-011, Cergy Pontoise, France) perfusion of livers from 160- to 200-g Wistar rats. Cell viability (by the trypan blue exclusion test) was higher than 85%. The hepatocytes were cultured in monolayer in Leibovitz medium (Gibco BRL 31415-029, Cergy Pontoise, France) supplemented with 5% fetal bovine serum (10%, v/v, Gibco BRL, Cergy Pontoise, France), insulin (4 μg/ml, I5500, Sigma, St. Quentin-Fallavier, France), hydrocortisone (10^{-5} M, H4001, Sigma, St. Quentin-Fallavier, France), and penicilline/streptomycin (1% v/v, Gibco BRL, 15070-022, Cergy Pontoise, France), at 37°C in a humidified atmosphere of 100% air. To each petri dish was added 2.5×10^6 cells suspended in 4 ml of medium. After a 4-h incubation period, the medium was replaced with serum-free medium supplemented with 9-*cis*-retinoic acid (R 4643, Sigma, St. Quentin-Fallavier, France) (10^{-6} M in DMSO) or the vehicle alone as a control. One group of hepatocytes from the hypothyroid rats was treated with 9-*cis*-retinoic acid (10^{-6} M in DMSO) and L-T_3 (sodium salt T 2752, Sigma, St. Quentin-Fallavier, France) (10^{-7} M dissolved in NaCl 0.9%). The time course from UGT mRNA accumulation was determined at 1, 6, 12, 16, and 24 h after treatment by cell harvest.

Quantification of mRNA

mRNA was quantified by reverse transcription and amplified by the polymerase chain reaction (RT-PCR). The values of the different isoforms of UGTs (A_{UGT}) were evaluated by comparison with the level of an internal standard, β-actin mRNA (A_{act}), which is known to be insensitive to nutritional and hormonal conditions (8,9), and was simultaneously amplified in the same test tube: A_{UGT}/A_{act}. A semiquantitative analysis was performed as previously described (4,5).

Extraction of RNA was performed using a RNAXEL kit (Eurobio, Les Ulis, France) according to the protocol suggested by the supplier. Complementary DNA was made by RT from 1 μg of total RNA for 60 min at 37°C using random primers.

Ten microliters of cDNA was then used for amplification in a Minicycler MJ Research (Watertown, NY). The reaction mixture contained 20 mM Tris-HCl buffer (pH 8.5), 16 mM $(NH_4)_2SO_4$, 150 μg/ml bovine serum albumin, 0.2 mM of each dNTP, 2U of Taq polymerase, 1.5 mM of $MgCl_2$, 25 pmol of each primer β-actin, and 50 pmol of primer UGT1A1, UGT1A2, UGT1A5, or UGT1A6 in a total volume of 50 μl. The annealing temperatures were optimized at 60°C, 52°C, and 56°C, respectively, for bilirubin UGTs and 55°C for 4-nitrophenol UGT. The number of cycles to obtain the values of target sequences and β-actin before the amplification reactions reached the plateau phase was determined. Absence of genomic contamination was verified by using a control tube supplemented with 10 μg RNase. The specificity of PCR products obtained was verified by sequencing (Genomexpress, Grenoble, France). The sequence of the primers used is indicated in Table I.

The PCR products were then electrophoresed in a 3% (w/v) agarose gel to which BET was added. The bands were quantified under UV light by densitometry Vilber-Lourmat (Marne-la-vallée, France). All peak areas were calculated using NIH System software (NIH, USA).

Statistical Data

For statistical comparisons, the data obtained in duplicate for the four groups of four animals were analyzed by the Student *t* test (8 data per kinetic point). Results with $p < 0.05$ were considered as significant.

RESULTS

The PCR coamplification of reverse transcribed hepatic UGT and β-actin mRNAs produced amplicons of the expected size: 249 bp for UGT1A1, 303 bp for UGT1A2, 568 bp for UGT1A5, 507 bp for UGT1A6, and 222 bp for the internal standard (β-actin). The kinetic of controls (hepatocytes from different thyroid statuses treated with NaCl 0.9% or DMSO) showed no variation over time.

Thyroid Status Modifies UGT mRNA Basal Level

Figure 1 presents the UGT isoforms mRNA basal levels in different thyroid statuses. The levels of mRNA encoding bilirubin and phenol UGT isoforms in rat hepatocytes were estimated in comparison to those of β-actin, a protein whose expression remains unmodified by nutritional and hormonal conditions. We observed that UGT1A1, the major bilirubin

Table I. Sequences of Nucleotide Primers for β-Actin, UGT1A1, UGT1A2, UGT1A5, and UGT1A6.

Primers	Sequences 5′3′	Complementary site
β-actin	TGCAGAAGGAGATTACTGCC	2818 to 2837
(Nudel *et al.,* 1983)	CGCAGCTCAGTAACAGTCC	3153 to 3135
UGT1A1	GTGATCCCCATAGATGGCAG	102 to 122
(Coffman *et al.,* 1995)	GTATGTTTTAACCACACGCAGCA	348 to 324
UGT1A2	GAAGAATATCAGCGGGAAATA	250 to 270
(Sato *et al.,* 1990)	CGGACATTGTGTAGCCTCA	555 to 537
UGT1A5	ACCCTGCAAGGATTAGCTGGG	16 to 36
(Personal comm.)	AGGTTCGGAATATAAGAGG	581 to 563
UGT1A6	TTGCCTTCTTCCTGCTGC	6 to 23
Iyanagi *et al.,* 1986)	TCTGAAGAGGTAGATGGAAGGC	513 to 492

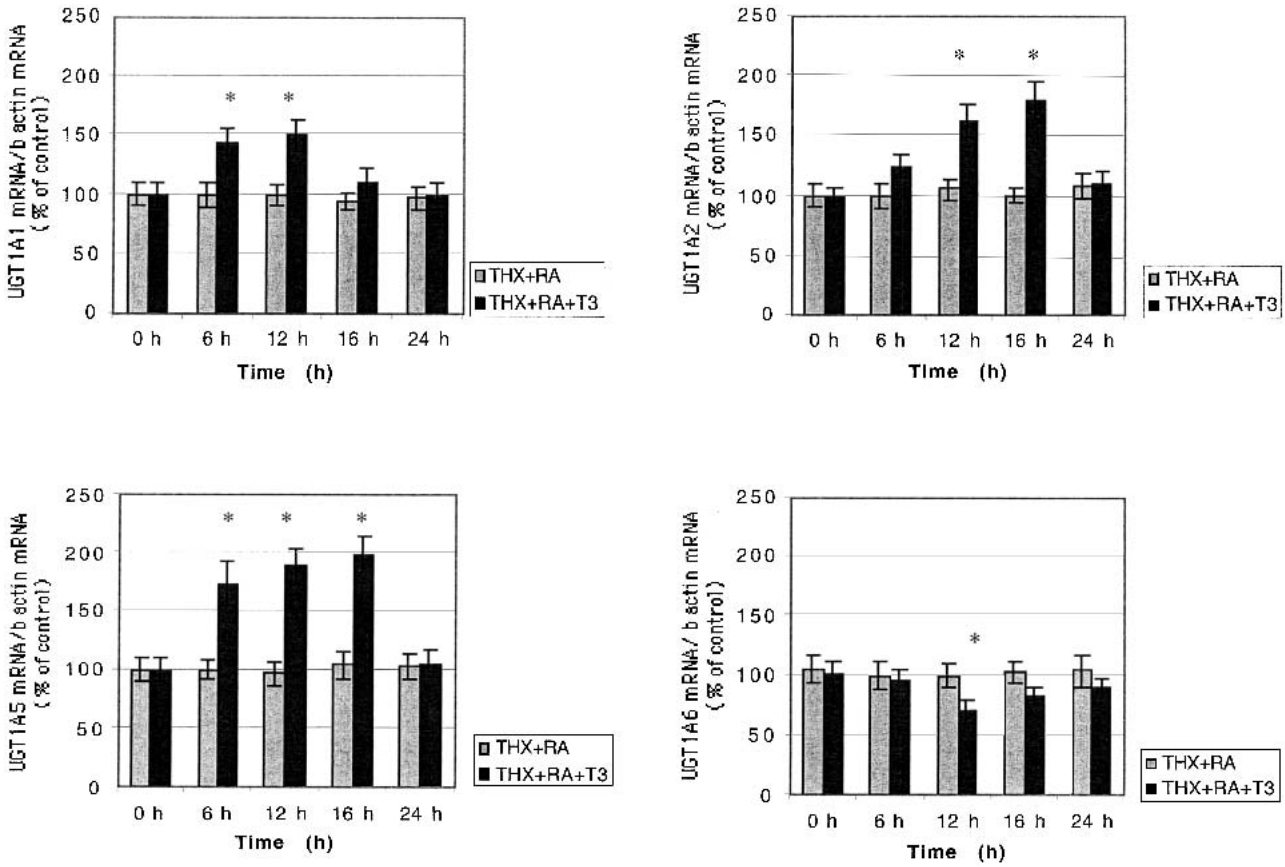

Fig. 1. Relative basal amount of UGT1A1, UGT1A2, UGT1A5, and UGT1A6 mRNA in cultured hepatocytes from thyroidectomized (TFX), normal (N), and treated with 3,3′,5-triiodo-L-thyronine (T_3). Cells were harvested and total RNA were extracted as described previously. UGT mRNA level quantified by densitometer scanning of PCR products. Data are expressed as a percentage of the value obtained with hepatocytes from normal rats. Data are expressed as the mean ± SEM of four experiments. *Significantly different ($p < 0.05$) from corresponding values measured in hepatocytes from euthyroid rats.

isoform, mRNA level was decreased by 32% in hepatocytes from hyperthyroid rats, whereas hepatocytes from hypothyroid rats exhibited a 25% increase in the UGT1A1 basal expression compared to that in hepatocytes from euthyroid animals. The amounts of both minor bilirubin UGT isoform mRNAs (UGT1A2 and UGT1A5) were about 50% lower in cells from hyperthyroid animals compared to that of normothyroid rats. Conversely, the levels of UGT1A2 and UGT1A5 mRNA were, respectively, 1.35- and 1.7-fold higher in hypothyroid rats. By contrast, the UGT1A6 mRNA level was decreased by 60% in hepatocytes from hypothyroid rats and 1.5 times higher in cells from hyperthyroid animals compared to that from normal rats.

The Kinetics of UGTs mRNA Accumulation in Cultured Hepatocytes from Rats with Various Thyroid Statuses Treated with 9-*cis*-Retinoic Acid

Hepatocytes from hypo- (THX), normo-, and hyperthyroid (T_3) rats were cultured in the presence of 1 μM of 9-*cis*-retinoic acid. The concentration of retinoic acid added was determined in a preliminary experiment by dose-dependent studies (data not shown). As shown in Figs. 2 and 3, the kinetics of UGT mRNA accumulation in cells treated with 9-*cis*-retinoic acid strongly depended on the presence or the absence of thyroid hormones. Figure 2A shows the levels of the major bilirubin isoform mRNA (UGT1A1) at different time points after treatment with 9-*cis*-retinoic acid in hepatocytes from animals with various thyroid states: hepatocytes from thyroidectomized, euthyroid or hyperthyroid rats. In hepatocytes from euthyroid animals, there is a gradual retinoic acid–induced increase in UGT1A1 mRNA with a maximum level found at 12 h (about 1.5-fold). This then falls, almost reaching control levels at 24 h after treatment. In hepatocytes from hypothyroid rats, major bilirubin isoform expression was not significantly modified, whereas there was a two-fold increase in hepatocytes from hyperthyroid rats with a maximum level at 16 h. DMSO alone has no effect on UGT mRNA accumulation during the experiment (data not shown).

As shown in Fig. 2B,C, the amounts of minor bilirubin isoforms, UGT1A2 and UGT1A5, increased by 1.9- and 2.2-fold, respectively, in euthyroid rats. This induced level remained high for up to 12 h and decreased thenafter to reach the starting mRNA level 24 h after the treatment. These isoform expressions were significantly modified in hepatocytes from hyperthyroid rats, too. The UGT1A2 and UGT1A5 mRNA levels were three times higher at 16 h. No difference was observed in the amount of either bilirubin minor isoform from hypothyroid rat hepatocytes.

In contrast, after the administration of retinoic acid, the amount of mRNA encoding the UGT1A6 isoform decreased

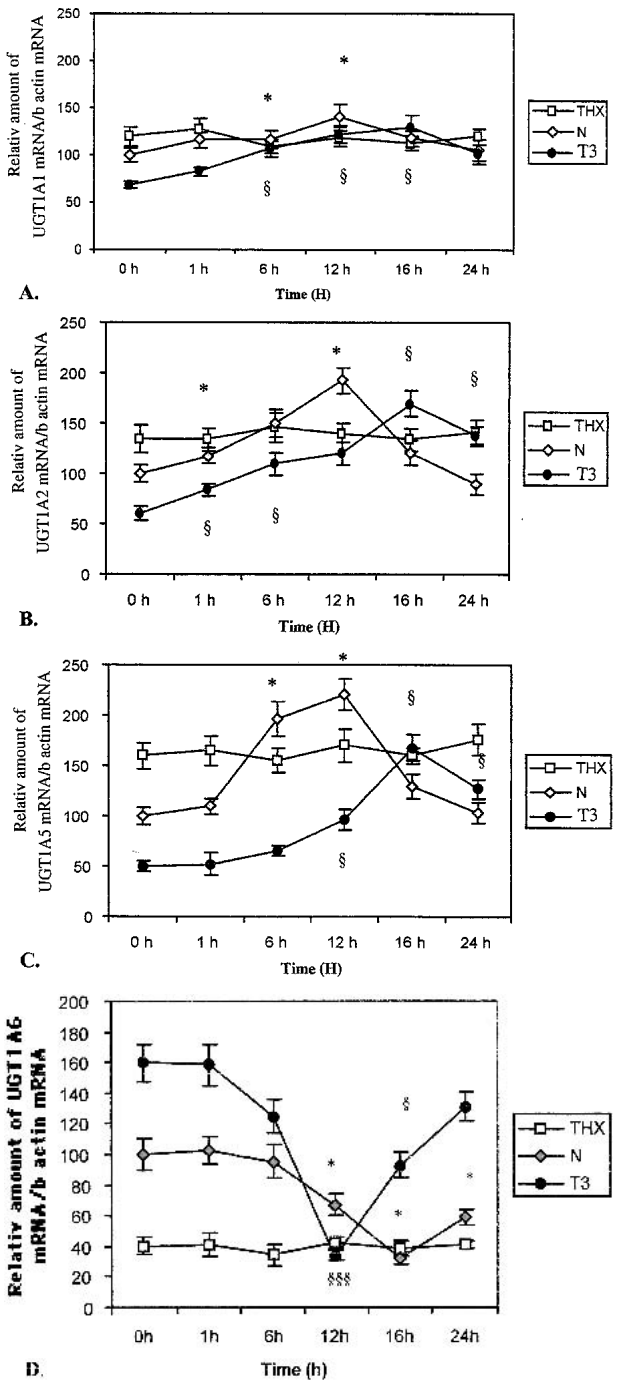

Fig. 2. Effects of 9-*cis*-retinoic acid on UGT1A1 mRNA (A), UGT1A2 mRNA (B), UGT1A5 mRNA (C), and UGT1A6 mRNA (D) accumulation in cultured hepatocytes from thyroidectomized, normal, and treated with 3,3′,5-triiodo-L-thyronine rats. After adherence, hepatocytes were cultured in Leibovitz in the presence of 1 μM of 9-*cis*-retinoic acid. Cells were harvested at different times and total RNA was extracted as described previously. UGT mRNA level quantified by densitometer scanning of PCR products. Data are expressed as the mean ± SEM of four experiments. *Significantly different ($p < 0.05$) from corresponding values measured at 0 h in hepatocytes from euthyroid rats; §significantly different ($p < 9.05$) from corresponding values measured at time 0 in hepatocytes from hyperthyroid rats.

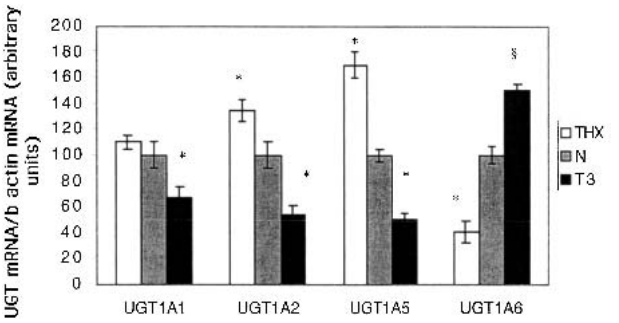

Fig. 3. Effects of coadministration of 9-*cis*-retinoic acid and L-triiodothyronine on UGT1A1, UGT1A2, UGT1A5, UGT1A6 mRNA accumulation in cultured hepatocytes from thyroidectomized rats. After adherence, hepatocytes were cultured in Leibovitz in the presence of 1 μM of 9-*cis*-retinoic acid and 10^{-7} M of L-T_3. Cells were harvested at different times, and total RNA was extracted as described previously. UGT mRNA level quantified by densitometer scanning of PCR products. Data are expressed as a percentage of the value obtained with hepatocytes from normal rats. Data are expressed as the mean ± SEM of four experiments. *Significantly different ($p < 0.05$) from corresponding values measured at 0 h in hepatocytes from hypothyroid rats treated by 9-*cis*-retinoic acid and L-T_3.

by 60% and 75% respectively in normal and hyperthyroid rats (Fig. 2D). Finally, phenol-UGT isoform expression was not significantly modified in hepatocytes from thyroidectomized rats.

The Effect of L-T_3 and 9-*cis*-Retinoic Acid Coadministration in Hepatocytes from Thyroidectomized Rats

In order to determine if the lack of UGT isoform responsiveness to 9-*cis*-retinoic acid stimulation observed in hepatocytes from thyroidectomized rats is caused by the absence of thyroid hormone, we determined the four UGT isoform mRNA levels in hepatocytes from hypothyroid rats treated with both 9-*cis*-retinoic acid and L-triiodothyronine. The dose of L-T_3 was chosen because it was previously found to be active on UGT gene expression without being toxic for the hepatocytes (6).

We observed first that this coadministration reestablished the retinoic acid effect observed previously in normal rats. Thus, expression of the three bilirubin isoforms increased by 1.5- to 2-fold, whereas UGT1A6 mRNA decreased by 25%. Inhibition of UGT1A6 is less pronounced in hepatocytes from hypothyroid rats treated with both retinoic acid and L-T_3 compared to normal rats treated with retinoic acid alone, whereas no variations were observed for bilirubin isoforms. Moreover, UGT expressions reach the maximum or minimum level 16 h after the treatment for minor bilirubin isoforms, and 12 h for major bilirubin isoforms.

DISCUSSION

In a previous study, we showed that vitamin A status influences *in vivo* the effect of a single injection of L-T_3 on the expression of various UGT genes (7). The major result was that the specific effect of L-T_3 on bilirubin (UGT1A1) and phenol (UGT1A6) isoforms was not observed in animals fed a vitamin A–deficient diet. This suggests the involvement of a

coordinated mechanism in the regulation of UGT expression by thyroid hormones and vitamin A. Therefore, we decided to use a hepatocyte model to test the effect on the expression of a specific UGT gene of the active vitamin A metabolite, 9-*cis*-retinoic acid, which is responsible for various cellular effects of vitamin A. We postulated that primary hepatocyte cultures from rats in various thyroid states present similar metabolism characteristics to those of hypo- or hyperthyroid animals.

Our first results substantiate this experimental model by showing that the basal expression of UGT1A1 and UGT1A6 mRNA is dependent on the type of hepatocyte, and corresponds to that observed previously *in vivo.* Thus, we observed that basal bilirubin UGT (UGT1A1) expression decreased and phenol UGT (UGT1A6) isoform increased in hepatocytes from hyperthyroid animals compared to those obtained in normal rats. The opposite was observed in hepatocytes from thyroidectomized rats. Results obtained with UGT1A1 were confirmed for UGT1A2 and UGT1A5, two minor bilirubin isoforms. Moreover, the mRNA level of the major bilirubin isoform (UGT1A1) is modified to a lesser degree compared to that of minor bilirubin isoforms (UGT1A2 and UGT1A5). These results suggest that in a hormonal perturbation, an adaptive mechanism could appear. Therefore, minor bilirubin isoforms (UGT1A2 and UGT1A5) may play a compensatory role when the function of major UGT1A1 isoform is not sufficient. These results are in accordance with those of Iyanagi *et al.* (24), which showed that UGT1A2 expression was highly induced in primary culture of rats hepatocytes.

Following the treatment of hepatocytes from hypothyroid rats with 9-*cis*-retinoic acid, we observed interestingly that bilirubin and phenol-UGT isoform expressions were not significantly modified: indeed, retinoic acid had no effect on UGTs in the absence of thyroid hormone. In contrast, in hepatocytes from normal and specially hyperthyroid rats treated with 9-*cis*-retinoic acid, both major and minor bilirubin isoform mRNA levels were significantly higher, whereas that of phenol isoform fell. This suggests that the effect of retinoic acid on UGT mRNA expression requires the presence of thyroid hormone. In a recent study, we found that *in vivo* vitamin A deprivation inhibited the L-T_3 effect on bilirubin and phenols UGT isoforms. Thus, the regulation of UGT gene expression appears to be dependent on the presence of both 9-*cis*-retinoic acid (as vitamin A) and thyroid hormones. In order to verify if the lack of UGT responsiveness to retinoic acid treatment is caused by the absence of thyroid hormone, we treated cultured hepatocytes from hypothyroid rats with both retinoic acid and L-T_3. We observed the full reestablishment of the effects of retinoic acid for bilirubin UGT isoform and only partial reestablishment for phenol UGT isoform. We also confirm that thyroid hormone deprivation inhibited the effect of retinoic acid and suggest that the sensitivity of phenol and bilirubin isoforms are different, probably because of the involvement of other factors in UGT1A6 mRNA regulation. Tzameli and Zannis (11) suggest that the general lack of responsiveness to the different hormonal signals may reflect altered DNA-protein and protein-protein interactions between the nuclear hormone receptors, other factors, and proteins of the basal transcription system (11). It had been originally assumed that retinoic acid mediates its diverse effects by modulating the expression of specific genes through binding to specific retinoic acid nuclear receptors (12–13), and thyroid hormones by binding to Thyroid Receptors (14). In a recent study, Coustaut *et al.* (1996) observed that hypothyroidism decreases the availability of retinol and/or its metabolism (16,17). The decrease in the level of retinol leads to a decrease in Retinoic Acid nuclear Receptor expression, which could explain the lack of retinoic acid response. Moreover, unoccupied thyroid receptors block the expression of RAR (15) and perhaps of RXR, the specific 9-*cis* retinoic acid receptor, which is the partner TR heterodimerization (18). Thus, the decrease, in RAR mRNA observed in hypothyroid animals (19), explains the absence of one effect of retinoic acid on UGT expression. Recently, Park *et al.* (1999) showed that unliganded TR and RAR interact with the co-repressors SMRT and N-CoR, which are components of co-repressor complexes that contain histone deacetylase and therefore induce transcriptional repression. The binding of hormone to these receptors causes dissociation of the corepressor complex and transcriptional activation (21). This could explain the re-establishment of the effect of retinoic acid, observed in the presence of L-T_3.

In summary, these results have established that i) hepatocytes from rats in various thyroid states are an interesting model that permits the study of the direct effect of vitamin A active metabolite, and reflects the similar *in vivo* thyroid situation, ii) 9-*cis* retinoic acid, regulates UGT isoform expression, and iii) thyroid hormones are required for this retinoic acid effect. Mechanisms involved in this co-regulation of UGT expression by retinoic acid and thyroid hormones have not yet been elucidated, however they probably involve specific nuclear receptors. Thus, further studies on the UGT gene promoter are required to elucidate these mechanisms.

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