Thyroid hormones act indirectly to increase sex hormone-binding globulin production by liver via hepatocyte nuclear factor-4α

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Abstract

Thyroid hormones increase hepatic sex hormone-binding globulin (SHBG) production, which is also regulated by hepatocyte nuclear factor-4α (HNF-4α) in response to changes in the metabolic state of the liver. Since the human *SHBG* promoter lacks a typical thyroid hormone response element, and because thyroid hormones influence metabolic state, we set out to determine whether thyroid hormones mediate *SHBG* expression indirectly via changes in HNF-4α levels in HepG2 human hepatoblastoma cells, and in the livers of transgenic mice that express a 4·3 kb human *SHBG* transgene under the control of its own 0·8 kb promoter sequence. Thyroid hormones (triiodothyronine (T_3) and thyroxine (T_4)) increase SHBG accumulation in HepG2 cell culture medium over 5 days, and increase cellular SHBG mRNA levels. In addition, T_4 treatment of HepG2 cells for 5 days increased HNF-4α mRNA and HNF-4α levels in concert with decreased cellular palmitate levels. Plasma SHBG levels were also increased in mice expressing a human *SHBG* transgene after 5 days treatment with T_3 along with increased hepatic HNF-4α levels. In HepG2 cells, the human *SHBG* promoter failed to respond acutely (within 24 h) to T_4 treatment, but a 4-day pre-treatment with T_4 resulted in a robust response that was prevented by co-treatment with HNF-4α siRNA, or by blocking the β-oxidation of palmitate through co-treatment with the carnitine palmitoyltransferase I inhibitor, etomoxir. These data lead us to conclude that thyroid hormones increase SHBG production indirectly by increasing *HNF-4α* gene expression, and by reducing cellular palmitate levels that further contribute to increased HNF-4α levels in hepatocytes.

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Introduction

Thyroid hormones influence plasma sex hormone-binding globulin (SHBG) levels under both normal and pathological conditions (Anderson 1974) by altering the production of SHBG by hepatocytes (Rosner *et al.* 1984). In addition, blood levels of SHBG in infants increase within the first 2–3 weeks after birth, and this has been attributed to a postnatal maturation of hepatic sensitivity to thyroid hormones (Leger *et al.* 1990). Plasma SHBG levels are also commonly elevated beyond normal reference ranges in patients with hyperthyroidism (Anderson 1974), but are not increased in individuals suffering from familial thyroid hormone resistance, despite their very high blood levels of thyroid hormones (Sarne *et al.* 1988). Since the latter is likely due to defects in thyroid hormone action in the liver, it has been proposed that plasma SHBG measurements can be used clinically to assess end-organ sensitivity to thyroid hormones (Sarne *et al.* 1988).

Human HepG2 hepatoblastoma cells have been widely used as a model for the studies of thyroid hormone action in the liver (Rosner *et al.* 1984, Raggatt *et al.* 1992, Kester *et al.* 2006). These cells express the type I iodothyronine deiodinase that converts the pro-hormone thyroxine (T_4) into the receptor-active triiodothyronine (T_3) in the normal liver, but they lack the type III iodothyronine deiodinase: a key enzyme responsible for deactivating thyroid hormones in the liver and other tissues (Kester *et al.* 2006). On the other hand, HepG2 cells express the glucuronidases responsible for the metabolic clearance of thyroid hormones in the liver (van Stralen *et al.* 1996). The human *SHBG* gene is also expressed in HepG2 cells and their production of SHBG is enhanced by thyroid hormone treatments in concert with an increase in SHBG mRNA levels (Rosner *et al.* 1984, Mercier-Bodard *et al.* 1991, Raggatt *et al.* 1992).

In earlier studies, increases in plasma SHBG have been noted in hypothyroid patients several days after the initiation of treatment with T_3 (Sarne *et al.* 1988), and this is consistent with studies in which treatment of HepG2 cells with thyroid hormones increase SHBG production after 2–3 days (Rosner *et al.* 1984, Raggatt *et al.* 1992). It has been assumed that these responses are mediated directly by the thyroid hormone receptor

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(TR) acting at the level of the human *SHBG* gene. Thyroid hormones exert their effects at the gene level via the TR, which generally binds as a heterodimer with the retinoid X receptor to *cis*-acting elements that are referred to as thyroid hormone response elements (TREs) within target gene promoters. A typical TRE is a tandem repeat of the consensus AGGTCA separated by four nucleotides (Zhang & Lazar 2000), and is classified as a direct repeat-4 (DR-4) element. It was therefore of interest that a bioinformatic scan failed to identify a DR-4-like element that might act as a TRE within the human *SHBG* promoter responsible for controlling its expression in the liver (Jänne & Hammond 1998).

Since thyroid hormones influence the metabolic state of the liver (Malik & Hodgson 2002) and because we have recently demonstrated that *SHBG* gene expression in hepatocytes is dynamically regulated by changes in their metabolic state (Selva *et al.* 2007), we set out to determine whether thyroid hormone-induced changes in the metabolic state of HepG2 cells and the liver could account for changes in *SHBG* expression.

Materials and methods

Cell culture experiments

Cell culture reagents were from Life Technologies Inc. (Invitrogen). HepG2 hepatoblastoma cells (catalog no. HB-8065; ATCC, Manassas, VA, USA) were routinely maintained in DMEM supplemented with 10% FBS and antibiotics (100 U penicillin/ml and 100 μg streptomycin/ml). For experiments, HepG2 cells were cultured to 30–50% confluence prior to the addition of supplements: glucose (Sigma–Aldrich Canada Ltd), T_3 (Sigma–Aldrich), T_4 (Sigma–Aldrich) or etomoxir (Sigma–Aldrich), as indicated. Palmitate levels in HepG2 cells were determined as described previously (Selva *et al.* 2007).

Transient transfections of human *SHBG* promoter-driven luciferase reporter plasmids together with a pCMVlacZ control plasmid (Jänne & Hammond 1998, Selva *et al.* 2007) were performed using the HiPerfect Transfection Reagent (Qiagen). The siRNA experiments were carried out using HiPerfect Transfection Reagent together with either a control siRNA (catalog 1022076) or an hepatocyte nuclear factor-4α (HNF-4α) siRNA (catalog 00161546) obtained from Qiagen. Two days after transfection, the cells were washed twice with PBS and harvested by scraping. After centrifugation, cell pellets were resuspended in 100 μl 250 mM Tris–Cl, pH 7·8, and cells were lysed by three freeze–thaw cycles. Appropriate aliquots of cell extracts were used for measurements of luciferase and β-galactosidase activity. To correct for transfection efficiency, light units from the luciferase assay were divided by the OD reading from the β-galactosidase assay.

SHBG measurements

Human SHBG levels in culture medium taken from HepG2 cells were measured using a time-resolved immunofluorometric assay (Niemi *et al.* 1988).

RNA analysis

Total RNA was extracted from HepG2 cells using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed at 42 °C for 50 min using 3 μg of total RNA and 200 U of Superscript II together with an oligo(dT) primer and reagents provided by Invitrogen. An aliquot of the RT product was amplified in a 35-μl reaction using PCR SuperMix (Invitrogen) with appropriate oligonucleotide primer pairs corresponding to human HNF-4α (forward primer 5′-GCTCCTCCTTC TGCTGCTGC and reverse primer 5′-GGAAGAGCTT GAGACAGGCC), SHBG (forward primer 5′-GTTGCT ACTACTGCGTCACAC and reverse primer 5′-GCC ATCTCCCATCATCCAGCCG), TR1-α (forward primer 5′-CCGCACAATCCAGAAGAACC and reverse primer 5′-GGCAATGTGGATCAGATCCC), and cyclophilin A (forward primer 5′-ATGGTCAACCCCACCGTG and reverse primer 5′-TGCAATCCAGCTAGGCATG). The PCR was performed for 40 cycles at 94 °C for 15 s, 57–65 °C for 30 s, and 72 °C for 1 min, and PCR products were resolved by electrophoresis in a 1% agarose gel.

Treatment of human *SHBG* transgenic mice with thyroid hormone

Mice that express a 4·3 kb human *SHBG* transgene in the liver (Jänne *et al.* 1998) were routinely maintained on standard laboratory chow and water provided *ad libitum*. The human *SHBG* transgene in these mice is expressed in the liver under the control of its own ~800 bp promoter sequence that corresponds to the same sequence we have studied in the context of a luciferase reporter gene (Jänne & Hammond 1998, Selva *et al.* 2007). For the experiment, female mice ($n=3$) were treated with water (controls) or water containing T_3 (0·5 mg/l) for 5 days. Blood samples were taken by saphenous vein sampling for measurements of plasma SHBG levels (Selva *et al.* 2007) immediately before the treatment, and on day 5 of treatment when livers were also taken for RNA and protein extraction (see below). Animals were weighed before and after treatment and no change in weights were observed in either group over

the 5 days of treatment. This animal protocol was approved by the Animal Use Subcommittee of the University Council on Animal Care at The University of British Columbia.

Total cellular and nuclear protein extracts

After treatments, mouse livers or HepG2 cells were harvested and homogenized in 50 mM Tris–HCl, pH 7·9, 300 mM KCl, 1·5 mM $MgCl_2$, 0·1% Nonidet P-40, and 20% glycerol supplemented with Complete protease inhibitor cocktail (Roche Diagnostics) at 4 °C, followed by centrifugation (11 000 ***g*** at 4 °C) for 10 min to obtain total protein extracts. For nuclear protein extracts, the HepG2 cells were first incubated on ice for 10 min with hypotonic buffer (10 mM HEPES, pH 7·9, 1·5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0·5 mM dithiothreitol (DTT), 0·2 mM phenylmethylsulfonyl fluoride (PMSF), and the Complete protease inhibitor cocktail). Samples were then homogenized and centrifuged at 2000 ***g*** for 10 min. Pellets were vortex mixed in resuspension buffer (20 mM HEPES, pH 7·9, 240 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0·5 mM DTT, 0·2 mM PMSF, and Complete protease inhibitor cocktail) and centrifuged through a 3 ml 1 M sucrose pad for 1 h to obtain nuclei. The nuclei were recovered in 200 µl resuspension buffer followed by the slow addition of 100 µl nuclei isolation buffer (20 mM HEPES, pH 7·9, 240 mM sucrose, 1·2 M KCl, 1 mM EDTA, 1 mM EGTA, 0·5 mM DTT, 0·2 mM PMSF, and Complete protease inhibitor cocktail) and further centrifugation (15 000 ***g*** at 4 °C) for 30 min to obtain a nuclear extract, which was dialyzed overnight against 20 mM Tris, pH 7·9, 100 mM KCl, 1 mM EDTA, 1 mM EGTA, and 0·5 mM DTT containing 10% glycerol.

Western blot analysis

Total cell protein and nuclear protein extracts were used for western blotting with antibodies against human HNF-4α (C-19; catalog sc-6556; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and human cyclophilin A (SA-296; BIOMOL Int., Plymouth Meeting, PA, USA). Specific antibody–antigen complexes were identified using an HRP-labeled goat anti-rabbit IgG or rabbit anti-goat IgG and chemiluminescent substrates (Pierce Biotechnology Inc., Rockfort, IL, USA) by exposure to X-ray film.

Statistical analyses

Data were analyzed using one-way ANOVA, and P values of $<0{\cdot}05$ were considered significant.

Results

Thyroid hormones only increase SHBG production by HepG2 cells after 2 days of treatment

We first examined the effects of daily supplementation with T_3 (100 nM) or T_4 (100 nM) on SHBG production by HepG2 cells over the course of 5 days, by comparing medium concentrations of SHBG on days 1 and 5 of thyroid hormone treatments. This showed that T_3 and T_4 stimulated SHBG production ($P<0{\cdot}001$), and that T_4 was more effective than T_3 (Fig. 1A). In addition, the amount of SHBG mRNA was clearly increased in relation to the cyclophilin A mRNA control in HepG2 cells after 5 days treatment with T_4, but this was not as obvious after treatment with T_3 (Fig. 1B).

The effect of T_4 on *SHBG* gene expression in HepG2 cells was then studied over a 3-day treatment period during which 100 nM T_4 was added daily to the cultures, coincident with the collection of culture media for measurements of SHBG production rates (Fig. 1C). This demonstrated that the T_4-induced increases in SHBG production are only apparent ($P<0{\cdot}05$) after 2 days of treatment, and increase substantially between treatment days 2 and 3 ($P<0{\cdot}001$). We also explored the possibility that the delayed response to thyroid hormone in HepG2 cells might be due to an increase in TR expression, but within the time frame of our experiments in which HepG2 cells were treated with T_4 there was no change in their TR1-α mRNA levels, as assessed by RT-PCR (Fig. 1D).

We next examined whether T_4 (100 nM) treatments influence SHBG production by HepG2 cells over a 5-day period when the cells were cultured in high concentrations of glucose. This demonstrated that T_4 increased SHBG production by HepG2 cells regardless of whether they were maintained in the presence of 100 µM glucose (control) or 10 mM glucose, added as daily supplements (Fig. 1E).

Human *SHBG* promoter lacks a TRE and only responds in HepG2 cells after several days pre-treatment with T_4

We have previously demonstrated that the 803 bp human *SHBG* promoter, which controls its activity in the liver, is influenced by a polymorphic TAAAA repeat sequence in the distal promoter sequence (Hogeveen *et al.* 2001) as well as a Sp1 site (Fig. 2A). However, when these upstream sequences are removed, the proximal 299 bp promoter sequence is not only generally more active in HepG2 cells (Jänne & Hammond 1998), but also retains its responsiveness to changes in metabolic state (Selva *et al.* 2007), or to treatments with drugs that act via the peroxisome proliferator receptor γ that binds to the DR-1 element in the 299 bp human

SHBG promoter and represses its transcriptional activity (Selva & Hammond 2009). This DR-1 sequence can also bind HNF-4α as well as COUP-TF, but does not appear to have much influence on the basal transcriptional activity of the human *SHBG* promoter in HepG2 cells (Jänne & Hammond 1998). By contrast, the 'TATA-less' human *SHBG* promoter (Jänne & Hammond 1998) contains another binding site for HNF-4α and COUP-TF, which is located in the position where a TATA box would normally be positioned (Fig. 2A), and this appears to serve as the major on–off switch for SHBG transcription: such that occupancy of this site by HNF-4α promotes transcription while COUP-TF binding represses transcription (Jänne & Hammond 1998, Selva *et al.* 2007). The 299 bp human *SHBG* promoter sequence also contains a binding site for USF transcription factors (Fig. 2A) that play a key role in controlling the expression of the *SHBG* gene in testicular cell types, but has no obvious role in hepatocytes (Selva *et al.* 2005).

Although an analysis of the 803 bp human *SHBG* promoter failed to identify a sequence that resembles a typical TRE (Zhang & Lazar 2000), a DR-8 sequence that has been reported to function as an atypical TRE (Bouterfa *et al.* 1995) overlaps the DR-1 element within the 299 bp *SHBG* proximal promoter (Fig. 2A). However, the half-site of this DR-8 sequence extends outside the DNase 1 footprinted region in which this DR-1 element is located (Jänne & Hammond 1998).

Since we found that T_4 was clearly more effective than T_3 in increasing SHBG production by HepG2 cells (Fig. 1), we used T_4 to treat HepG2 cells in subsequent experiments to explore its mechanism of action. When the activities of the 803 and 299 bp human *SHBG* promoter sequences were tested in the context of a luciferase reporter gene assay after treatment with

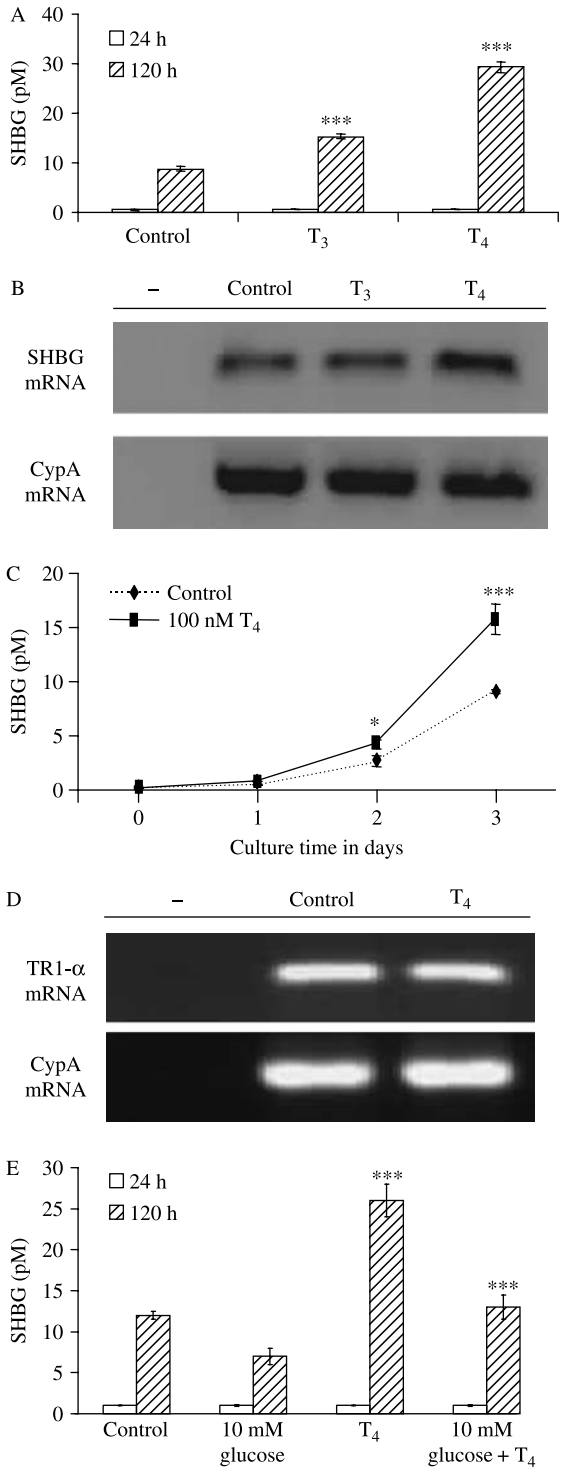

Figure 1 Increases in human SHBG production by HepG2 cells occur after 2 days treatment with thyroid hormone. (A) HepG2 cells were cultured in medium containing FBS for 5 days and treated daily with or without 100 nM T_3 or T_4. Accumulation of human SHBG in the medium was measured using an immunofluorometric assay. Data points are shown as mean±S.D. of triplicates. ***$P<0{\cdot}001$ compared with the control. (B) Semi-quantitative RT-PCR analysis of SHBG mRNA levels in HepG2 cells cultured for 5 days with daily addition of 100 μM glucose and PBS vehicle (control), 100 nM T_3 or T_4 (100 nM). Cyclophilin A (CypA) mRNA was amplified as an internal control. The negative control for the RT-PCR (−) lacked RNA template. (C) HepG2 cells were cultured in medium containing FBS for 3 days and treated daily with thyroid hormone (100 nM T_4 in PBS) or PBS control. Accumulation of human SHBG in the medium was measured every 24 h using an immunofluorometric assay. Data points are mean±S.D. of triplicate measurements. *$P<0{\cdot}05$ and ***$P<0{\cdot}001$ compared with the control. (D) Semi-quantitative RT-PCR analysis of TR1-α mRNA levels in HepG2 cells cultured for 5 days with PBS vehicle (control) or 100 nM T_4. Cyclophilin A (CypA) mRNA was amplified as an internal control. The negative control (−) lacked RNA template. (E) HepG2 cells were cultured in medium containing FBS for 5 days and treated daily with 100 μM glucose (control) or 10 mM glucose in the presence or absence of 100 nM T_4. Accumulation of human SHBG in the medium was measured using an immunofluorometric assay. Data points are shown as mean±S.D. of triplicates. ***$P<0{\cdot}001$ compared with the control.

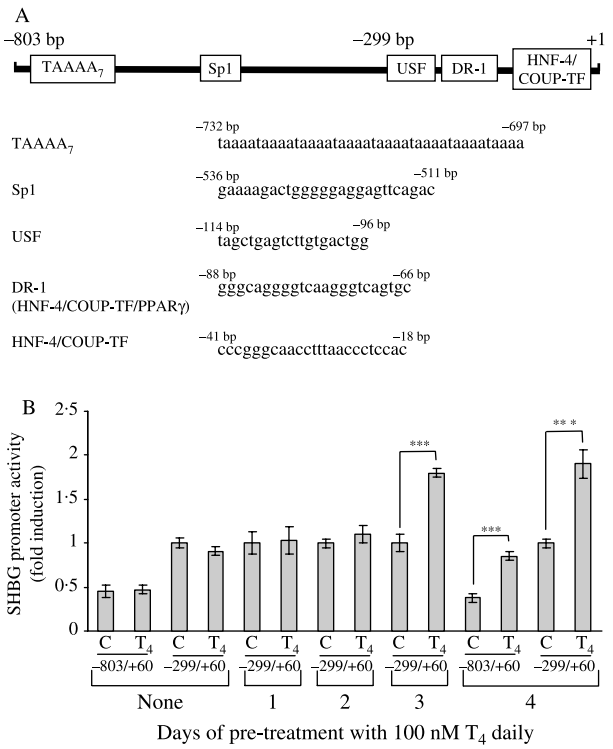

Figure 2 Human *SHBG* promoter only responds to thyroid hormone after a 3–4 day pre-treatment. (A) The *SHBG* promoter sequence contains several *cis* elements that are footprinted by liver nuclear proteins (Jänne & Hammond 1998) and their relative positions within the 803 bp promoter sequence are shown as boxes, with their actual sequences listed below together with the identities of their known binding proteins (Jänne & Hammond 1998, Selva *et al.* 2005, Selva & Hammond 2009). (B) The effect of daily addition of 100 nM T_4 on human *SHBG* promoter activity was analyzed in HepG2 cells in the context of a luciferase reporter gene assay. Two different promoter sequences (−803/+60) and (−299/+60) were analyzed. Data points are mean ± S.D. of triplicate measurements. ***$P<0·001$ compared with the control (C).

100 nM T_4 for 24 h, we observed no differences when compared with their activities in untreated HepG2 cells (Fig. 2B). We therefore examined the 299 bp *SHBG* promoter activity in HepG2 cells pre-treated daily for 1–4 days with 100 nM T_4 or the vehicle control. In this experiment, a significant difference (twofold, $P<0·001$) in the relative luciferase activity was only observed in cells pre-treated with T_4 for 3 or 4 days (Fig. 2B). A similar increase in the activity of the 803 bp promoter was observed in HepG2 cells after a 4-day pre-treatment with 100 nM T_4 (Fig. 2B).

Thyroid hormones influence hepatic SHBG production indirectly by increasing HNF-4α levels

Because HNF-4α plays a key role in the transcriptional activity of the human SHBG promoter (Jänne & Hammond 1998), we examined HNF-4α mRNA levels in HepG2 cells after a 5-day treatment with T_4. This indicated that the amounts of HNF-4α mRNA were substantially increased after treatment with 100 nM T_4 (Fig. 3A), and that this resulted in an increase in HNF-4α in total cell protein extracts as well as a substantial increase in HNF-4α within nuclear extracts, as demonstrated by western blotting (Fig. 3B). To confirm that this effect of thyroid hormone on increasing the production of SHBG is associated with an increase in hepatic HNF-4α levels *in vivo*, we treated mice expressing a human *SHBG* transgene in the liver with T_3 by adding it to their drinking water. This almost doubled the plasma levels of human SHBG ($P<0·05$) after 5 days of T_3 treatment (Fig. 4A), and resulted in a similar increase in hepatic HNF-4α levels ($P<0·01$) as assessed by western blotting (Fig. 4B).

In order to demonstrate that the thyroid hormone effect on *SHBG* expression in HepG2 cells is mediated through increasing HNF-4α levels, we pre-treated the cells daily for 4 days with or without 100 nM T_4, and examined the activity of the 299 bp human *SHBG* promoter in the context of a luciferase reporter gene assay after co-transfection with either a control siRNA or a siRNA for HNF-4α. The results clearly indicate that the increased *SHBG* promoter activity seen after 4 days pre-treatment with 100 nM T_4 can be abrogated ($P<0·001$) by treatment with the siRNA for HNF-4α (Fig. 5A). As expected, the siRNA for HNF-4α also reduced *SHBG* promoter activity in untreated HepG2 cells ($P<0·01$), but the magnitude of the decrease was less than that seen it the T_4-treated cells (Fig. 5A). As a control for the effectiveness of the siRNA treatment,

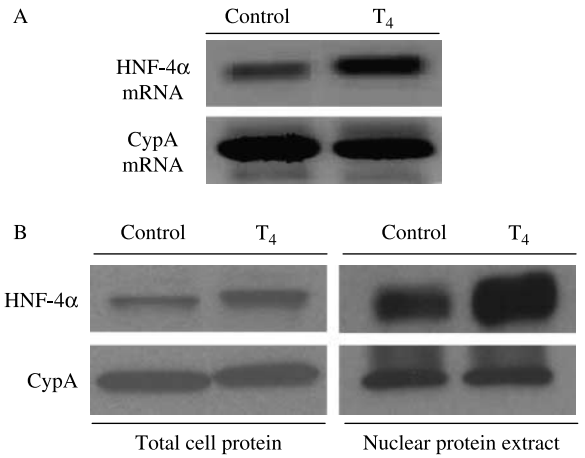

Figure 3 Thyroid hormone increases HNF-4α mRNA and HNF-4α levels in HepG2 cells. (A) Semi-quantitative RT-PCR analysis of HNF-4α mRNA levels in HepG2 cells cultured for 5 days and treated daily with 100 nM T_4 or PBS as vehicle control. Cyclophilin A (CypA) mRNA was amplified as an internal control. (B) Western blot of HNF-4α and cyclophilin A in total cell protein and nuclear protein extracts of HepG2 cells harvested after the 5-day treatment period with PBS (control) or 100 nM T_4.

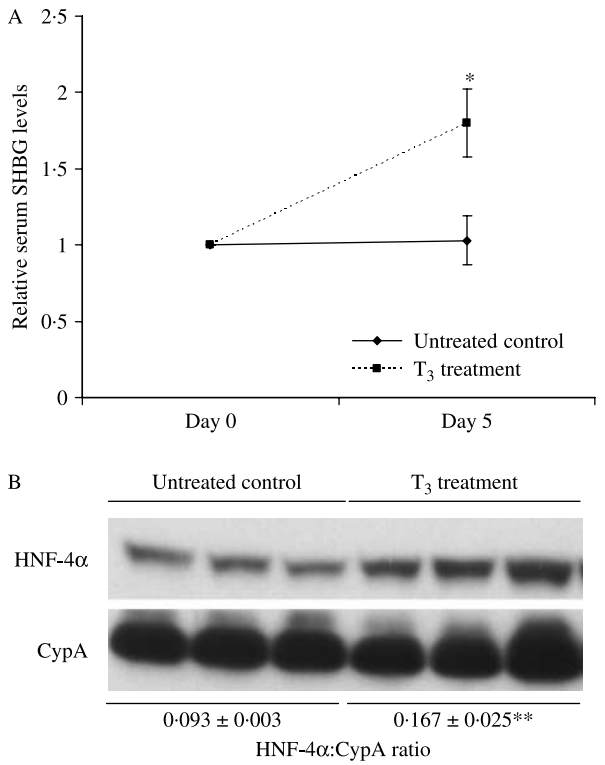

Figure 4 Treatment with T_3 increases plasma levels of human SHBG in mice that express a human *SHBG* transgene (A) in concert with an increase in hepatic HNF-4α levels (B). Female mice were either untreated ($n=3$) or treated with T_3 (0·5 mg/l) in the drinking water ($n=3$) for 5 days. Blood samples for measurements of blood levels of human SHBG (Selva *et al.* 2007) were taken on the day T_3 treatment began and on day 5, when livers were taken for protein extraction and measurements of HNF-4α levels by western blotting using cyclophilin A (CypA) as a housekeeping reference protein (Selva *et al.* 2007). $^{**}P<0·01$.

western blots from nuclear extracts of cells treated under the same conditions reveal a substantial increase in nuclear HNF-4α protein levels when the HepG2 cells were treated with 100 nM of T_4, while treatment with siRNA for HNF-4α reduces the protein levels of HNF-4α in cells pre-treated with either the vehicle control or 100 nM T_4 (Fig. 5B).

Inhibition of β-oxidation blocks the thyroid hormone effect on SHBG production and HNF-4α levels in HepG2 cells

Recently, we have demonstrated that HNF-4α levels in HepG2 cells decrease in response to increased lipogenesis and cellular palmitate levels, and that this leads to a reduction in *SHBG* gene expression (Selva *et al.* 2007). We therefore measured the palmitate content of HepG2 cells treated for 5 days with 100 nM T_4 versus untreated cells, and found lower levels of palmitate in the T_4-treated cells (4·0 μg/10^6 cells), when compared with the untreated cells (6·4 μg/10^6 cells). Since thyroid hormones increase metabolic rate and influence cellular levels of carnitine palmitoyltransferase I (CPTI), the rate-controlling enzyme in the fatty acid oxidation pathway (McGarry & Brown 1997), we performed an experiment in which HepG2 cells were treated with 100 nM of T_4 or vehicle control alone or in the presence of etomoxir, a CPTI inhibitor (Murthy & Pande 1990). This showed that treatment with 5 or 10 μM etomoxir did not influence SHBG production by control cells, but reduced ($P<0·05$ and $P<0·01$ respectively) the increase in SHBG production by T_4-treated HepG2 cells (Fig. 6A), as well as the increase in HNF-4α protein levels that occurs in response to T_4 treatment (Fig. 6B).

Discussion

T_4 increases the production and secretion of SHBG by HepG2 cells, and this response is only apparent after 2–5 days of treatment, as reported previously (Rosner *et al.* 1984). In our experiments, equimolar amounts of T_4 were consistently more effective than T_3 in these *in vitro* experiments, and this may be due to differences in their transport into cells in culture when compared with the liver *in vivo*, as well as differences in their metabolism in HepG2 cells versus normal hepatocytes. By contrast, our experiments using a transgenic mouse

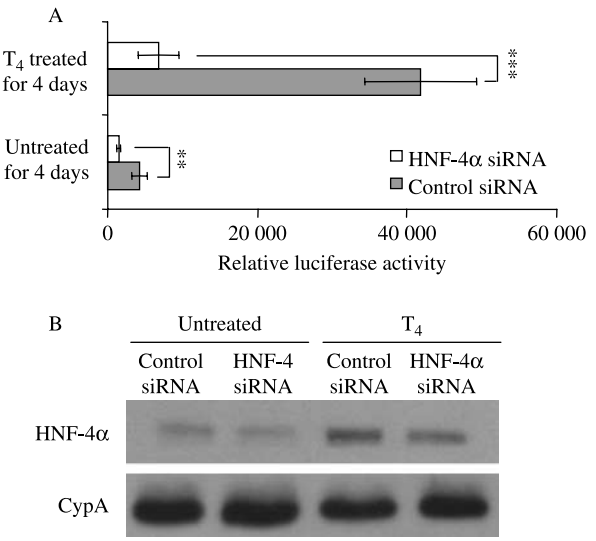

Figure 5 HNF-4α siRNA treatment blocks the thyroid hormone increase in SHBG promoter activity. (A) Co-transfection of HepG2 cells with HNF-4α siRNA reduced human *SHBG* promoter activity in a luciferase reporter gene assay after 4 days pre-treatment with or without thyroid hormone (T_4 at 100 nM) when compared with a control siRNA. Data points are shown as mean ± S.D. of triplicates. $^{**}P<0·01$ and $^{***}P<0·001$ compared with the control siRNA. (B) Western blot of HNF-4α and cyclophilin A (CypA) in nuclear extracts of HepG2 cells cultured as in (A).

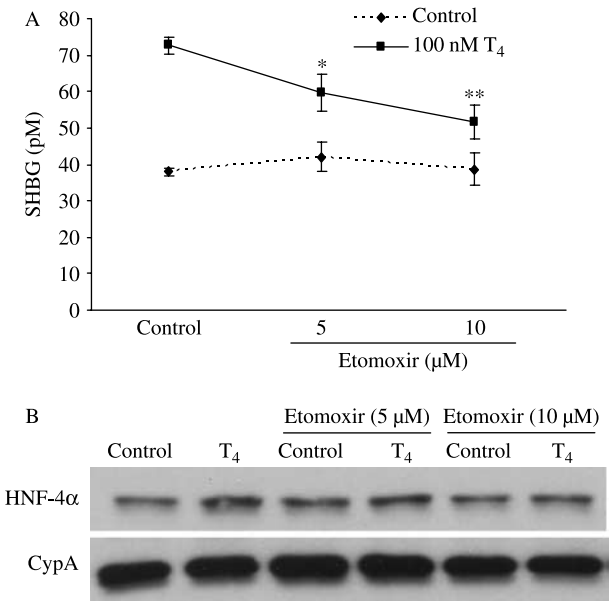

Figure 6 Thyroid hormone-induced increase in human SHBG production by HepG2 cells can be reduced by etomoxir treatment. (A) HepG2 cells were cultured in medium containing FBS for 5 days and treated daily with or without thyroid hormone (T_4 at 100 nM) in the presence or absence of etomoxir (5 or 10 μM). Accumulation of human SHBG in the medium was measured using an immunofluorometric assay. Data points are shown as mean±S.D. of triplicates. *$P<0{\cdot}05$ and **$P<0{\cdot}01$ compared with the control. (B) Western blot of HNF-4α and cyclophilin A (CypA) in total protein extracts of HepG2 cells cultured for 5 days as in (A).

model (Jänne *et al.* 1998) indicated that treatment with T_3 was much more effective than treatment with T_4 in terms of increasing plasma levels of human SHBG when these hormones were added to the drinking water (data not shown), and this likely reflects the greater bioavailability of T_3 versus T_4 due to their differential binding to thyroxin-binding globulin in the blood.

To determine how T_4 might influence transcription of the human *SHBG* gene, we examined the *SHBG* promoter sequence that controls its expression in the liver (Jänne & Hammond 1998, Jänne *et al.* 1998). When analyzed in the context of a luciferase reporter gene assay in HepG2 cells, the activity of the 803 bp 'full-length' human *SHBG* promoter is consistently lower than that of the 299 bp 'proximal' promoter sequence, and this has been attributed to the effects of upstream binding sites for Sp1 and a TAAAA pentanucleotide repeat on transcription (Jänne & Hammond 1998, Hogeveen *et al.* 2001). Consistent with the time-dependent effects of thyroid hormone on the secretion of SHBG from HepG2 cells, the full-length (803 bp) and proximal (299 bp) human *SHBG* promoter sequences failed to respond acutely to thyroid hormone treatments, but they both exhibit robust responses after cells are pre-treated with T_4 for 3–4 days. Most importantly, these results demonstrate that the effect of thyroid hormone on *SHBG* gene expression only requires sequences within the proximal promoter, and we found this to be remarkable because it lacks a typical TRE. Moreover, the fact that the *SHBG* promoter failed to respond to either T_3 or T_4 within 48 h suggests that the thyroid hormone-induced increase in *SHBG* gene expression is mediated indirectly through some other mechanism, and we excluded the possibility that this might involve thyroid hormone-mediated changes in the expression of the TR itself.

We have recently found that the human *SHBG* gene in HepG2 cells, as well as in a transgenic mouse model, responds to changes in metabolic state brought about by increased lipogenesis after treatment with glucose or fructose (Selva *et al.* 2007). Importantly, these studies demonstrated that this effect is mediated by alterations in cellular HNF-4α levels, which acts as a key regulator of *SHBG* transcription (Jänne & Hammond 1998). Since HNF-4α responds to and modulates the metabolic state of the liver (Sladek 1993, Jump 2004), and because thyroid hormones increase the basal metabolic rate of the liver (Malik & Hodgson 2002), we explored the possibility that the changes in *SHBG* expression in HepG2 cells after 5 days treatment with thyroid hormones involves changes in HNF-4α expression and/or its accumulation. Our results support this because we observed an increase in cellular HNF-4α mRNA content, as well as an increase in nuclear HNF-4α levels, in concert with an increase in SHBG mRNA levels after the cells were treated with T_4, and the T_4-mediated increase in SHBG production by HepG2 cells occurs regardless of whether the cells were cultured in the presence of low or high levels of glucose. We also found that the addition of T_3 to the drinking water of mice that express a human *SHBG* transgene in their liver results in an increase in the blood levels of human SHBG in these mice, and that this occurs in parallel with an increase in hepatic HNF-4α levels in the same animals. While there is no evidence that thyroid hormones directly regulate the *HNF-4α* gene in the liver, a change in the metabolic state of the liver after T_4 treatment could in turn influence *HNF-4α* expression.

The essential role of HNF-4α in enhancing *SHBG* transcription in HepG2 cells in response to thyroid hormones was demonstrated by blocking the increase in cellular HNF-4α levels by using a siRNA approach. It was considered important to do this because thyroid hormones could increase the expression of other transcription factors that might influence *SHBG* expression, or coactivators, such as Trp-3, PGC-1α, Src-1, and Src-3 (Zhang & Lazar 2000) that influence the activities of transcription factors, including HNF-4α (Wang *et al.* 1998, Yoon *et al.* 2001, Iwahashi *et al.* 2002). We therefore also studied the effects of the thyroid hormone treatments on the levels of Trp-3, PGC-1α,

and Src-3 mRNA levels in the HepG2 cells, and failed to observe any changes in semi-quantitative RT-PCR assays (data not shown). Moreover, our results imply that the effect of thyroid hormone on human *SHBG* expression is entirely due to the increase in HNF-4α levels, because siRNA suppression of the T_4-induced HNF-4α levels in HepG2 cells completely blocked the T_4-induced increase in *SHBG* promoter activity.

It is possible that HNF-4α levels in HepG2 cells are also influenced by their overall metabolic state because we have previously observed that HNF-4α levels are reduced in concert with increases in the cellular content of palmitate after treatment of HepG2 cells with either monosaccharides or palmitoyl CoA (Selva *et al.* 2007). Our present experiments further support this by showing that a reduction in cellular palmitate levels after T_4 treatment occurs in concert with an increase in HNF-4α levels. It is also known that thyroid hormones increase the expression of CPTI (Mynatt *et al.* 1994), and this likely explains the reduced palmitate levels in HepG2 cells treated with T_4. Taken together therefore, these observations have led us to conclude that the lipid content of the cells must in some way influence the production and/or the accumulation of HNF-4α, which in turn plays a pivotal role in controlling *SHBG* gene expression. This is further supported by our experiment in which HepG2 cells were treated with T_4 in the presence or absence of a CPTI inhibitor to block palmitate oxidation, which resulted in a loss of the thyroid hormone stimulated increase in HNF-4α levels and a concomitant reduction in production of SHBG by HepG2 cells.

In conclusion, our studies demonstrate that the well-known effect of thyroid hormones on increasing plasma SHBG levels reflects an alteration in the metabolic state of hepatocytes resulting in increased nuclear HNF-4α levels, which in turn act on the *SHBG* promoter to increase its transcriptional activity. It remains to be determined whether thyroid hormones act directly or indirectly to alter *HNF-4α* gene expression in hepatocytes, but our data do imply that changes in metabolic state contribute in part to an increase in HNF-4α levels within hepatocytes treated with thyroid hormones.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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