Monosaccharide-induced lipogenesis regulates the human hepatic sex hormone–binding globulin gene

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The liver produces plasma sex hormone–binding globulin (SHBG), which transports sex steroids and regulates their access to tissues. In overweight children and adults, low plasma SHBG levels are a biomarker of the metabolic syndrome and its associated pathologies. Here, we showed in transgenic mice and HepG2 hepatoblastoma cells that monosaccharides (glucose and fructose) reduce human SHBG production by hepatocytes. This occurred via a downregulation of hepatocyte nuclear factor–4α (HNF-4α) and replacement of HNF-4α by the chicken OVA upstream promoter–transcription factor 1 at a _cis_-element within the human _SHBG_ promoter, coincident with repression of its transcriptional activity. The dose-dependent reduction of HNF-4α levels in HepG2 cells after treatment with glucose or fructose occurred in concert with parallel increases in cellular palmitate levels and could be mimicked by treatment with palmitoyl-CoA. Moreover, inhibition of lipogenesis prevented monosaccharide-induced downregulation of HNF-4α and reduced _SHBG_ expression in HepG2 cells. Thus, monosaccharide-induced lipogenesis reduced hepatic HNF-4α levels, which in turn attenuated _SHBG_ expression. This provides a biological explanation for why SHBG is a sensitive biomarker of the metabolic syndrome and the metabolic disturbances associated with increased fructose consumption.

Introduction

Body mass index is a major determinant of sex hormone–binding globulin (SHBG) concentrations in the blood of men and women (1–3). Low serum SHBG levels in overweight individuals are a biomarker for the metabolic syndrome (4–7) and are predictive of type 2 diabetes (8–10) and cardiovascular disease risk (11, 12). Conversely, high serum SHBG levels in children (13) and women (14) with anorexia nervosa decrease during weight gain, and SHBG measurements represent an index of nutritional status in this type of eating disorder (14). The reason why plasma SHBG levels are invariably low in obese individuals of all ages and sex has been the subject of much debate. There is no reason to suspect that the plasma clearance of SHBG differs in normal-weight and obese individuals, and current evidence favors a mechanism involving reduced SHBG production by hepatocytes in response to a metabolic disturbance associated with excess body mass index and especially visceral adiposity (15, 16). To date, the widely held explanation for this is that elevated insulin in obese and insulin-resistant individuals somehow acts on the liver to decrease hepatic SHBG production (16). Although it has been reported that insulin reduces SHBG production by human HepG2 hepatoblastoma cells (17), a subsequent study indicated that this effect is not specific and may be attributed to a global reduction in protein secretion (18). In view of earlier reports that plasma SHBG levels are negatively correlated with glucose, as well as insulin (19), we have explored the hypothesis that expression of the human _SHBG_ gene in the liver responds to increased exposures to monosaccharides rather than insulin. In addition, because one of the major dilemmas of high-carbohydrate diets in the management of obesity and type 2 diabetes is increased hepatic lipogenesis (20), we have examined whether this contributes to the regulation of _SHBG_ gene expression in the liver.

Results

Human SHBG expression in transgenic mouse livers is decreased by carbohydrates and not by insulin. We first manipulated the diets of mice expressing different human _SHBG_ transgenes (21): one line contains the 4.3-kb transcription unit expressed in liver and kidney (22), while the other contains a larger, 11-kb region of human _SHBG_ that is also expressed in male germ cells (23). When male mice expressing an 11-kb human _SHBG_ transgene were fed a high-sucrose diet, human SHBG levels in the blood were reduced by about 50% after 1 week (Figure 1A). Serum SHBG levels in these animals increased to normal levels within 1 week after they were returned to an isocaloric basal diet but decreased again when the animals were fed the high-sucrose diet in a third week of treatment (Figure 1A). When female mice carrying the same transgene were treated first with the basal diet and then fed the high-sucrose diet, a similar reduction in serum SHBG levels was observed, which returned to normal when this group of animals was returned to the basal diet (Figure 1A). In addition, human SHBG mRNA levels were 4-fold lower in the livers of male and female mice fed the high-sucrose diet when compared with the basal diet but were unchanged in the kidney (Figure 1B shows data for male mice only). Similar results were obtained using mice carrying a 4.3-kb human _SHBG_ transgene mutated to disrupt an upstream stimulatory factor (USF) binding site (24) in the proximal promoter (Figure 1C). This USF binding site is adjacent to a hepatocyte nuclear
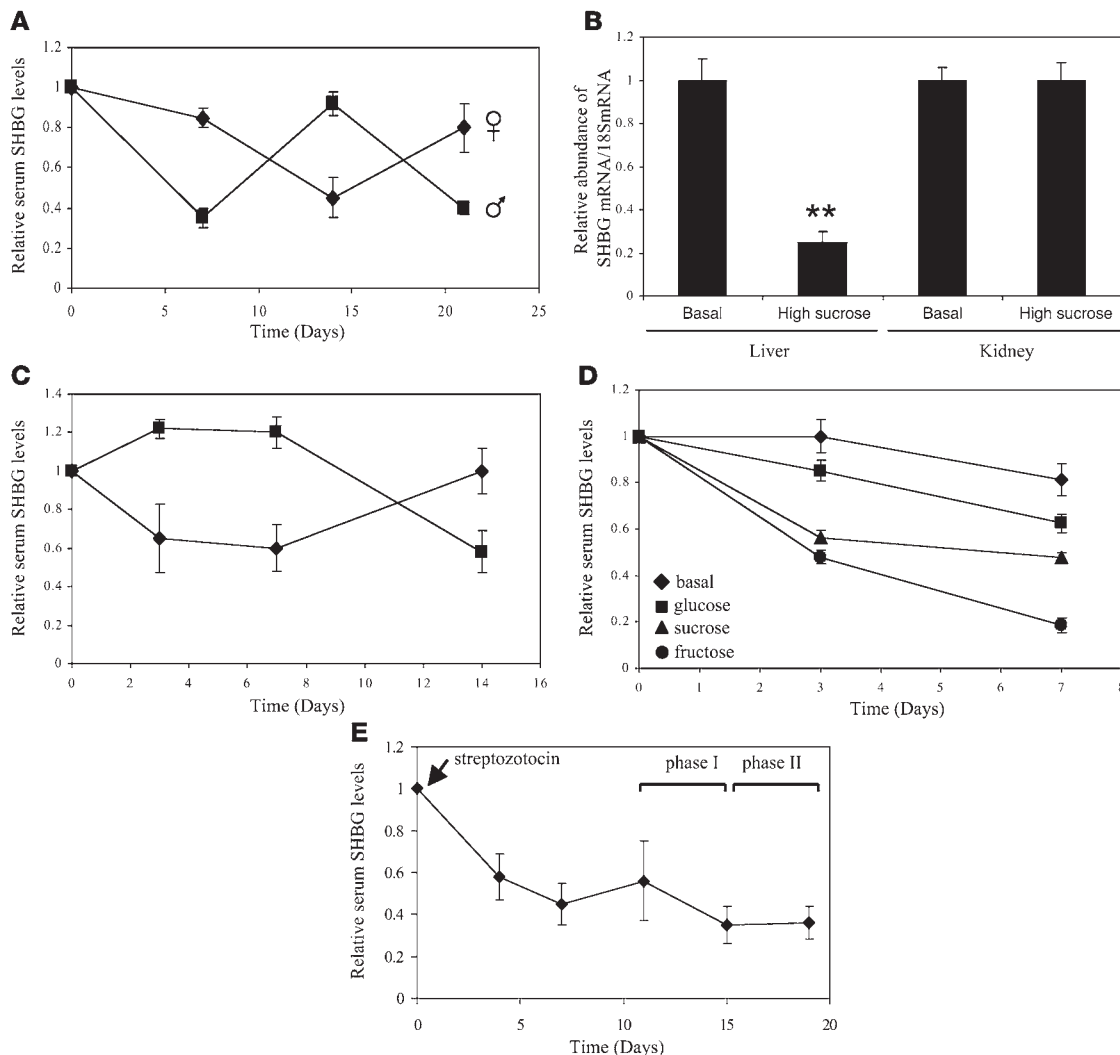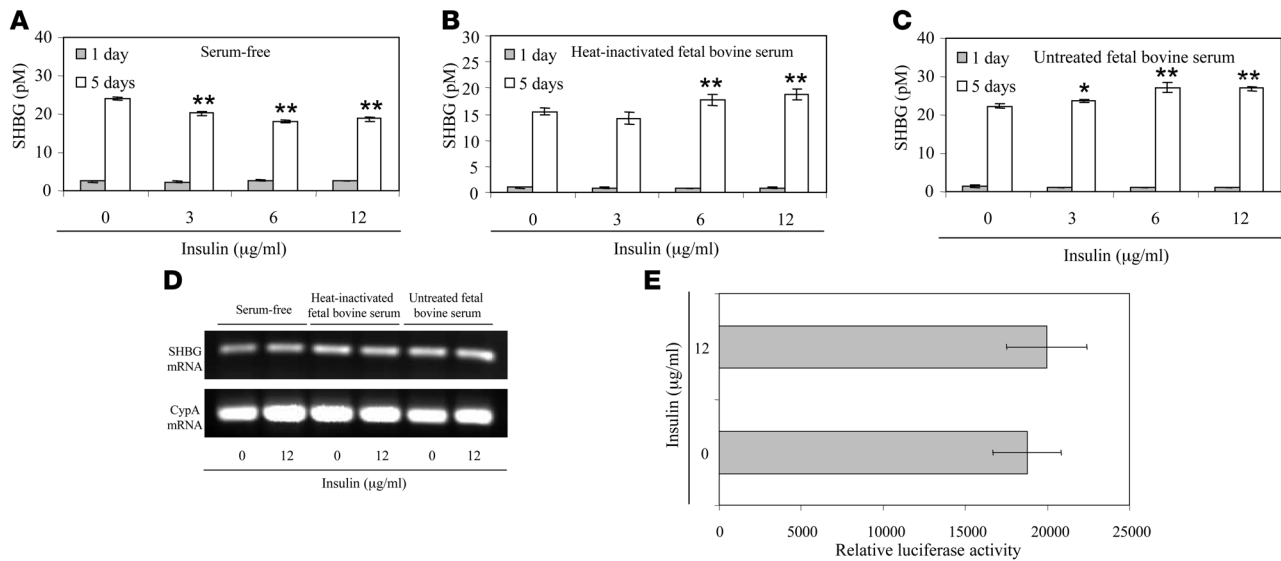
Figure 1
Hepatic production of SHBG in mice expressing human *SHBG* transgenes (21) is reduced after feeding diets with high monosaccharide content or increasing blood glucose levels by streptozotocin treatment. (**A** and **B**) Mice were fed high-sucrose or isocaloric basal diets for 7 days (3 per group), and the diets were then reversed in two 7-day cycles. Serum SHBG levels are expressed as mean ± SEM relative to pretreatment levels to compensate for between-animal variability (**A**). At day 21, human SHBG mRNA abundance was determined in relation to 18S RNA (mean ± SEM) in liver and kidney; **P < 0.01 compared with basal diet values (**B**). (**C**) Serum SHBG levels in mice expressing a human *SHBG* transgene lacking a USF-binding site in the promoter (24) were reduced by feeding a high-sucrose diet. Animals (3 per group) were fed a basal diet (squares) or a high-sucrose diet (diamonds) for 7 days, and diets were then reversed for 7 days. Serum SHBG measurements (mean ± SEM) are expressed relative to pretreatment levels. (**D**) Serum SHBG levels (mean ± SEM) were reduced relative to pretreatment levels in human *SHBG* transgenic mice (3–4 per group) fed equicaloric diets containing high glucose, sucrose, or fructose, when compared with a basal diet. (**E**) Human *SHBG* transgenic mice treated with streptozotocin were maintained on a basal diet for 11 days, followed by a high-sucrose diet (phase I) and the basal diet (phase II). Serum SHBG levels (mean ± SEM) are expressed relative to pretreatment values.

factor–4α (HNF-4α) binding site, and this particular combination of *cis*-elements has been proposed to constitute a carbohydrate response element in the promoters of other genes (25, 26).

Sucrose, a major dietary disaccharide, is hydrolyzed to glucose and fructose prior to absorption. Therefore, to isolate the potential effects of glucose and fructose, we repeated the experiment using isocaloric diets prepared to contain identical amounts of sucrose, glucose, or fructose as the carbohydrate source. Furthermore, because fructose does not acutely stimulate pancreatic β cell insulin release (27, 28), this experimental paradigm allows us to distinguish between the effects of monosaccharides and insulin on

hepatic metabolism. Transgenic mice fed all 3 high-carbohydrate diets for 1 week showed decreased serum SHBG levels, when compared with mice fed the isocaloric basal diet (Figure 1D). Remarkably, the high-fructose diet was the most effective and decreased the SHBG levels by about 80%, while equicaloric diets with glucose and sucrose resulted in 40% and 50% decreases, respectively (Figure 1D). More importantly, these effects occurred within 3 days of feeding fructose or sucrose (Figure 1D).

To further determine how changes in insulin and blood glucose levels influence human SHBG production by the liver, a similar experiment was performed on animals that were first made diabet-

Figure 2
Insulin does not influence SHBG production by HepG2 cells. (**A**–**D**) HepG2 cells were cultured in serum-free medium, inactivated FBS, or untreated FBS for 5 days and treated daily with insulin at different concentrations. Accumulation of human SHBG in the medium was measured at timed intervals using an immunofluorometric assay (46) over a 5-day treatment period. Data points are shown as mean ± SD of triplicates; *$P < 0.05$, **$P < 0.01$ compared with no insulin treatment (**A**–**C**). On day 5 cells were taken for human SHBG mRNA and cyclophilin A mRNA measurements by RT-PCR analyses (**D**). (**E**) The effect of insulin (12 μg/ml) on human *SHBG* promoter activity was analyzed in HepG2 cells in the context of a luciferase reporter gene assay. Data points are mean ± SD of triplicate measurements.

ic by a single injection of streptozotocin (29). These animals were then fed a high-sucrose diet for 5 days (phase I), followed by the basal diet for 4 days (phase II). Failure of pancreatic β cell function was confirmed by an increase in blood glucose levels to greater than 400 mg/dl (normal value, 120 mg/dl) within 4 days of streptozotocin treatment. Serum SHBG levels decreased by 40%–50% within 4–7 days of treatment with streptozotocin, but no further decrease in serum SHBG levels occurred when these mice were fed a high-carbohydrate diet, and no increase occurred when they were returned to the basal diet (Figure 1E).

Monosaccharides reduce SHBG production by HepG2 cells irrespective of the presence of insulin. To explore the mechanism(s) responsible for the reduced hepatic production of SHBG in response to monosaccharides observed in vivo, we first reexamined the effects of insulin on SHBG production by HepG2 cells. In the most widely cited report that insulin reduces SHBG production by HepG2 cells (17), the experiments were conducted using serum-free medium without defining the glucose levels. To separate the effects of glucose and insulin, we first examined the effects of 3 different concentrations of insulin on SHBG secretion by HepG2 cells cultured in low-glucose medium, in the absence or presence of fetal FBS or heat-inactivated FBS. As reported previously (17), the amounts of SHBG secreted by HepG2 cells cultured in serum-free medium decrease by about 20% after insulin treatment (Figure 2A). However, since culture of HepG2 cells in serum-free medium results in a global decrease in protein secretion, the observed change is most likely nonspecific, as noted by others (18). By contrast, there were small increases in the amounts of SHBG secreted by insulin-treated HepG2 cells cultured in the presence of heat-inactivated FBS (Figure 2B) or untreated FBS (Figure 2C), but the SHBG mRNA levels did not change in the HepG2 cells cultured under these conditions, even at the highest concentration of insulin used

(Figure 2D). To further assess whether insulin influences human *SHBG* expression, we studied the transcriptional activity of a luciferase reporter gene under the control of the human *SHBG* promoter (–299/+60 bp relative to the major transcription start site) in HepG2 cells (22) and found no insulin-dependent difference in the reporter gene response (Figure 2E).

We next studied the effects of monosaccharides on SHBG production by HepG2 cells. When HepG2 cells were cultured in the presence of FBS with 10 mM glucose or fructose for 5 days, SHBG accumulation in the medium was reduced by 50% (Figure 3A) in concert with reduced cellular SHBG mRNA levels (Figure 3B). Because HNF-4α plays a key role in the transcriptional activity of the human *SHBG* promoter (22), we examined HNF-4α mRNA levels in these cells and found they were substantially reduced after treatment with glucose or fructose (Figure 3B). In addition, a Western blot of cell extracts revealed lower HNF-4α levels after treatment with the monosaccharides (Figure 3C). We also examined human *SHBG* promoter activity in the context of a luciferase reporter gene in HepG2 cells cultured for 4 days in the presence or absence of 10 mM glucose or fructose and found a 3- to 4-fold reduction in reporter gene expression (Figure 3D).

To determine whether insulin influences the downregulation of *SHBG* gene expression by monosaccharides in HepG2 cells, we measured the SHBG production by cells grown for 5 days in low-glucose medium containing FBS or in the same medium supplemented daily with glucose, insulin, or both (Figure 4A). In this experiment, SHBG levels in the culture medium were reduced by about 50% when cells were grown in the presence of glucose (1 mM or 10 mM), but insulin alone had no effect. Moreover, when insulin was added together with 1 mM or 10 mM glucose, no additional reductions in SHBG concentrations in the medium were observed. Similarly, the levels of HNF-4α in HepG2 cells cultured under the

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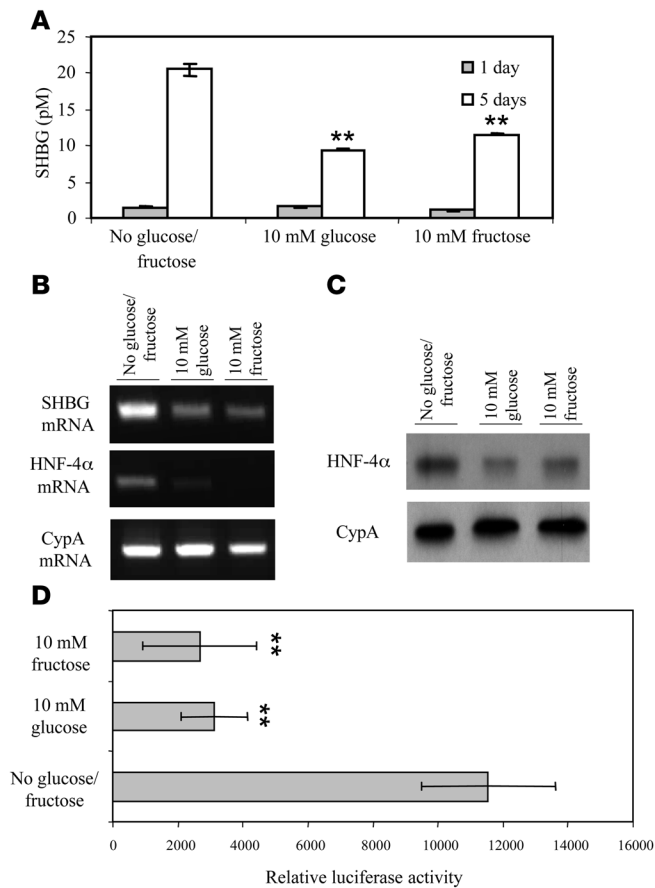
Figure 3

Monosaccharides decrease human SHBG production by HepG2 cells in concert with reduced cellular HNF-4α levels. (**A**) Human SHBG accumulation in the medium of HepG2 cells treated daily with 10 mM of glucose or fructose was substantially reduced. Data points are shown as mean ± SD of triplicates; **$P < 0.01$ compared with no glucose/fructose supplementation. (**B**) Human SHBG and HNF-4α mRNA levels determined by RT-PCR in HepG2 cells cultured for 5 days as in **A**. Cyclophilin A (CypA) mRNA was amplified as in internal control. (**C**) HNF-4α and cyclophilin A levels measured by Western blotting in HepG2 cells cultured for 5 days as in **B**. (**D**) Human *SHBG* promoter activity was measured in the context of a luciferase reporter gene assay in HepG2 cells cultured for 4 days in the presence or absence of 10 mM glucose or fructose. Data points are shown as mean ± SD of triplicates; **$P < 0.01$ compared with no glucose/fructose supplementation.

approach to mimic the reduction in HNF-4α levels observed in HepG2 cells after treatment with glucose or fructose and found that this reduced the activity of the human *SHBG* promoter (Figure 5A). Importantly, this effect was evident in the construct containing less than 300 bp of the promoter (Figure 5A). We then performed a chromatin immunoprecipitation (ChIP) assay using DNA/protein complexes extracted from HepG2 cells cultured in low (0.1 mM) or high (10 mM) glucose or fructose to determine whether HNF-4α and COUP-TF1 bind differentially to sites within the *SHBG* proximal promoter (22). The results indicate that in the presence of low monosaccharide concentrations, HNF-4α rather than COUP-TF1 bound this region of the *SHBG* promoter, while binding of COUP-TF1 was more evident when cells were grown in medium containing 10 mM glucose or fructose (Figure 5B). To further demonstrate that interactions between HNF-4α and COUP-TF1 at this site control the human *SHBG* promoter, we treated HepG2 cells maintained in low glucose-containing medium with an siRNA to reduce COUP-TF1 mRNA levels (Figure 5C). Under these conditions, the HNF-4α/COUP-TF1 binding site in the human *SHBG* promoter was largely occupied by HNF-4α (Figure 5B), and it was therefore not unexpected

same conditions were uniformly reduced by supplementation with glucose and not influenced by the presence of insulin (Figure 4B). To confirm that the insulin-signaling cascade was functional in the HepG2 cells grown under these conditions, we measured the insulin-induced phosphorylation of the ribosomal protein S6 (30) in the cells and found that it increased only after treatment with insulin in the presence of glucose (Figure 4C) — a finding that casts doubt on whether insulin functions at all in HepG2 cells maintained under conditions in which glucose will be depleted, as in previous reports (17, 18, 31).

Reduced SHBG production by monosaccharides involves competition between HNF-4α and chicken OVA upstream promoter–transcription factor 1 (COUP-TF1) for binding sites within the *SHBG* promoter. Overexpression of HNF-4α in HepG2 cells increases human *SHBG* transcription by binding a *cis*-element within the human *SHBG* promoter, and COUP-TF1 modulates this through competitive binding to the same site (22). We therefore first used an siRNA

Figure 4

Insulin does not alter human SHBG production or HNF-4α protein levels in HepG2 cells cultured in the presence of high glucose. (**A**) Human SHBG accumulation in the medium is decreased in HepG2 cells treated daily with 1 mM or 10 mM glucose in the absence or presence of 6 μg insulin/ml for 5 days. Data points are shown as mean ± SD of triplicates; **$P < 0.01$ compared with no glucose supplementation. (**B**) Western blots of HNF-4α and cyclophilin A in HepG2 cells treated as in **A** for 5 days. (**C**) Western blots of phospho-S6 and cyclophilin A in HepG2 cells treated as in **A**.

A

B



C

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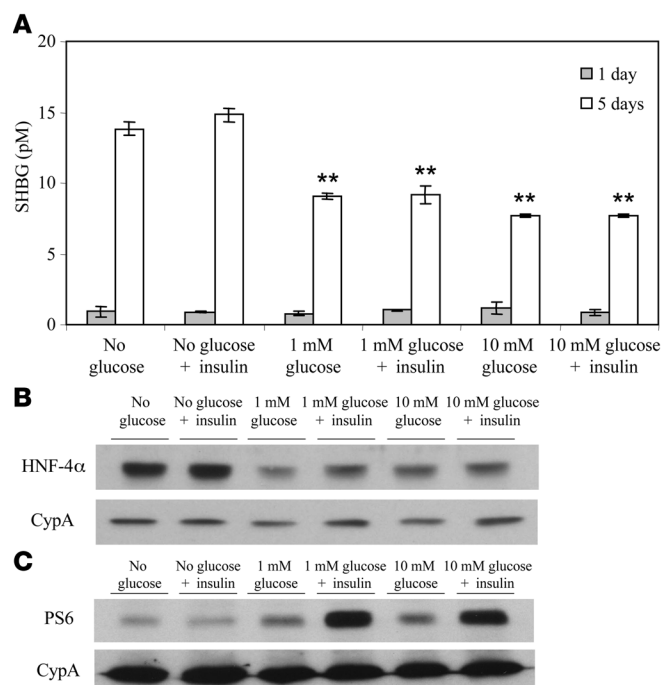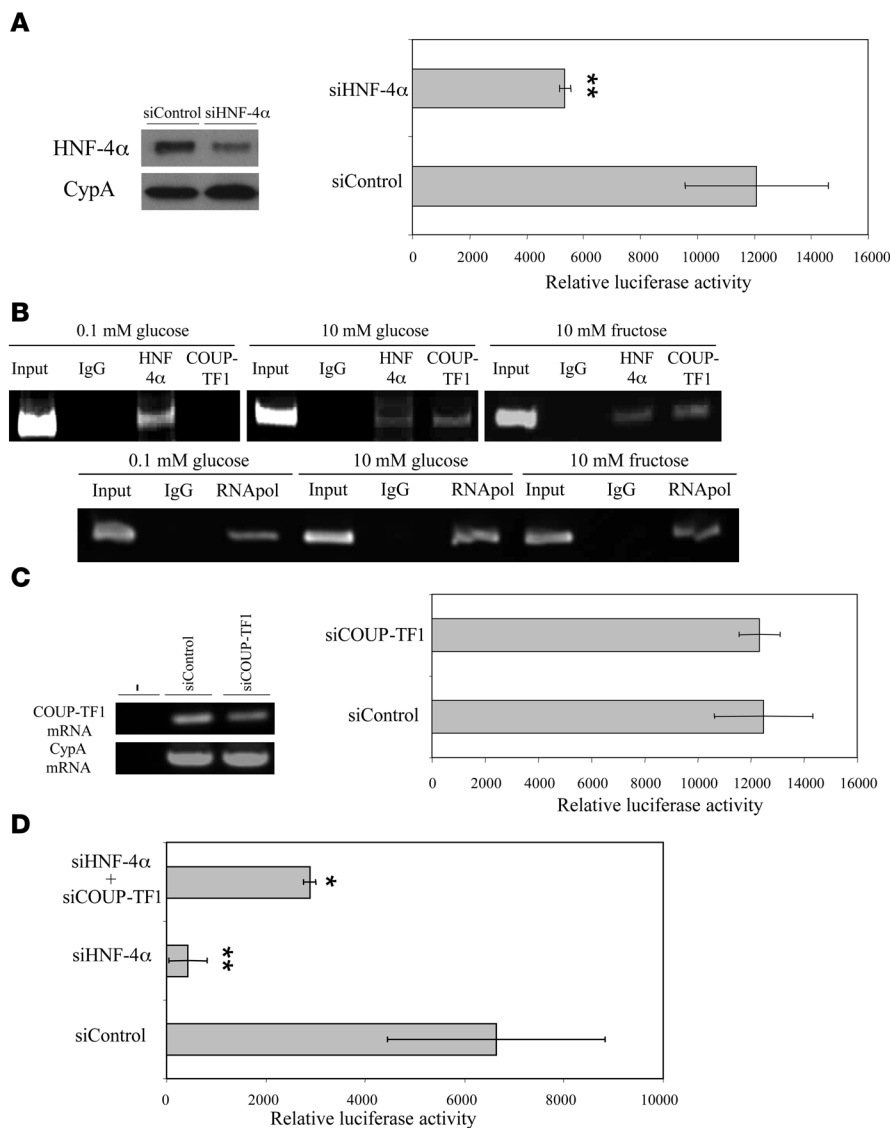
Figure 5
Competition between HNF-4α and COUP-TF1 at a *cis*-element within the human *SHBG* promoter regulates its activity. (**A**) HNF-4α levels were reduced in HepG2 cells after transient transfection of an HNF-4α siRNA versus a control siRNA oligonucleotide (left), and siRNA-mediated downregulation of HNF-4α reduced human *SHBG* promoter activity in a luciferase reporter gene assay (right). Data points are shown as mean ± SD of triplicates; **$P < 0.01$ compared with cells treated with an siRNA control. (**B**) ChIP assays of HNF-4α and COUP-TF1 binding to the human *SHBG* promoter (top). As a control for the ChIP, anti–RNA polymerase (RNApol) antibodies were used with human-specific oligonucleotide primers to PCR amplify the *GAPDH* promoter (bottom). A nonspecific mouse IgG was used in ChIP reactions to control for nonspecific immunoprecipitation. Positive PCR controls of sheared genomic DNA templates indicated the integrity of the input DNA used in the ChIP reactions. (**C**) Reduction of COUP-TF1 mRNA in HepG2 cells after siCOUP-TF1 treatment (left) did not influence the activity of the human *SHBG* promoter under basal conditions (right). Data points are shown as mean ± SD of triplicates. (**D**) Reduction of human *SHBG* promoter activity after treatment with siHNF-4α was mitigated by cotreatment with siCOUP-TF1. Data points are shown as mean ± SD of triplicates; *$P < 0.05$, **$P < 0.01$ compared with cells treated with an siRNA control.

that this siCOUP-TF1 treatment did not influence the transcriptional activity of the human *SHBG* promoter (Figure 5C). As noted above (Figure 5A), when the HepG2 cells were treated under these conditions with an siRNA against HNF-4α mRNA (siHNF-4α) there was a substantial reduction in human *SHBG* promoter activity (Figure 5D), but coadministration of siCOUP-TF1 together with siHNF-4α abrogated this (Figure 5D).

Monosaccharide-induced lipogenesis acts via HNF-4α to downregulate SHBG in HepG2 cells. Since fructose enters the glycolytic pathway at dihydroxyacetone phosphate via fructose-6-phosphate, it bypasses key regulatory steps of glycolysis and markedly increases lipogenesis and triglyceride synthesis. This suggested that *SHBG* gene expression might be coordinately regulated with lipogenic activity, and we set out to test this in HepG2 cells. We first performed an experiment to assess the effects of glucose or fructose supplementation in relation to fatty acid synthesis and changes in SHBG production by HepG2 cells. To do this, we determined the levels of palmitate in HepG2 cells cultured in the absence or presence of 0.1–10 mM glucose or fructose for 5 days and found modest but significant increases in cellular palmitate levels even after treat-

ment with 0.1 mM glucose or fructose that were also associated with small decreases in SHBG production (Figure 6A). However, we observed substantial, 2- to 3-fold increases in cellular palmitate levels after 5 days supplementation with 1–10 mM glucose or fructose, and these were associated with maximal reductions in SHBG production that also occurred after the 1 mM monosaccharide supplementations (Figure 6A).

To further demonstrate that increases in palmitate as a result of de novo lipogenesis reduce SHBG in HepG2 cells, we treated HepG2 cells cultured in the presence of 10% delipidated FBS with 1–10 μM palmitoyl-CoA for 5 days. This resulted in a dose-dependent decrease in HepG2 production of SHBG (Figure 6B) that was accompanied by reductions in cellular SHBG mRNA and HNF-4α mRNA (Figure 6C) and immunoreactive HNF-4α (Figure 6D), as well as reductions in the transcriptional activity of the human *SHBG* promoter (Figure 6E). In addition, we cocultured cells treated with 10 mM glucose or fructose with the fatty acid synthase inhibitor cerulenin (32) and found that this prevented the monosaccharide-dependent reductions in SHBG production (Figure 7A) and cellular HNF-4α levels (Figure 7B).
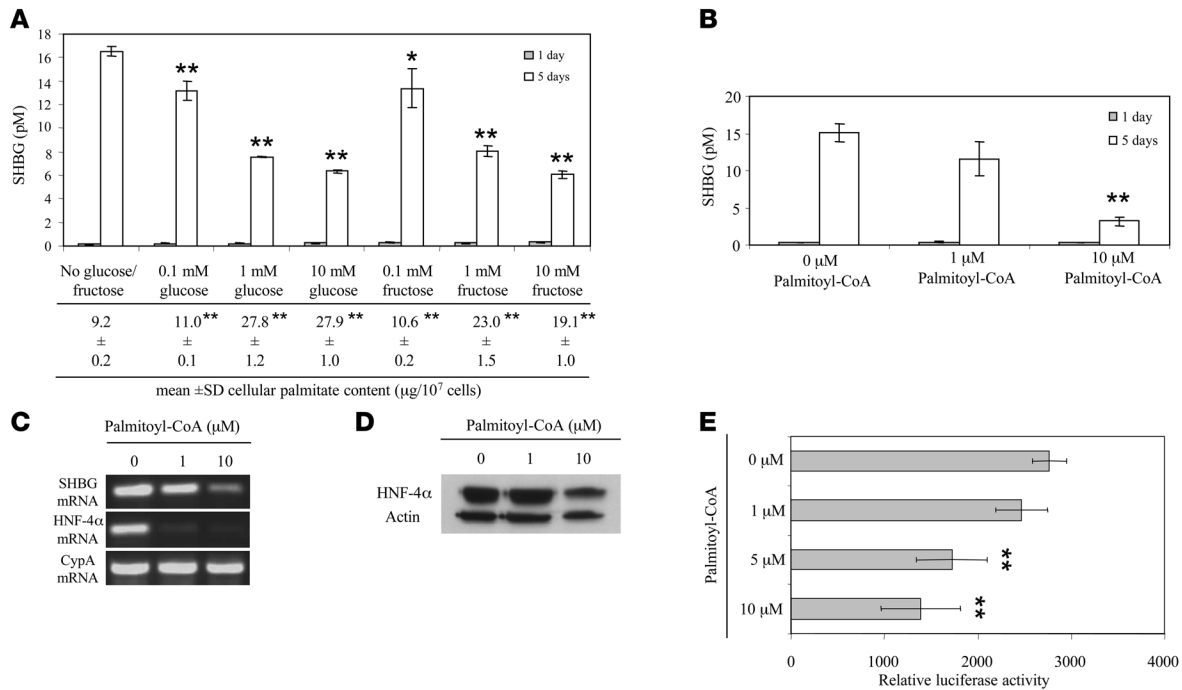
Figure 6
Increased palmitate levels in HepG2 cells as a result of monosaccharide or palmitoyl-CoA supplementation reduce HNF-4α levels and SHBG production. (**A**) Concentrations of human SHBG in the medium from untreated HepG2 cells and HepG2 cells after 5 days supplementation with 0.1–10 mM glucose or fructose. Data points are shown as mean ± SD of triplicates; *$P < 0.05$, **$P < 0.01$ compared with no glucose/fructose supplementation. In addition, the cellular content of palmitate was measured in cells after 5-day treatment with monosaccharides as indicated; **$P < 0.01$ compared with no glucose/fructose supplementation. (**B**) Concentrations of SHBG in medium from untreated HepG2 cells and HepG2 cells treated for 5 days with 1 μM or 10 μM palmitoyl-CoA. **$P < 0.01$ compared with no treatment. (**C**) HNF-4α and SHBG mRNA levels determined by RT-PCR in untreated HepG2 cells and HepG2 cells treated for 5 days with 1 μM or 10 μM palmitoyl-CoA. Cyclophilin A mRNA was amplified as an internal control. (**D**) Western blots of HNF-4α and actin in extracts of untreated HepG2 cells and HepG2 cells treated for 5 days with 1 μM or 10 μM palmitoyl-CoA. (**E**) The effects of 1–10 μM palmitoyl-CoA on human *SHBG* promoter activity was analyzed in HepG2 cells in the context of a luciferase reporter gene. Data points are mean ± SD of triplicate measurements; **$P < 0.01$ compared with no treatment.

Discussion

Low plasma SHBG levels are a biomarker of the metabolic syndrome and a harbinger of its associated pathologies. In insulin-resistant individuals, elevated insulin levels have been linked to low plasma SHBG levels, but hyperglycemia is also usually present in conjunction with hypertriglyceridemia. To discriminate the direct effects of insulin and blood sugars on human *SHBG* gene expression in vivo, we used mice in which human *SHBG* transgenes are expressed under the control of their own regulatory sequences in an appropriate tissue-specific manner (21, 22). Previous studies have defined the transcription unit responsible for human *SHBG* gene expression in the liver (21) and mapped key regulatory elements within a proximal promoter sequence (22), which includes a binding site for USF transcription factors (24) that we previously assumed might represent part of a carbohydrate response element (22). However, removal of this USF binding site from a human *SHBG* transgene has no impact on the rapid reduction of human SHBG levels in the blood of mice after feeding a high-sucrose diet.

Increasing blood glucose levels through feeding high amounts of sucrose (a disaccharide of glucose and fructose) or glucose resulted in decreased human SHBG levels in the blood of mice expressing human *SHBG* transgenes in their livers, but both treatments increased insulin levels. However, human SHBG levels in the blood were reduced even more effectively when these transgenic mice

were fed high concentrations of fructose rather than an equicaloric amount of glucose, and this occurred within just 3 days. Fructose, unlike glucose, fails to acutely stimulate pancreatic β cell insulin release (27), and we therefore suspected that insulin might not play a significant role in reducing the hepatic production of SHBG. Moreover, since the plasma half-life of SHBG is about 38 hours (33), these results imply that the effects of dietary monosaccharides on *SHBG* gene expression in the liver occur very rapidly.

To further determine whether insulin represses human *SHBG* gene expression in vivo, we took a second approach that involved using streptozotocin to induce diabetes in the same transgenic mouse model. Remarkably, serum levels of human SHBG declined rapidly as the mice became diabetic and their blood glucose levels rose and were not further reduced upon feeding a high-sucrose diet. Clearly, then, insulin is not directly responsible for repressing human SHBG production, since otherwise its blood levels should have increased as the animals became insulin deficient. Instead, these results are consistent with a rapid repression of hepatic SHBG production in response to increased blood glucose levels, as in our dietary studies. These in vivo studies prompted us to reevaluate whether insulin directly regulates *SHBG* expression in HepG2 cells. In a comprehensive series of experiments, we demonstrate that the *SHBG* gene in HepG2 cells responds similarly to the human *SHBG* transgene in livers of mice; i.e., it is not regulated by insulin but is repressed by monosaccharides.

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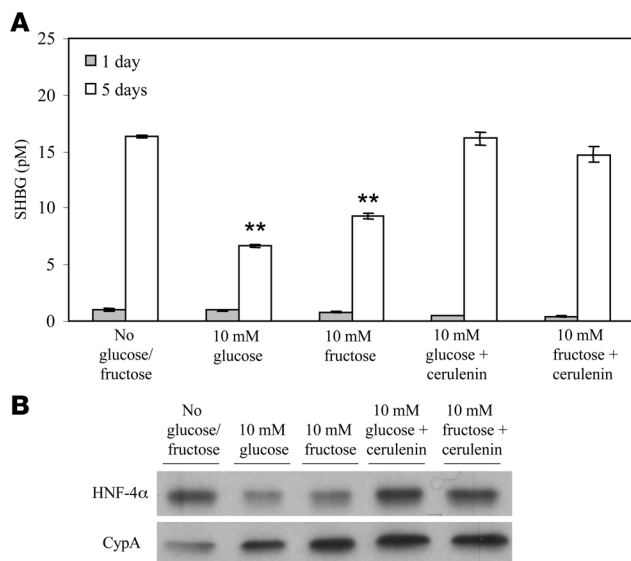
Figure 7
Monosaccharide-induced reductions of SHBG production by HepG2 cells are blocked by inhibiting fatty acid synthase. (**A**) Reduction of human SHBG accumulation in HepG2 medium treated daily with 10 mM glucose or fructose was blocked by cotreatment of the cells with cerulenin (10 μg/ml) over 5 days. Data points are shown as mean ± SD of triplicates; **P < 0.01 compared with no glucose/fructose supplementation. (**B**) Reduction of HNF-4α levels in HepG2 grown for 5 days in the presence of 10 mM glucose or fructose was also blocked by cerulenin cotreatment.

Several aspects of the experimental design distinguish our work from previous studies. First, previous studies used HepG2 cells grown in serum-free medium for 1 week or more during insulin treatment, without replacing or supplementing the medium in order to allow the accumulation of SHBG to be measured (17, 18, 31). When we replicated these experiments, a modest decline in the accumulation of SHBG in the medium was observed. However, under these conditions the cells are not only serum starved, but the culture medium is depleted of nutrients, and this causes a nonspecific reduction in protein biosynthesis/secretion (18). This conclusion is supported by our observation that insulin fails to reduce steady-state SHBG mRNA levels or the transcriptional activity of the *SHBG* promoter in HepG2 cells grown in serum-free medium or in medium supplemented with untreated or heat-inactivated FBS. Second, a series of studies with HepG2 cells maintained in 10% FBS to simulate more physiologically relevant conditions provided additional evidence that monosaccharides directly regulate *SHBG* gene expression in these cells.

Reduced SHBG secretion in response to supplementation with glucose or fructose was accompanied by reductions in SHBG mRNA and the activity of the *SHBG* promoter in HepG2 cells. Under these conditions, *HNF-4α* expression was also reduced at the mRNA and protein levels in the cells, and we considered this important, because HNF-4α plays a key role in controlling human *SHBG* gene expression in the liver (22). This was further illustrated by showing that a similar reduction in *SHBG* promoter activity occurs in HepG2 cells when the cellular content of HNF-4α is reduced by treatment with siRNA to about the same extent as by monosaccharide treatment.

We have shown previously that *SHBG* gene transcription in HepG2 cells is under the control of a TATA-less promoter (22). In that report, we demonstrated that HNF-4α binds to a site just upstream from the major transcription start site, where it substitutes for TBP in engaging the transcriptional machinery. We have also demonstrated that competition between HNF-4α and COUP-TF1 occurs at this site, with HNF-4α promoting transcription and COUP-TF1 repressing it (22). We therefore explored whether the reduction of HNF-4α levels in HepG2 cells after exposure to monosaccharides is accompanied by a relative increase in COUP-TF1 binding in this region of human *SHBG* promoter. The results of

a ChIP analysis revealed that this indeed occurs, and further support for this mechanism was obtained in an experiment in which cotreatment with a COUP-TF1 siRNA mitigated the monosaccharide-induced reduction of *SHBG* promoter activity in HepG2 cells.

Excess dietary carbohydrate is metabolized to fatty acids, with the lipogenic response to fructose being greater than that of glucose when administered in vivo (34). There are several reasons for this. First, the peripheral utilization of glucose is substantially greater than that of fructose, because glucose uptake by adipose and muscle via the GLUT4 transporter increases in response to insulin, while fructose is mainly taken up along with glucose by the GLUT2 transporter in the liver (35). In addition, fructose is a more efficient substrate for lipogenesis than glucose because it bypasses key regulatory steps in glycolysis. Although fructose is likely metabolized in HepG2 cells in much the same way, our data indicate that fructose and glucose are equipotent when administered to these cells with respect to their ability to increase lipogenesis and regulate *SHBG* gene expression. The most likely explanation for this is that the GLUT1 transporter, which takes up glucose but not fructose (35), is expressed at high levels in HepG2 cells, while expression of the GLUT2 transporter, which takes up both glucose and fructose (35), is relatively low in these cells (data not shown). Thus, despite the additional metabolic steps necessary for glucose to enter lipogenesis, it is likely that HepG2 cells take up glucose more efficiently than fructose and therefore both monosaccharides have equivalent effects on lipogenesis in vitro.

Long-chain fatty acids, including palmitic acid, have been identified as endogenous HNF-4α ligands that may influence its transcriptional activity (36, 37). In our experiments, palmitate levels increased substantially in HepG2 cells treated with glucose and fructose in concert with reductions in the expression of both *HNF-4α* and *SHBG*. In addition, inhibition of the regulatory enzyme complex of de novo fatty acid synthesis (38) by cerulenin not only blocked glucose or fructose from reducing SHBG production by HepG2 cells, but also prevented the reduction in cellular HNF-4α levels. These data are also consistent with a reduced loading of HNF-4α onto a *cis*-element in the human *SHBG* promoter that is shared with COUP-TF1, and with our finding that cotreatment with COUP-TF1 siRNA mitigates the effects of reduced HNF-4α mRNA levels on *SHBG* expression. Although decreases in HNF-4α levels in the liver in response to increased de novo lipogenesis provide an explanation for why serum SHBG levels are such a sensitive biomarker of metabolic disturbance, we do not know how increases in lipogenesis reduce *HNF-4α* expression in HepG2 cells. However, the *HNF-4α* promoter utilized in these cells also contains a shared binding site for HNF-4α and COUP-TF (39), and it is possible that long-chain fatty acid ligands of HNF-4α influence *HNF-4α* gene expression at this level.

In conclusion, our findings that monosaccharides regulate human *SHBG* gene expression by altering hepatic HNF-4α levels provide a mechanism to explain the link between plasma levels of SHBG and

the metabolic extremes in obese and anorexic individuals. As far as we know, no one else has noted that monosaccharides influence HNF-4α expression in hepatocytes via their conversion to fatty acids, and this is potentially important because *HNF-4α* gene variants have been implicated in type 2 diabetes (40). Finally, our finding that hepatic *SHBG* gene expression is remarkably responsive to fructose implies that plasma SHBG levels represent a useful biomarker of metabolic disturbances associated with excess sugar consumption (28, 41).

Methods

Animals. Mice expressing human *SHBG* transgenes have been characterized previously (21). Mice were maintained under standard conditions with food and water provided ad libitum, and the experimental procedures were approved by the Institutional Animal Use Subcommittees of University of Western Ontario and University of British Columbia and conducted in accordance with the Canadian Council on Animal Care.

In vivo experiments. Transgenic mice containing human *SHBG* transgenes were maintained on a basal diet containing 53% carbohydrate (PicoLab Mouse Diet 20) and where indicated were fed an isocaloric high-carbohydrate diet (70% sucrose; TD 98029; Harlan-Teklad) or a semisynthetic diet containing casein as the source of protein and in which sucrose, glucose, or fructose were exchanged while maintaining essential nutrient density across all diets (42). Diabetes in mature mice was induced by a single intraperitoneal injection of 200 mg/kg streptozotocin (Sigma-Aldrich) in a 50-mM citrate buffer, pH 4.5 (29). Diabetes in these animals was confirmed by the presence of elevated blood glucose levels (>400 mg/dl) measured by Fast-Take glucose strips (LifeScan Inc.).

Cell culture experiments. All cell culture reagents were from Life Technologies Inc. HepG2 hepatoblastoma cells (catalog no. HB-8065; ATCC) were routinely maintained in low-glucose DMEM (catalog 11885-084) 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 with low-glucose DMEM alone or in DMEM supplemented with 10% untreated or heat-inactivated FBS, or delipidated FBS (Cocalico Biologicals), prior to the addition of supplements (glucose, fructose, insulin, palmitoyl-CoA, or cerulenin), as indicated. Palmitate levels in HepG2 cells were determined by addition of a known amount of heptadecanoate (17:0) followed by direct methylation (43) and GLC for the separation and quantification of fatty acids (44).

Transient transfections of human *SHBG* promoter reporter plasmids together with a pCMVlacZ control plasmid (22) were carried out using LipofectAMINE reagent (according to Life Technologies Inc.) or HiPerfect Transfection Reagent (according to QIAGEN). The siRNA experiments were carried out using HiPerfect Transfection Reagent according to QIAGEN, using a control siRNA (catalog 1022076), HNF-4α siRNA (catalog 00161546), or COUP-TF1 siRNA (catalog 00079877). Two days after transfection, the cells were washed twice with PBS and harvested by scraping. After centrifugation, cell pellets were resuspended in 100 μl of 0.25 M Tris-Cl, pH 7.8, and cells were lysed by 3 cycles of freeze-thawing. Appropriate aliquots of cell extracts were used for measurements of luciferase and β-galactosidase activity (22). To correct for transfection efficiency, light units from the luciferase assay were divided by the OD reading from the β-galactosidase assay.

Human SHBG measurements. To determine the human SHBG concentrations in the serum of transgenic mice, we used a saturation binding capacity assay with [³H]5α-dihydrotestosterone as the labeled ligand (45). Human SHBG accumulation in culture medium from HepG2 cells was measured using a time-resolved immunofluorometric assay (46).

RNA analysis. Total RNA was extracted from HepG2 cells using TRIZOL reagent (Invitrogen). RT was performed at 42°C for 50 minutes 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 5′-GCTCCTCCTTCTGCTGCTGC-3′ and reverse 5′-GGAAGAGCTTGAGACAGGCC-3′), SHBG (forward 5′-GTTGCTAC-TACTGCGTCACAC-3′ and reverse 5′-GCCATCTCCCATCATCCAGCCG-3′), COUP-TF1 (forward 5′-AGTGCCTCAAAGTGGGCATGAGG-3′ and reverse 5′-AGCGCCTTGAGCTTCTCCACC-3′), and cyclophilin A (forward 5′-ATGGTCAACCCCACCGTG-3′ and reverse 5′-TGCAATCCAGC-TAGGCATG-3′) mRNA sequences. The PCR was performed for 40 cycles at 94°C for 15 seconds, 57–65°C for 30 seconds, and 72°C for 1 minute, and PCR products were resolved by electrophoresis in a 1% agarose gel.

Western blot analysis. Soluble protein was extracted from HepG2 cells by homogenization in 50 mM Tris-HCl, pH 7.9, 300 mM KCl, 1.5 mM MgCl₂, 0.01% Nonidet P-40, 20% glycerol supplemented with protease inhibitors (Roche Diagnostics) at 4°C, and used for Western blotting with antibodies against human HNF-4α (C-19; catalog sc-6556; Santa Cruz Biotechnology Inc.), human cyclophilin A (SA-296; BIOMOL Int.), human actin (C-11; catalog sc-1615; Santa Cruz Biotechnology Inc.), and human phospho-S6 ribosomal protein (catalog 2211; Cell Signaling Technology Inc.). Specific antibody-antigen complexes were identified using an HRP-labeled goat anti-rabbit IgG or rabbit anti-goat IgG and chemiluminescent substrates (Pierce) by exposure to x-ray film.

ChIP assays. We performed ChIP assays with a ChIP-IT kit (Active Motif Inc.) following the manufacturer's instructions. Briefly, HepG2 cells were cross-linked with 1% formaldehyde for 10 minutes at room temperature. After washing and treatment with glycine Stop-Fix solution, the cells were resuspended in lysis buffer and incubated for 30 minutes on ice. The cells were homogenized, and nuclei were resuspended in shearing buffer and subjected to optimized ultrasonic disruption conditions to yield 100- to 400-bp DNA fragments. The chromatin was precleared with protein G beads and incubated (overnight at 4°C) with 1 μg of each of the following specific antibodies: negative control mouse IgG and positive control anti-RNA polymerase (provided in the ChIP-IT kit), anti–COUP-TF1 (N-19; catalog sc-6575; Santa Cruz Biotechnology Inc.), and anti–HNF-4α (C-19; catalog sc-6556; Santa Cruz Biotechnology Inc.). Protein G beads were then added to the antibody/chromatin incubation mixtures and incubated for 1.5 hours at 4°C. After extensive washings, the immunoprecipitated DNA was removed from the beads in an elution buffer. To reverse cross-links and remove RNA, 5 M NaCl and RNase were added to the samples and incubated for 4 hours at 65°C. The samples were then treated with proteinase K for 2 hours at 42°C, and the DNA was purified using gel exclusion columns. The purified DNA was subjected to PCR amplification (1 cycle of 95°C for 2 minutes, 40 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for 30 seconds) using specific forward (5′-GATCCCCAGAGGGGTGATAGC-3′) and reverse (5′-GGGTA-AAGGAAACAGGGGCAC-3′) primers designed to amplify a 153-bp region in the human *SHBG* promoter (22). As a control, PCR amplification (1 cycle of 95°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds) of the human *GAPDH* promoter was performed using forward (5′-TACTAGCGGTTTTACGGGCG-3′) and reverse (5′-TCGAACAGGAGGAGCAGAGAGAGCGA-3′) primers. The PCR products were resolved by electrophoresis in a 6% acrylamide gel and visualized after ethidium bromide staining.

Statistics. Data were analyzed using 1-way ANOVA, and *P* values of < 0.05 were considered significant.

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