# Potential Role of Tumor Necrosis Factor-α in Downregulating Sex Hormone–Binding Globulin

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Low plasma sex hormone–binding globulin (SHBG) levels are associated with obesity and predict the development of type 2 diabetes. The reason why obese individuals have low circulating SHBG has been attributed to hyperinsulinemia, but no mechanistic evidence has been described. The aim of the current study is to explore whether tumor necrosis factor-α (TNF-α) rather than insulin could be the main factor accounting for low SHBG levels in obesity. We performed in vitro and in vivo studies using human HepG2 cells and human *SHBG* transgenic mice. In addition, a cross-sectional study to explore the relationship between TNF-α and SHBG in obese patients and an interventional study to examine the effect of insulin administration on circulating SHBG in type 2 diabetic patients were performed. We provide evidence that TNF-α, but not insulin, is the main factor by which SHBG is reduced in obesity. Plasma SHBG was significantly increased rather than decreased after insulin treatment in diabetic patients. TNF-α–induced reduction of *SHBG* expression was mediated by downregulating *HNF4A*. Finally, a negative and independent correlation was found between plasma TNF-α receptor 1 and SHBG levels in obese patients. Our results suggest that TNF-α plays an important role downregulating SHBG in chronic low-grade inflammatory diseases such as obesity and type 2 diabetes. ***Diabetes*** **61:372–382, 2012**

Sex hormone–binding globulin (SHBG) is produced and secreted by the human liver, and it binds androgens and estrogens with high affinity. In blood, SHBG acts as a carrier of these sex steroids and regulates their bioavailability (1). Low plasma SHBG levels are associated with obesity, abdominal adiposity, and metabolic syndrome and predict the development of type 2 diabetes (2–4). In addition, an inverse relationship between plasma SHBG levels and risk of cardiovascular disease has been reported (5,6).

BMI is considered a major determinant of SHBG plasma concentrations (7,8). Obese individuals of all ages have low plasma SHBG levels (7,8). Although low plasma SHBG levels in obese individuals have been attributed to hyperinsulinemia (9,10), we have recently demonstrated that excessive intake of monosaccharides leads to lower human SHBG production by the liver by reducing hepatocyte nuclear factor 4-α (HNF4-α) (11), a key transcription factor that regulates *SHBG* expression in the liver (12). In addition, it has been reported that low concentrations of SHBG are strongly associated with increased risk of developing metabolic syndrome independently of insulin resistance (2). Furthermore, Peter et al. (13) have recently shown that SHBG is not related to fasting insulinemia or insulin secretion. All these findings suggest that other mechanisms unrelated to insulin signaling pathways should be involved in the low levels of plasma SHBG observed in obesity.

Accumulating evidence over the past decade points to inflammation as one of the critical processes associated with the development of obesity, insulin resistance, and diabetes (14,15). In fact, obesity is considered a state of chronic low-grade inflammation (16). A robust inverse correlation has recently been found between testosterone and SHBG levels with C-reactive protein levels (17). The authors suggested a potential role of androgens in inflammatory processes, but owing to the cross-sectional nature of the study, the alternative hypothesis that low testosterone and SHBG could be a consequence of inflammation should not be ruled out (17). The proinflammatory cytokine tumor necrosis factor-α (TNF-α) is elevated in obese patients (18) and in other inflammatory diseases (19,20). In obese subjects, TNF-α is mainly produced by macrophages that infiltrate the expanded adipose tissue, and its levels correlate to the degree of adiposity and insulin resistance (21). Evidence for a key role of TNF-α in obesity-related insulin resistance comes from studies showing that deletion of TNF-α or TNF-α receptors (TNF-α-Rs) results in significantly improved insulin sensitivity in both diet-induced obese mice and leptin-deficient *ob*/*ob* mice (22,23). TNF-α signals through two cell-surface receptors, TNF-α-R1 and TNF-α-R2, and membranous shedding of these receptors reflects activation of the TNF system (24,25). In fact, increased plasma levels of TNF-α-Rs are found in obese individuals (25). The half-life of TNF-α is only 4.6 min, and its circulating levels are highly variable (26). By contrast, soluble (s)TNF-α-Rs are more stable proteins, remaining elevated in systemic circulation for longer periods of time and, therefore, are better markers for the activation of the TNF-α system than TNF-α itself (27).The TNF-α actions in the liver mainly occur through TNF-α-R1 (28), and they are mediated by nuclear factor-κB (NF-κB), Jun $NH_2$-terminal kinase, and p38 kinase (29).

Given that an inverse relationship has been reported between TNF-α and SHBG plasma levels in several chronic inflammatory diseases (19,20), it is plausible that TNF-α could be the reason for low plasma SHBG levels that exist in obesity. However, to the best of our knowledge, this hypothesis has never been tested. To shed light on this issue, we have addressed the question of whether human *SHBG* expression in the liver is regulated by TNF-α and/or insulin and which signaling pathways are involved. For these purposes, we have performed in vitro studies using human HepG2 hepatoblastoma cells, as well as in vivo studies using

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human *SHBG* transgenic mice that harbor the complete transcription unit responsible for human SHBG production by hepatocytes. This is the only mouse model available to study *SHBG* expression in vivo because adult mice do not express *SHBG* in their livers. Finally, we have performed two studies in humans, one addressed to investigating the effect of insulin administration on serum SHBG levels in type 2 diabetic patients and the other to assessing whether measurements of TNF-α-R1 are independently related to plasma SHBG levels in obese patients.

## RESEARCH DESIGN AND METHODS

### Subjects and samples

***Interventional study: insulin administration in type 2 diabetic patients.*** Serum samples from 20 consecutive type 2 diabetic men who failed to respond to oral antidiabetes agents and who were admitted to the hospital to initiate treatment with insulin were selected. These patients were hepatitis C virus (HCV) negative, had a C-peptide <1.2 ng/mL (0.5 ± 0.48) (thus providing an insulinopenic state), and in all cases, hepatic steatosis was excluded by ultrasonography.

During admission, they received the same type of diet (carbohydrates constituted 55% of total caloric content). In all cases, patients were treated with both long-acting insulin (0.4 UI/kg/day) and short-acting insulin administered before breakfast, lunch, and dinner (0.1, 0.15, and 0.15 UI/kg, respectively). Total daily insulin dose was adjusted every day according with the values from the nine-point glucose profile obtained the day before. After 7–10 days of insulin treatment (insulin dose mean: 60 ± 24 UI/day), another serum sample was drawn. The following parameters were compared before and after insulinization: BMI, glucose, fructosamine, $HbA_{1c}$, total cholesterol, triglycerides, sTNF-α-R1, and SHBG.

***Cross-sectional study.*** We recruited 97 consecutive obese subjects (BMI 44.9 ± 6.3 kg/m$^2$; waist circumference >102 cm in men and >88 cm in women) of Caucasian origin attended in the University Hospital Vall d'Hebron Obesity Unit. Approximately half of the recruited subjects (46.3%) were taking antihypertensive medications, and 18.5% were taking lipid-lowering drugs.

Exclusion criteria were *1*) diabetic patients; *2*) history of cardiovascular disease; *3*) treatment with estrogens, androgens, corticosteroids, antibiotics, or anti-inflammatory agents; and *4*) hospitalization in the preceding 2 months.

Diabetes was defined on the basis of a history of therapy with oral hypoglycemic agents or insulin at the time of inclusion. In all patients not previously diagnosed, the criteria recommended by the Expert Committee on the Diagnosis and Classification of Diabetes were used (30). Thus, diabetes was diagnosed if the fasting blood glucose was ≥7 mmol/L (126 mg/dL) on two separate occasions, and impaired fasting glucose (IFG) was diagnosed if the fasting blood glucose was between 5.6 mmol/L (100 mg/dL) and 6.9 mmol/L (125 mg/dL).

Informed written consent was obtained from all participants, and the study was approved by the human ethics committee from the Hospital Vall d'Hebron.

**Serum analysis.** All laboratory measurements were performed on fasting blood samples. Serum glucose, fasting insulin, fructosamine, $HbA_{1c}$, total cholesterol, and triglycerides were measured by standard laboratory techniques used in clinical chemistry laboratories. Insulin resistance was determined by the homeostasis model assessment (HOMA-IR).

SHBG and TNF-α-R1 were determined by enzyme-linked immunosorbent assay (ELISA; Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany, and Leti Diagnostics, Badalona, Spain).

**Animals.** Mice C57BL6 expressing human *SHBG* transgenes have been characterized previously (12). Mice were maintained under standard conditions with food (Global Diet 2018; Harlan Interfauna Iberica, Barcelona, Spain) and water provided ad libitum and a 12-h light/dark cycle. Experimental procedures were approved by the institutional animal use subcommittees of Hospital Vall d'Hebron Research Institute and the Universitat Autònoma Barcelona.

**In vivo experiments.** Male mice were treated with daily intraperitoneal injections of phosphate-buffered saline (PBS), 5 μg TNF-α (Miltenyi Biotech S.L., Madrid, Spain), or 5 μg TNF-α and 1 μg NF-κB inhibitor QNZ (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) for 5 days. Blood samples were taken by saphenous vein for measurements of plasma SHBG levels immediately before the treatment and on days 3 and 5 when livers were taken for RNA and protein extraction. Treatments did not affect animal weight.

**Cell culture experiments.** Cell culture reagents were from Life Technologies Inc. (Invitrogen SA, Barcelona, Spain). HepG2 hepatoblastoma cells (catalog no. HB-8065; American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics. For experiments, HepG2 cells were cultured to 50–70% confluence prior to the addition of vehicle (PBS or DMSO), TNF-α (Miltenyi Biotech S.L.), insulin (Sigma-Aldrich, Madrid, Spain), SP600125 (Sigma-Aldrich), and SB203580 (Sigma-Aldrich).

Stable transfections with the *HNF4A* expression vector were performed using Lipofectamine 2000 (Invitrogen SA) and G418 (Invitrogen SA).

**SHBG measurements.** Human SHBG levels in culture medium taken from HepG2 cells or from mice plasma were measured using an ELISA (Demeditec Diagnostics GmbH).

**ELISAs.** Plasma soluble TNF-α-R1 levels were measured using Quantikine Immunoassay (MRT10; R&D Systems Europe, Ltd., Abingdon, U.K.).

**RNA analysis.** Total RNA was extracted from HepG2 cells and mice livers using TRIzol reagent (Invitrogen SA). Reverse transcription was performed at 42°C for 50 min using 3 μg of total RNA and 200 units of Superscript II together with an oligo-dT primer and reagents provided by Invitrogen. An aliquot of the reverse-transcription product was amplified in a 25-μL reaction using SYBRGreen (Invitrogen SA) with appropriate oligonucleotide primer pairs corresponding to human HNF4-α, mouse HNF4-α, human SHBG, human 18S, and mouse 18S (Supplementary Table 1). Results were analyzed using the 7000 SDS program (Applied Biosystems).

**Western blot analysis.** After treatments, mouse livers or HepG2 cells were homogenized in radioimmunoprecipitation assay buffer with Complete protease inhibitor cocktail (Roche Diagnostics, Barcelona, Spain). Protein extracts were used for Western blotting with antibodies against human HNF4-α (C-19; catalog sc-6556; Santa Cruz Biotechnology Inc., Heidelberg, Germany), human phospho–NF-κB (sc-33039; Santa Cruz Biotechnology Inc.), phospho–IRS-1 (insulin receptor substrate 1) (sc-101712; Santa Cruz Biotechnology Inc.), phospho-S6 ribosomal protein (catalog 2211; Cell Signaling Technology Inc.), and human PPIA (SA-296; BIOMOL Int., Madrid, Spain). Specific antibody-antigen complexes were identified using a horseradish peroxidase–labeled goat anti-rabbit IgG or rabbit anti-goat IgG and chemiluminescent substrates (Pierce Biotechnology Inc., Barcelona, Spain) by exposure to X-ray film.

**Chromatin immunoprecipitation assays.** After treatment, HepG2 cells and mice livers were used to perform chromatin immunoprecipitation (ChIP) assays with a ChIP-IT kit (Active Motif Inc.) as described previously (11). The purified DNA was subjected to PCR amplification using specific primers designed to amplify a 306–base pair of the human *SHBG* promoter.

**Statistical analyses.** Normal distribution of the variables was evaluated using the Kolmogorov-Smirnov test. Comparison of quantitative variables was performed by either Student *t* test or Mann-Whitney *U* test according to the data distribution. All data are presented as means ± SD or medians [range]. Spearman correlation coefficients were used to establish the association between SHBG levels and the other parameters. For graphics, a linear regression test was applied.

Stepwise multiple regression analysis was performed to explore the variables independently related to SHBG levels. The variables that significantly correlated with SHBG levels in univariate analysis and those with clinical relevance (i.e., age and BMI) were included as independent variables in multivariate analysis. All *P* values were based on a two-sided test of statistical significance. Significance was accepted at the level of $P < 0.05$. Statistical analyses were performed with the SPSS statistical package (SPSS Inc, Chicago, IL).

## RESULTS

**TNF-α decreases SHBG production by HepG2 cells, whereas insulin does not.** We first examined the effects of daily supplementation of different doses of insulin (0, 20, 100, or 200 μU/mL) or TNF-α (0, 50, 100, or 200 ng/mL) on SHBG production by HepG2 cells over the course of 5 days. This was done by comparing medium concentrations of SHBG on days 1 and 5 of vehicle and different doses of insulin or TNF-α. As expected, insulin treatment induced an increase in phosphorylation of IRS-1 and PS6 proteins (Fig. 1*A*). However, the different concentrations of insulin did not change SHBG protein levels (Fig. 1*B*) or *SHBG* mRNA levels (Fig. 1*C*). By contrast, TNF-α treatments reduced SHBG production (Fig. 1*D*) and *SHBG* mRNA levels (Fig. 1*E*) in HepG2 cells.

We next studied the daily supplementation with TNF-α on SHBG production by HepG2 cells grown in the presence or absence of insulin over the course of 5 days. These treatments showed that cells treated with TNF-α with or without insulin had a reduced SHBG production ($P < 0.01$) when compared with vehicle- or insulin alone–treated cells

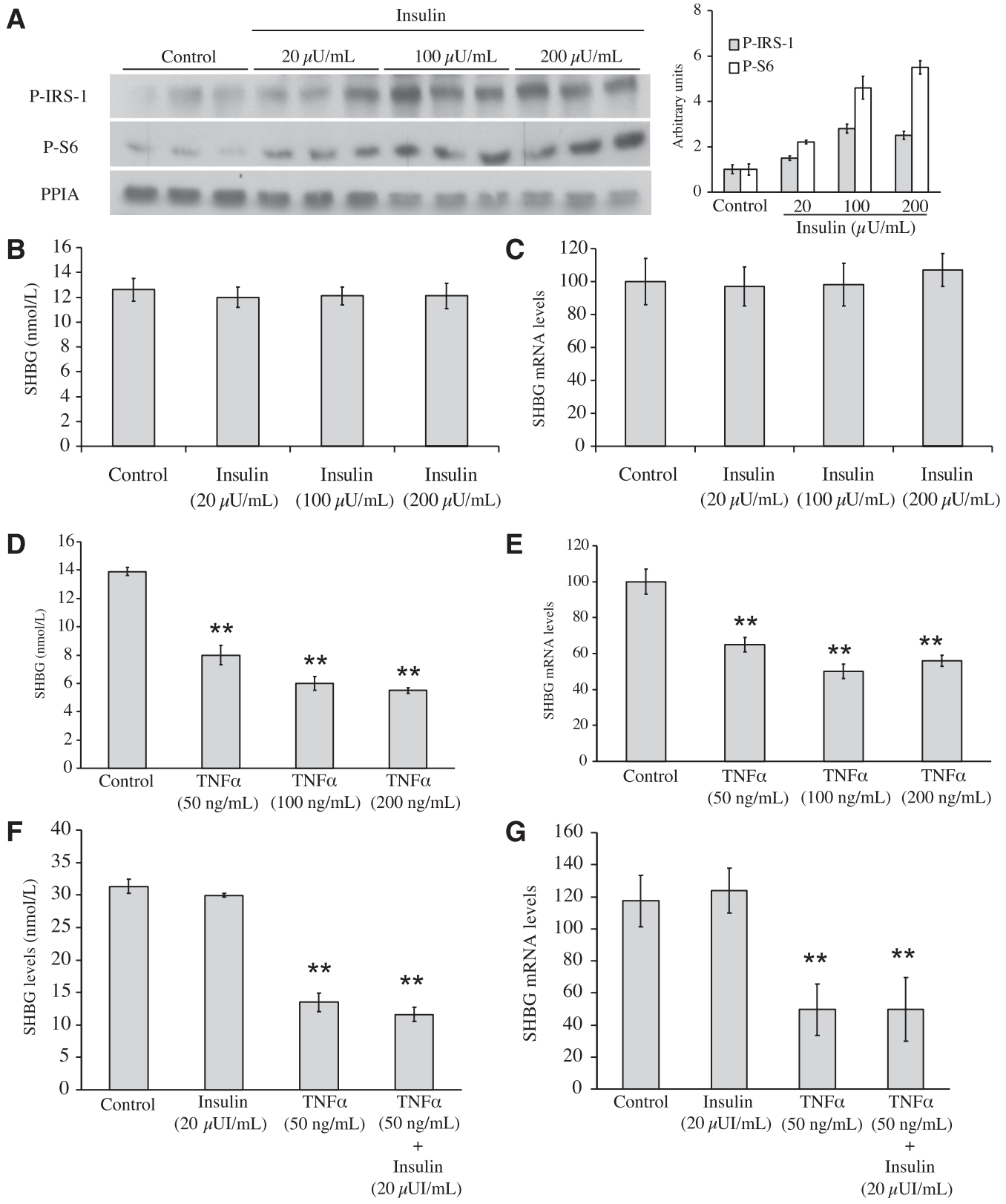


**FIG. 1. Daily treatment with TNF-α, but not insulin, decreases SHBG production over 5 days in HepG2 cells. *A*: HepG2 cells were treated daily with vehicle and different doses of insulin for 5 days. P-IRS-1 and P-S6 protein levels were measured by Western blotting using PPIA as a housekeeping reference protein in HepG2 cells. *B*: SHBG accumulation in the medium was measured using an ELISA in HepG2 cells treated as in (*A*). Data points are mean ± SD of triplicate measurements. *C*: Analysis of *SHBG* mRNA levels in HepG2 cells treated as in (*A*). Human 18S mRNA was amplified as a control. Data points are shown as mean ± SD of triplicates. *D*: HepG2 cells were treated daily with vehicle and different doses of TNF-α for 5 days. SHBG accumulation in the medium was measured using an ELISA. *E*: Analysis of *SHBG* mRNA levels in HepG2 cells treated as in (*D*). Human 18S mRNA was amplified as a control. Data points are shown as mean ± SD of triplicates. *F*: HepG2 cells were treated daily with vehicle, insulin, TNF-α, or TNF-α plus insulin for 5 days. SHBG accumulation in the medium was measured using an ELISA. *G*: Analysis of *SHBG* mRNA levels in HepG2 cells treated as in (*F*). Human 18S mRNA was amplified as a control. Data points are shown as mean ± SD of triplicates. ***P* < 0.01 compared with the control.**

(Fig. 1*F*). In addition, the amount of SHBG mRNA in HepG2 cells after 5 days of treatment with TNF-α with or without insulin was significantly ($P < 0.01$) decreased in relation to the 18S mRNA control when compared with vehicle- or insulin alone–treated cells (Fig. 1*G*).

Finally, it should be noted that TNF-α treatment for 5 days did not affect the viability or survival of the HepG2 cells when compared with vehicle-treated cells assessed by transferase-mediated dUTP nick-end labeling assays (Supplementary Fig. 1).

**Insulin administration does not downregulate SHBG production in type 2 diabetic patients.** To confirm that insulin does not play an essential role in reducing SHBG production, we conducted an interventional study in type 2 diabetic patients requiring insulin treatment. For this purpose, we collected serum samples from 20 consecutive type 2 diabetic men who had failed to respond to oral antidiabetes agents before and after 7–10 days of initiating insulin treatment. The SHBG significantly increased rather than decreased after insulin treatment (Table 1). In addition, a correlation between the decrease of blood glucose levels and the increase of SHBG was found ($r = 0.59$; $P = 0.019$). Apart from demonstrating that insulin does not downregulate SHBG production, we examined the effect of insulin administration on sTNF-α-R1 and its relationship with SHBG. As shown in Table 1, a significant reduction of sTNF-α-R1 was detected after insulin treatment (1.46 ± 0.96 vs. 0.88 ± 0.77 ng/mL; $P = 0.003$), and the decrease was observed in 17 of 20 cases. Finally, it should be stressed that BMI was very similar at patient admission and discharge, thus ruling out this variable as a confounding factor.

**TNF-α influences hepatic SHBG production indirectly by decreasing HNF4-α levels through activation of NF-κB.** Given that HNF4-α plays a key role in the transcriptional activity of the human *SHBG* promoter (12), we examined HNF4-α protein levels in HepG2 cells after 5 days of treatment with vehicle or insulin, TNF-α, and TNF-α/insulin. These treatments showed that cells treated with TNF-α with or without insulin had reduced HNF4-α protein levels ($P < 0.01$) when compared with vehicle- or insulin alone–treated cells (Fig. 2*A*).

To study by which signaling pathway TNF-α was reducing *SHBG* expression, we treated HepG2 cells with QNZ (10 nmol/L to 1 μmol/L), SP600125 (500 nmol/L), and SB203580 (500 nmol/L), which are inhibitors of NF-κB, Jun $NH_2$-terminal kinase, and p38, respectively. After 5 days of treatment with QNZ, the HepG2 cells died (data not shown). Treatments with the other inhibitors did not influence or block the reduction in SHBG production after cotreatment with TNF-α (50 ng/mL) in HepG2 (Fig. 2*B*), although they inhibited phosphorylation of p38 or c-Jun induced by the TNF-α treatment (Supplementary Fig. 2). Moreover, HNF4-α protein levels were also reduced when HepG2 cells were treated with TNF-α (50 ng/mL) in the presence of SP600125 (500 nmol/L) or SB203580 (500 nmol/L) when compared with the vehicle and of SP600125 (500 nmol/L) or SB203580 (500 nmol/L) alone (Fig. 2*C*).

We next explored the possibility of preventing TNF-α–induced downregulation of SHBG by stably transfecting an HNF4-α expression vector. HNF4-α overexpression in TNF-α–treated cells increased SHBG production and SHBG mRNA levels to the levels of untreated HepG2 cells transfected with an empty vector (EV) (Fig. 2*D* and *E*). Furthermore, HNF4-α overexpression increased HNF4-α mRNA and protein in TNF-α–treated HepG2 cells to the levels of untreated HepG2 cells transfected with an EV (Fig. 2*E* and *F*).

**TNF-α decreases SHBG production independently of de novo lipogenesis.** To examine whether *HNF4A* downregulation observed in HepG2 cells after TNF-α treatment could be attributed to lipid accumulation (31,32), we treated HepG2 cells with TNF-α in the presence of cerulenin, a fatty acid synthase inhibitor (33). Cerulenin treatment did not block the reduction of SHBG production caused by TNF-α in HepG2 cells (Fig. 3*A*). Moreover, cerulenin treatment did not block the downregulation of SHBG and HNF4-α mRNA levels (Fig. 3*B*) or the reduction in HNF4-α protein levels (Fig. 3*C*). Furthermore, using oil red staining, we found no evidence of increased lipid accumulation in HepG2 cells treated with TNF-α for 5 days when compared with vehicle-treated cells (Fig. 3*D*).

**Daily TNF-α treatment decreases SHBG production via NF-κB in human SHBG transgenic mice.** To mimic the situation of chronic low-grade inflammation, we performed an experiment in which mice were treated daily with an injection of 5 μg i.p. of TNF-α for 5 days. Blood samples were collected immediately before treatment and at days 3 and 5 of treatment. The results indicated that plasma SHBG levels were reduced after days 3 and 5 of TNF-α treatment, whereas no changes in SHBG levels were observed in the vehicle-treated mice (Fig. 4*A*). In addition, SHBG and HNF4-α mRNA levels (Fig. 4*B*) and HNF4-α protein levels (Fig. 4*C*) were significantly reduced in the livers of TNF-α–treated mice. Moreover, TNF-α–treated mice had NF-κB activation in their livers because there was a significant increase in P-p65 levels when compared with the vehicle-treated mice (Fig. 4*C*). ChIP assays using DNA/protein complexes extracted from the livers of mice treated with TNF-α or vehicle revealed that in TNF-α–treated mice, there was a reduction of HNF4-α binding in the *SHBG* proximal promoter when compared with vehicle-treated mice (Fig. 4*D*).

We next measured the sTNF-α-R1 levels in the blood of untreated and TNF-α–treated mice. The results showed that daily TNF-α treatment produced a significant increase in TNF-α-R1 levels at days 3 and 5 when compared with the vehicle-treated mice (Fig. 4*E*). It should be noted that at day 5, TNF-α-R1 levels of mice treated with TNF-α (1138 ± 47) were almost double the levels of vehicle-treated mice (700 ± 50).

To investigate whether the effect of TNF-α on SHBG was via NF-κB in our in vivo system, human *SHBG* transgenic

TABLE 1
Main clinical and biochemical features of type 2 diabetic patients of the prospective study

| | Baseline | Day 7–10 | *P** |
|---|---|---|---|
| BMI (kg/m$^2$) | 29.8 ± 7.3 | 29.0 ± 7.5 | NS |
| Plasma glucose (mmol/L) | 10.8 ± 3.5 | 7.7 ± 3.9 | 0.045 |
| Fructosamine | 418.5 ± 89.2 | 358.6 ± 67.7 | 0.017 |
| $HbA_{1c}$ (%) | 9.9 ± 1.8 | 9.4 ± 1.2 | 0.043 |
| Total cholesterol (mg/dL) | 199 ± 59 | 187 ± 48 | NS |
| Triglycerides (mg/dL) | 203 [69–398] | 174 [68–295] | 0.021 |
| sTNF-α-R1 (ng/mL) | 1.46 ± 0.96 | 0.88 ± 0.77 | 0.003 |
| SHBG (pmol/mL)** | 29.8 [5.3–70.6] | 37 [12.8–66.8] | 0.039 |

Data are mean ± SD or median [range]. *Between subjects at the beginning (baseline) and the end of the insulin treatment (day 7–10). **SHBG normal levels of males 39.7 ± 12.8 and females 55.9 ± 12.5.

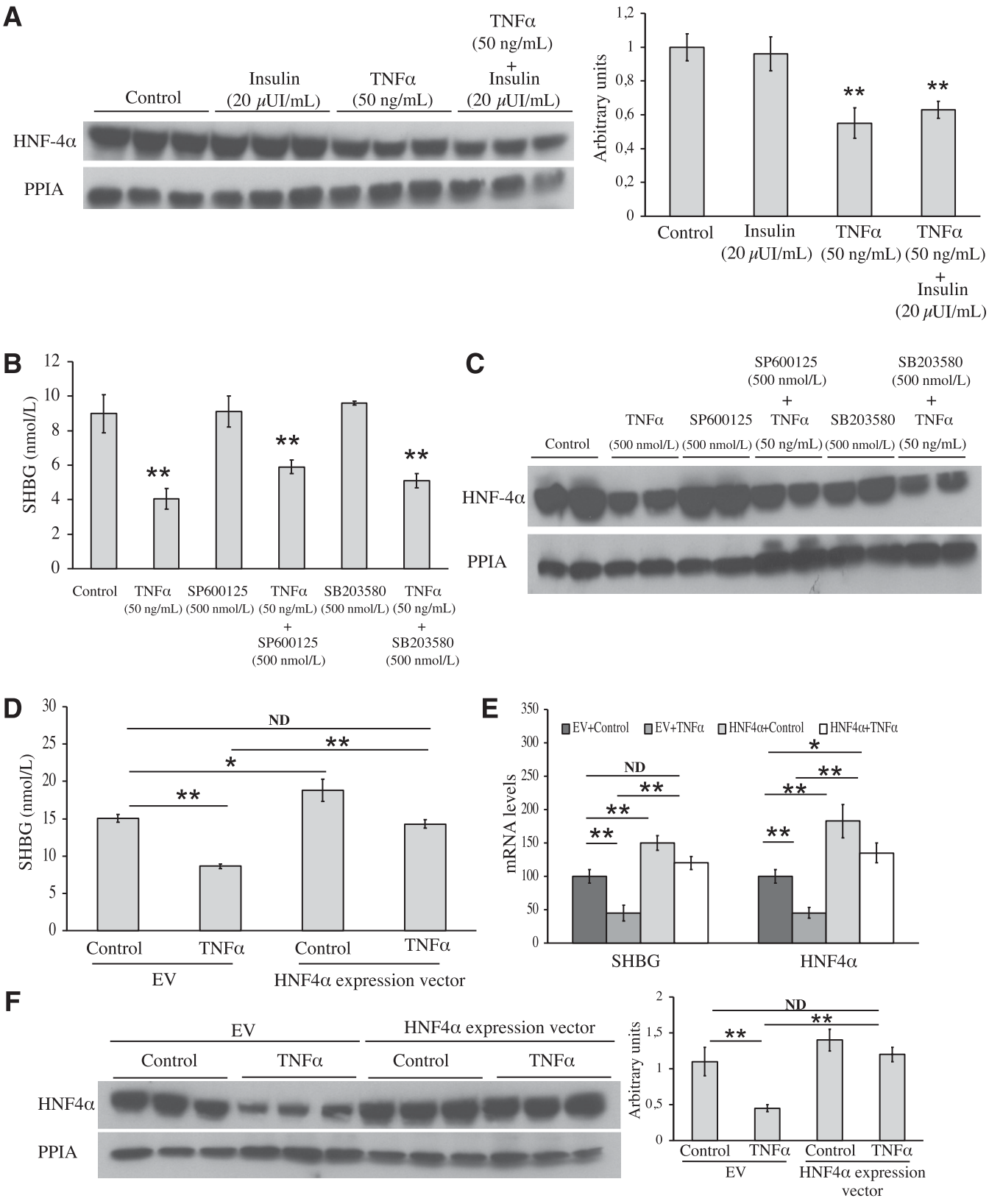


**FIG. 2. TNF-α influences hepatic SHBG production indirectly by decreasing HNF4-α levels through activation of NF-κB in HepG2 cells. *A*: Western blot of HNF4-α and PPIA in total cell protein extracts of HepG2 cells treated with vehicle, insulin, TNF-α, or TNF-α plus insulin for 5 days. *B*: HepG2 cells were treated daily with vehicle, TNF-α, SP600125, SP600125 plus TNF-α, SB203580, or SB203580 plus TNF-α. SHBG accumulation in the medium was measured using an ELISA. *C*: Western blot of HNF4-α and PPIA in total cell protein extracts of HepG2 cells treated as in (*B*). *D*: HepG2 cells stably transfected with an EV or an HNF4-α expression vector were daily treated with vehicle or TNF-α (50 ng/mL). SHBG accumulation in the medium was measured using an ELISA. *E*: Analysis of *SHBG* and *HNF-4α* mRNA levels in HepG2 cells treated as in (*D*). Human 18S mRNA was amplified as a control. Data points are shown as mean ± SD of triplicates. *F*: Western blot of HNF4-α and PPIA in total cell protein extracts of HepG2 cells treated as in (*D*). *$P < 0.05$ and **$P < 0.01$ compared with the control.**

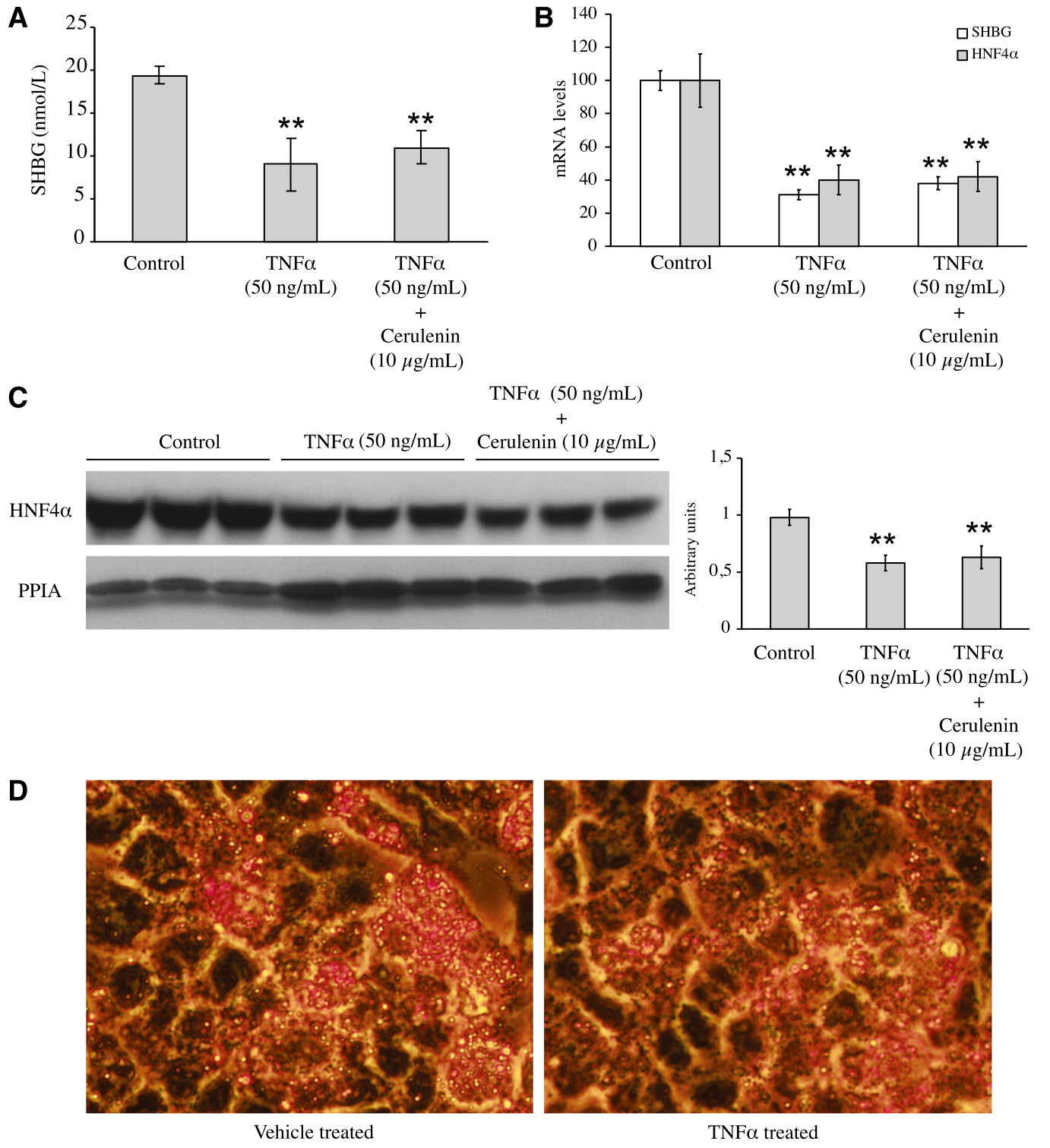


**FIG. 3. TNF-α did not reduce SHBG production by increasing in de novo lipogenesis in HepG2 cells. *A*: Cerulenin, an inhibitor of the fatty acid synthase, did not block the reduction of SHBG production caused by TNF-α in HepG2 cells measured by ELISA. *B*: Cerulenin treatment also did not block the reduction of *SHBG* or *HNF4-α* mRNA levels caused by the TNF-α treatment. *C*: Cerulenin treatment also did not block the reduction of HNF4-α protein levels caused by the TNF-α treatment. *D*: Daily treatment with TNF-α (50 ng/mL) produces no lipid accumulation in HepG2 cells when compared with vehicle-treated cells. After treatments, HepG2 cells were washed with PBS twice and formalin fixed for 5 min. After several washes, the cells were stained with fresh Oil Red O for 15 min and then rinsed in water. **$P < 0.01$ compared with the control. (A high-quality digital representation of this figure is available in the online issue.)**

mice were treated with daily intraperitoneal injections of TNF-α (5 μg) or TNF-α (5 μg) plus the NF-κB inhibitor QNZ (1 μg) for 5 days. Blood samples were collected immediately before treatment and at days 3 and 5. The results indicated that plasma SHBG levels were reduced after 5 days of treatment with TNF-α and that cotreatment with QNZ was able to block the reduction in plasma SHBG levels caused by TNF-α (Fig. 5*A*). The QNZ cotreatment also was

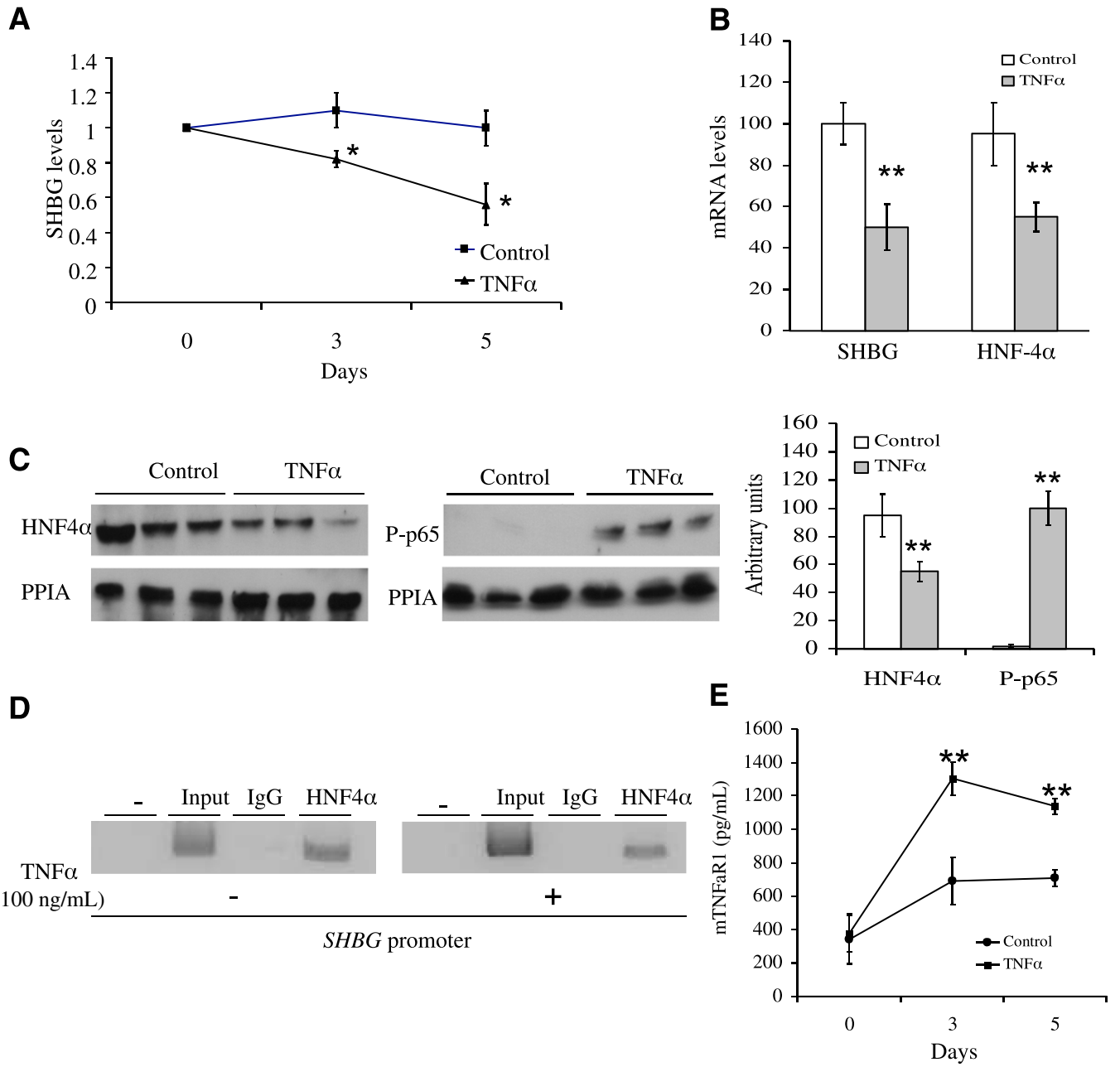


**FIG. 4. Daily treatment with TNF-α reduces plasma SHBG levels in human SHBG transgenic mice. *A*: Human *SHBG* transgenic mice ($n$ = 3–4) were treated over 5 days with intraperitoneal injections of PBS or TNF-α (5 μg), and blood SHBG levels were measured by ELISA. The SHBG levels are expressed as mean ± SD relative to pretreatment levels to compensate for between-animal variability. *B*: *SHBG* and *HNF4-α* mRNA abundance was determined in relation to 18S RNA (mean ± SD) in liver of mice treated with vehicle ($n$ = 3) and TNF-α ($n$ = 4). *C*: Liver HNF4-α and P-p65 protein levels were measured by Western blotting using PPIA as a housekeeping reference protein. Data points are mean ± SD of triplicate measurements. *D*: Plasma TNF-α-R1 levels were measured by ELISA at days 0, 3, and 5 of mice treated with vehicle ($n$ = 3) and TNF-α ($n$ = 4). *E*: ChIP assays using DNA/protein complexes extracted from livers of mice treated with TNF-α or vehicle. The TNF-α–treated mice had a reduction of HNF4-α binding in the *SHBG* proximal promoter when compared with the vehicle-treated mice [forward (5′-CCCGGTACCTCTAGACCTCAGGCCTGTG-3′) and reverse (5′-CCCA AGCTTGGCAGGCAGCC TTGCGTGTGG-3′) primers designed to amplify a 306–base pair region in the human *SHBG* promoter]. *$P$ < 0.05 and **$P$ < 0.01 compared with the control. (A high-quality color representation of this figure is available in the online issue.)**

able to block the downregulation of *SHBG* and *HNF4-α* mRNA levels (Fig. 5*B*) and the reduction of HNF4-α protein levels (Fig. 5*C*).

We did not find hepatic fatty acid accumulation in the livers of mice treated with TNF-α (Supplementary Fig. 3). Furthermore, no evidence of liver apoptosis in TNF-α–treated mice was detected when analyzed by transferase-mediated dUTP nick-end labeling assay (data not shown). These findings exclude the possibility that the reduction of plasma SHBG levels after TNF-α treatment could be attributed to liver damage.

**TNF-α-R1, but not insulin, is independently related to plasma SHBG levels in obese patients.** To explore whether TNF-α is a major factor accounting for the low levels of SHBG detected in obesity, we measured serum TNF-α-R1, a well-established surrogate of TNF-α activation, in 97 consecutive obese subjects (see the inclusion criteria in RESEARCH DESIGN AND METHODS). The main clinical

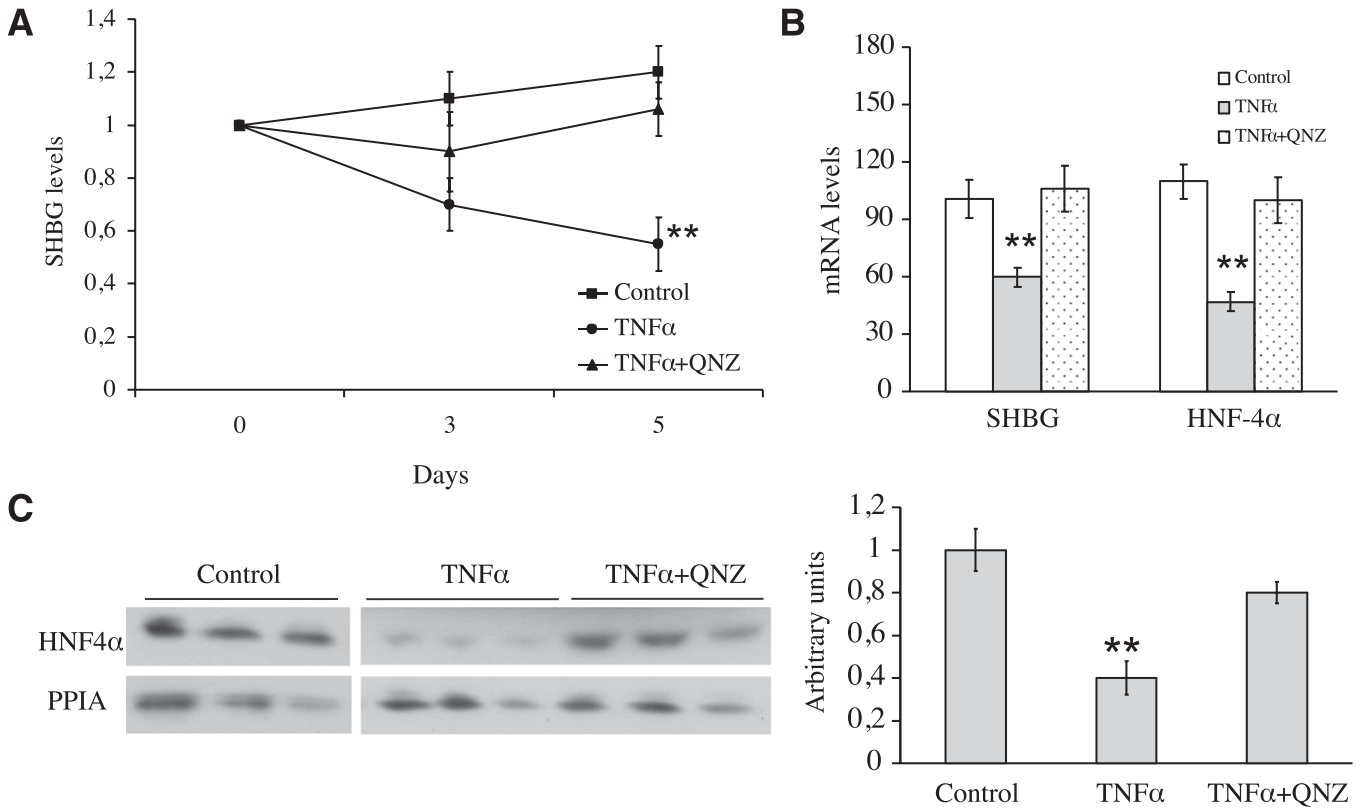


**FIG. 5. Daily treatment with QNZ blocked the TNF-α–induced reduction of plasma SHBG levels in human SHBG transgenic mice. *A*: Human *SHBG* transgenic mice ($n$ = 3) were treated over 5 days with intraperitoneal injections of vehicle, TNF-α (5 μg), or TNF-α (5 μg) plus QNZ (1 μg). Blood SHBG levels were measured by ELISA. The SHBG levels are expressed as mean ± SD relative to pretreatment levels to compensate for between-animal variability. *B*: *SHBG* and *HNF4-α* mRNA abundance was determined in relation to 18S RNA (mean ± SD) in liver of mice treated with vehicle ($n$ = 3), TNF-α ($n$ = 3), and TNF-α plus QNZ ($n$ = 3). *C*: Liver HNF4-α protein levels were measured by Western blotting using PPIA as a housekeeping reference protein. Data points are mean ± SD of triplicate measurements. **$P$ < 0.01 compared with the control.**

and analytical features of obese subjects included in the study are shown in Table 2. Although obese patients with glucose abnormalities were significantly older ($P$ = 0.018), and had higher HOMA-IR ($P$ = 0.006) than patients without glucose abnormalities, plasma SHBG levels were similar between groups (Table 2). In this cohort of obese subjects, we found no differences in SHBG levels between men and women, and for this reason, the results are presented together. Nevertheless, results according to sex are also displayed in Fig. 6.

In the univariate analysis, plasma SHBG was inversely correlated with plasma sTNF-α-R1 ($r$ = −0.546; $P$ < 0.001) (Fig. 6*A*). However, no correlation between SHBG and insulin ($r$ = −0.140; $P$ = 0.199) or HOMA-IR ($r$ = −0.110; $P$ = 0.314) was observed. In the multiple regression analysis, sTNF-α-R1 was the only variable that was independently related to plasma SHBG levels and explained the 29.8% of SHBG variation. When a separate analysis was performed according to the presence or absence of IFG, the results were very similar (data not shown).

TABLE 2
Main clinical and biochemical features of obese patients of the cross-sectional study

| | Subjects | | | |
|---|---|---|---|---|
| | Total | With IFG | Without glucose abnormalities | $P$* |
| $n$ | 97 | 54 | 43 | — |
| BMI (kg/m$^2$) | 44.9 ± 6.3 | 45.3 ± 5.9 | 44.3 ± 6.4 | 0.495 |
| Sex (M/F) | 35/62 | 19/37 | 18/25 | 0.196 |
| Age (years) | 47.1 ± 9.8 | 50.9 ± 8.5 | 45.5 ± 9.7 | 0.018 |
| Fasting glucose (mmol/L) | 5.6 ± 1.1 | 6.1 ± 0.4 | 5.3 ± 0.4 | <0.001 |
| Fasting insulin (pmol/L) | 21.8 ± 12.9 | 24.7 ± 14.3 | 20.5 ± 9.6 | 0.118 |
| HOMA-IR | 5.2 [0.9–23.4] | 6.5 [1.5–23.4] | 4.8 [0.9–9.9] | 0.006 |
| sTNF-α-R1 (ng/mL) | 2.4 [1.1–4.1] | 2.4 [1.2–3.5] | 2.4 [1.1–4.1] | 0.959 |
| SHBG (pmol/mL)** | 29.5 [8.0–120.5] | 28.8 [9.3–115.6] | 30.2 [8.0–120.5] | 0.635 |

Data are mean ± SD or median [range]. *Between subjects with IFG and subjects without glucose abnormalities. **SHBG normal levels of males 39.7 ± 12.8 and females 55.9 ± 12.5.

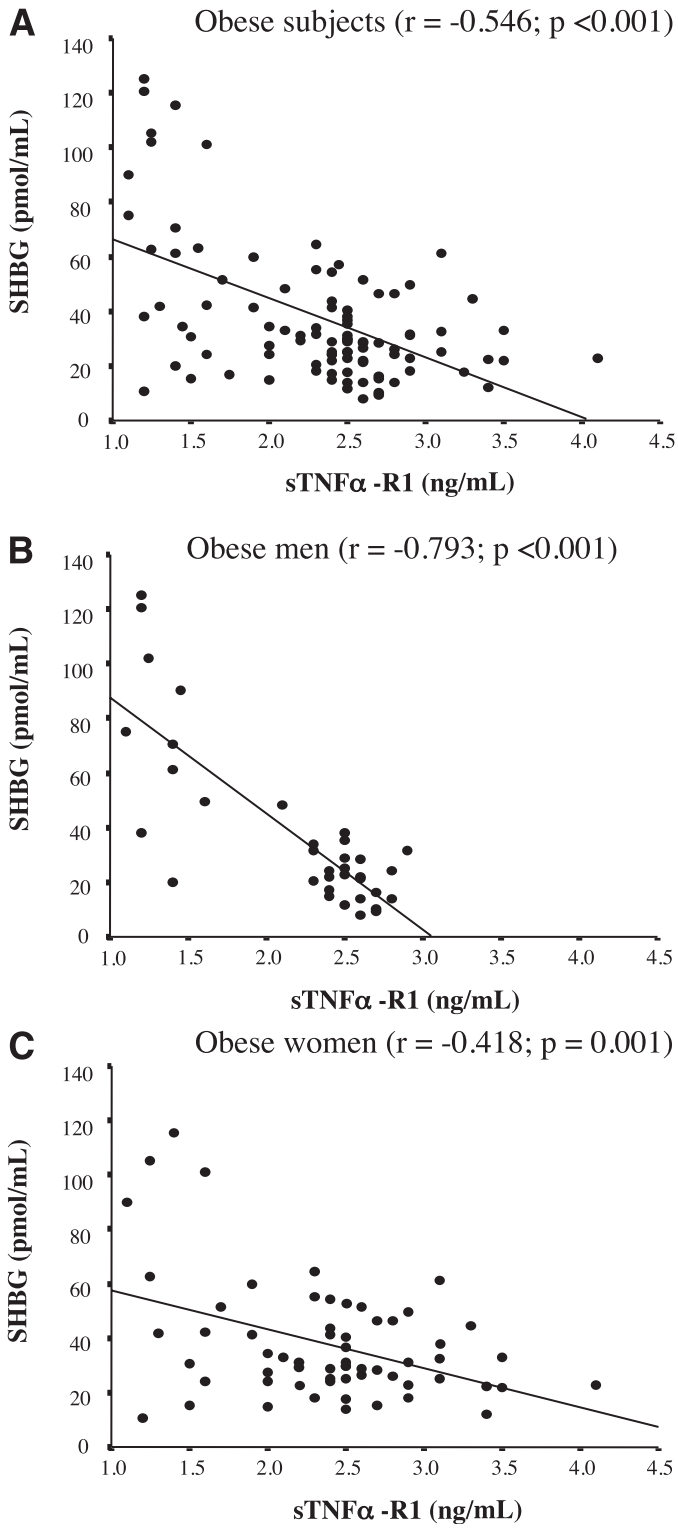


**FIG. 6. Negative correlation between plasma SHBG levels and sTNF-α-R1 in the whole population of obese subjects included in the study (*A*), as well as according to sex (*B* and *C*).**

## DISCUSSION

Low plasma levels of SHBG are associated with obesity and are a risk factor for the development of both type 2 diabetes and cardiovascular disease (2–5). The low plasma SHBG levels found in obesity have been largely attributed to hyperinsulinemia, even though there is no obvious mechanism to account for this (9). Recently, we have presented evidence that casts doubt on this widely held assumption and have shown that excessive exposure to glucose or fructose downregulates *SHBG* expression in the liver both in vivo and in vitro by reducing *HNF4A* expression (11). In the current study, we have found that daily treatment with TNF-α reduces SHBG production in HepG2 cells independently of the presence or absence of

insulin. Moreover, only cells treated with TNF-α have reduced levels of *SHBG* mRNA when compared with the cells treated with vehicle or insulin alone. In the clinical setting, we have demonstrated that TNF-α-R1, but not insulin, is independently related to circulating SHBG in obese subjects. In addition, we have shown a significant increase rather than decrease in SHBG plasma levels after initiating insulin treatment in type 2 diabetic patients. Taken together, these data strongly argue against the hypothesis that hyperinsulinemia is responsible for the decline in SHBG levels in humans and point to TNF-α as a key factor accounting for the low plasma levels of SHBG that exist in low-grade chronic inflammatory diseases characterized by high levels of TNF-α, such as obesity and type 2 diabetes.

We have recently found that the human *SHBG* gene in HepG2 cells, as well as in a transgenic mouse model, responds to thyroid hormones (34). Of import, because the human *SHBG* promoter lacks a thyroid hormone response element, these studies demonstrate that the thyroid hormone effects are mediated by alterations in cellular HNF4-α levels, which act as a key regulator of *SHBG* transcription (12). We therefore explored the possibility that the decrease in SHBG production in HepG2 cells after 5 days of treatment with TNF-α involves changes in hepatic HNF4-α levels. In this regard, it has been previously reported that TNF-α appears to inhibit *HNF4A* gene expression (35) and that TNF-α, through NF-κB, inhibits *HNF4A* transcriptional activity (36). Our results support this because we observed a decrease in cellular HNF4-α protein levels in concert with a decrease in SHBG production in the cells treated with TNF-α.

Because obese patients have chronic high levels of TNF-α (18), we treated the mice daily with intraperitoneal injection of TNF-α over 5 days, and we found clear reductions in both *HNF4-α* mRNA and protein levels in the liver, as well as in plasma SHBG levels. These TNF-α effects were mediated by NF-κB because it was phosphorylated in the livers of mice treated with TNF-α, and these effects were blocked by cotreatment of QNZ. Obesity is a state of chronic low-grade inflammation, and the high levels of TNF-α derived from visceral adipose tissue play an essential role in this process (21,37,38). Therefore, our findings provide the mechanism by which TNF-α could lead to low plasma levels of SHBG in obese patients. It should be emphasized that TNF-α-R1 levels at day 5 of the mice treated with TNF-α were almost double the levels of vehicle-treated mice, thus providing evidence of activation of the TNF-α system. It is interesting that a similar increase of circulating TNF-α-R1 levels has been reported in obese patients with IFG when compared with lean controls (39). These findings suggest that the results obtained in our experimental model could be transferred to the events that are taking place in humans and not only point to TNF-α as a crucial suppressor of *SHBG* expression but also open up a new mechanism linking obesity with the deleterious consequences arising from lower levels of SHBG/sex steroids.

It could be argued that the downregulation of *SHBG* was due to hepatotoxicity or fatty liver disease mediated by TNF-α. However, we found neither steatosis nor apoptosis in the livers of *SHBG* transgenic mice treated with TNF-α for 5 days. Moreover, daily treatment with TNF-α did not produce any lipid accumulation in HepG2 cells when compared with vehicle-treated cells. Furthermore, after blocking de novo lipogenesis by using cerulenin (an inhibitor of the fatty acid synthase), the reduction in SHBG production by TNF-α was unaffected. These findings suggest that fatty liver disease is not the primary event in the downregulation of *SHBG* mediated by TNF-α. Nevertheless, our results do not deny the possibility that when fatty liver disease is also present, the downregulation of *SHBG* could be accelerated. In this regard, we have previously described the role of de novo lipogenesis in the downregulation of hepatic *SHBG* expression (11), and a strong association between fatty liver disease and low circulating SHBG levels has been recently reported (13).

Apart from obesity and type 2 diabetes, there are other chronic inflammatory diseases, such as rheumatoid arthritis and osteoarthritis, in which both TNF-α activation and low SHBG levels have been reported (19,20). In addition, it has been shown that TNF-α blockade in patients with rheumatoid arthritis (40) and psoriatic arthritis (41) leads to an increase in blood levels of SHBG. Therefore, plasma SHBG levels could serve as a biomarker for diseases with chronic inflammation and be an indicator of a good response to treatment aimed at decreasing TNF-α levels or blocking TNF-α actions. Finally, our results point to the downregulation of *SHBG* mediated by an increase of TNF-α as one explanation for the low levels of total sexual steroids that exist in chronic inflammatory diseases such as obesity and type 2 diabetes (42).

In conclusion, our studies suggest that insulin is not a suppressor of SHBG production. By contrast, TNF-α plays an essential role in the downregulation of SHBG production by decreasing hepatic production of HNF4-α through NF-κB activation. In addition, we provide evidence that TNF-α is essential in accounting for the low levels of SHBG detected in obesity.

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R.S. researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. A.B.-D., A.L., and C.H. researched data, contributed to discussion, and reviewed and edited the manuscript. D.M.S. researched data, contributed to discussion, and wrote, reviewed, and edited the manuscript. D.M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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