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Hyporesponsiveness to Glucocorticoids in Mice Genetically Deficient for the Corticosteroid Binding Globulin

Helle Heibroch Petersen,[1] Thomas K. Andreassen,[2] Tilman Breiderhoff,[1] Jan Hinrich Bräsen,[3] Herbert Schulz,[1] Volkmar Gross,[1] Hermann-Josef Gröne,[4] Anders Nykjaer,[2,5] and Thomas E. Willnow[1,5]*

Max Delbrueck Center for Molecular Medicine[1] and Charite,[3] University Medical School, Berlin, and German Cancer Center, Heidelberg,[4] Germany, and Department of Medical Biochemistry, University of Aarhus,[2] and ReceptIcon Aps,[5] Aarhus, Denmark

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Corticosteroid binding globulin (CBG) is the carrier for glucocorticoids in plasma. The protein is believed to keep the steroids inactive and to regulate the amount of free hormone acting on target tissues (free hormone hypothesis). Here, we generated a mouse model genetically deficient for CBG to test the contribution of the carrier to glucocorticoid action and adrenocortical stress response. The absence of CBG resulted in a lack of corticosterone binding activity in serum and in an ~10-fold increase in free corticosterone levels in CBG-null mice, consistent with its role in regulation of circulating free hormone levels. Surprisingly, *cbg*$^{-/-}$ animals did not exhibit features seen in organisms with enhanced glucocorticoid signaling. Rather, the mice exhibited increased activity of the pituitary axis of hormonal control, normal levels of gluconeogenetic enzymes, and fatigue, as well as an aggravated response to septic shock, indicating an inability to appropriately respond to the excess free corticosterone in the absence of CBG. Thus, our data suggest an active role for CBG in bioavailability, local delivery, and/or cellular signal transduction of glucocorticoids that extends beyond a function as a mere cargo transporter.

Corticosteroid binding globulin (CBG) is the major transport protein for glucocorticoids in plasma of mammalian species, with more than 90% of circulating corticosteroid molecules being bound by this carrier (2, 11, 24). CBG is a 55-kDa monomeric glycoprotein that is secreted mainly by the liver but is also produced in the lung, kidney, and testis (11). Remarkably, CBG shares little sequence similarity with other steroid carriers, such as the vitamin D binding protein, sex hormone binding globulin, or α-fetoprotein. Rather, the protein exhibits structural homology to members of the superfamily of serine protease inhibitors (serpins). Similar to other serpins, CBG can be cleaved by proteases, inducing conformational changes in tertiary protein structure and abrogating the ability of the carrier to bind steroids (23).

CBG plays an important role in the metabolism and action of glucocorticoids. Most notably, its role in transport of otherwise insoluble steroid hormones is understood. According to the free hormone hypothesis, CBG provides a reservoir of circulating protein-bound steroids that are biologically inactive, and it regulates the amount of free hormones that are available for entry into target tissues (17). In line with this hypothesis, the concentration of CBG in human plasma correlates inversely with metabolic clearance rates for cortisol (28) while various stressors decrease CBG expression so as to increase free glucocorticoid levels during physiological stress responses (8, 21, 32).

Hereditary forms of CBG deficiency have been identified in a number of families. Known mutations in the human *cbg* gene result in amino acid changes that significantly reduce cortisol binding affinity (CBG Lyon, transcortin Leuven) (4, 31, 37) or that cause stop mutations, leading to premature termination of translation and the absence of CBG immunoreactivity in plasma (35). Individuals that are homozygous for the null mutation have drastically reduced total cortisol levels (1.8 μg/dl; reference range, 5 to 14 μg/dl), and exhibit a prevalence of fatigue (35). Unfortunately, the small number of null homozygotes precludes the firm establishment of the causal role of CBG deficiency in these phenotypes and its consequences for adrenocorticol stress response.

While the role of CBG in systemic transport of glucocorticoids is well documented, a number of additional functions for this protein have been proposed which still warrant rigorous experimental testing. In particular, more direct roles for the carrier in local delivery of glucocorticoids to target tissues is being discussed. Thus, the release of bound cortisol following proteolytic cleavage of CBG suggests a scenario whereby neutrophil elastase secreted by activated neutrophils may serve to induce local release of hormones from CBG at sites of inflammation (12, 23). An alternative mode of local delivery of steroid hormones is suggested by the identification of surface binding sites for CBG in a number of steroid target tissues, such as liver, endometrium, and spleen (14, 15, 30, 34), suggesting the existence of membrane receptors for cellular uptake and/or transmembrane signaling of CBG/steroid complexes. Finally, a role for the protein in embryonic development of the kidney and liver has been proposed based on the spatially and temporarily restricted expression of the carrier in these tissues during development (5, 26, 27).

In this study, we generated a mouse model of CBG deficiency and characterized the consequences of the carrier gene defect for the systemic and cellular actions of glucocorticoids.

* Corresponding author. Mailing address: Max Delbrueck Center for Molecular Medicine, Robert-Roessle-Strasse 10, D-13125 Berlin, Germany. Phone: 49-30-9406-2569. Fax: 49-30-9406-3382. E-mail: willnow@mdc-berlin.de.

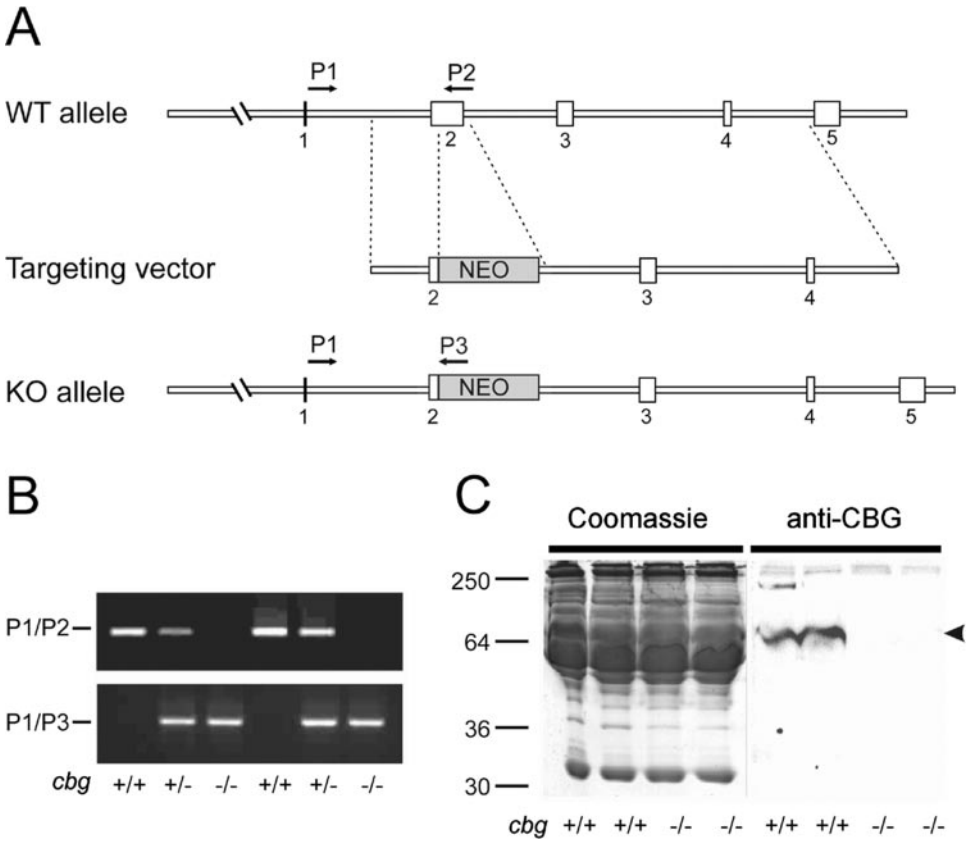

FIG. 1. Disruption of the murine *cbg* gene locus. (A) Organization of the murine *cbg* gene region (wild-type [WT] allele) indicating the five exons (open boxes) and the short and long gene homology regions (dotted lines) used to construct the targeting vector. Following homologous recombination, exon 2 is replaced in part by the pol2neobpA selection cassette (NEO) in the targeted *cbg* allele (knockout [KO] allele). The localization of primers used for PCR genotyping of the wild-type (P1 and P2) and the disrupted (P1 and P3) gene loci is indicated. (B) PCR-based genotyping of adult mice that were wild-type (+/+), heterozygous (+/−), or homozygously deficient (−/−) for the *cbg* gene. (C) Parallel plasma samples (0.5 μl) of mice of the indicated genotypes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie (loading control) or immunodetection with anti-CBG antiserum. The arrowhead indicates CBG immunoreactivity in plasma samples of +/+ but not −/− mice.

These studies enabled us to test in vivo the many biological functions that have been assigned to this protein.

MATERIALS AND METHODS

Materials and general methods. Polyclonal antiserum against murine CBG was produced by immunization of rabbits with recombinant protein expressed and purified from bacteria. Corticosterone (C2505) and [1,2,6,7-^{3}H]corticosterone (TRK406) were purchased from Sigma and Amersham, respectively. Experiments involving recombinant DNA technology and protein chemistry were performed according to standard protocols. Statistical evaluation was performed by Student's *t* test unless indicated otherwise.

Targeting of the murine *cbg* gene locus. A 1.3-kb and a 7.2-kb fragment of the murine *cbg* gene were PCR amplified from mouse genomic DNA using the primers forward-SA-CBG (5′-GGC CGC TCT AGA ACT AGT GGA TCC C-3′), reverse-SA-CBG (5′-GGC GTT TGT ACA AGT TGA AGG CAA A-3′), forward-LA-CBG (5′-GCC ACC CTC ATC CTG ATC AAC TAC ATC TTC CTC AAA GG-3′), and reverse-LA-CBG (5′-TTG CAG CAT GGC CTT GTG GAG T-3′), respectively. The fragments were used as short (SA) and long (LA) homology regions for the targeting construct and fused to the 5′ and 3′ ends of the pol2neobpA expression cassette (*neo*) (see Fig. 1A), resulting in replacement of exon 2-encoding sequences of the *cbg* gene locus by *neo*. Electroporation of the targeting vector into embryonic stem cell line ICp4 and derivation of germ line chimeras by blastocyst injections were performed according to standard procedures.

Animal experimentation. Studies used CBG-deficient ($cbg^{-/-}$) and wild-type control ($cbg^{+/+}$) male mice from individual lines established from heterozygous breeding of $cbg^{+/-}$ mice. Where indicated, studies were reproduced in $cbg^{+/-}$ and $cbg^{-/-}$ littermates from heterozygous breeding to exclude effects of genetic background (129SvEmcTer × C57BL/6N). The animals were kept on standard dark-light cycles (light on at 6 a.m., out at 6 p.m.), with all blood sampling and animal experimentation done between 8 a.m. and 10 a.m.. Experiments involving animals were performed according to institutional guidelines after approval by local ethic committees. Animals were fed ad libitum a normal mouse chow (4% fat, V1126 Extrudat diet; Ssniff, Soest, Germany) or a high-fat diet (26% crude fat; Ssniff). Plasma turnover of [^{3}H]corticosterone was determined by intravenous (i.v.) or intraperitoneal (i.p.) injection of 3×10^6 cpm tracer (25 pmol) in isotonic saline solution and by blood sampling at various time points by retroorbital bleeding. For histopathology, tissues were dissected from embryos, neonates, or adults of the genotypes indicated in Fig. 5, fixed in 4% neutral-buffered formalin, and subjected to standard histological sectioning (3- to 5-μm sections) and staining with hematoxylin and eosin.

For telemetry, 12-week-old male mice were anesthetized with isoflurane (CuraMed Pharma GmbH, Karlsruhe, Germany). The pressure-sensing catheter (TA11PA-C20 blood pressure device; Data Sciences Int., St. Paul, Minn.) was advanced via the right femoral artery into the abdominal aorta, and the transmitter was placed in a subcutaneous pocket along the right flank. All mice were allowed 10 days' recovery from surgery before baseline blood pressure and heart rate were recorded for 3 days via radio frequency signals and collected using the Dataquest A.R.T. system, version 2.1 (Data Sciences Int.). The data were sampled every 5 min for 10 s continuously day and night, with a sampling rate of 1,000 Hz. Heart rate was computed from the pulse intervals of the blood pressure recordings. Activity was monitored as changes in transmitter signal strength due to murine (transmitter) locomotion.

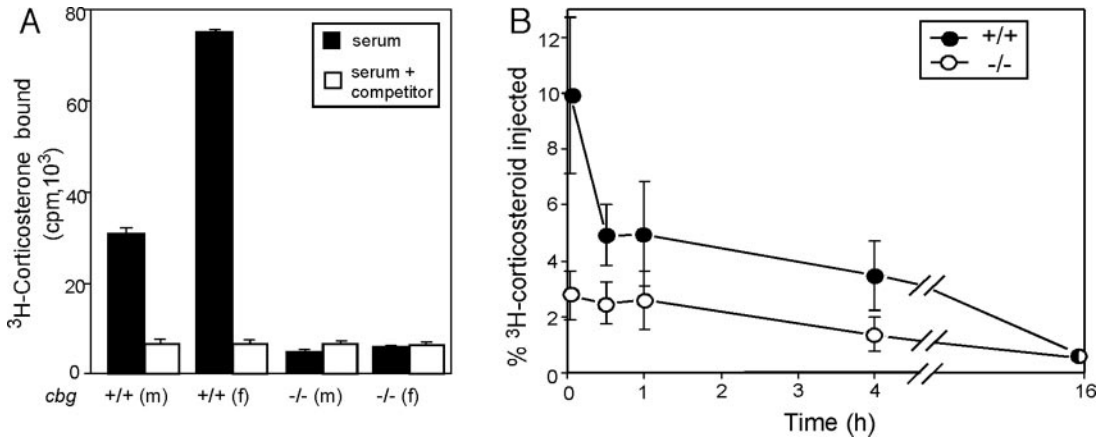

FIG. 2. Corticosterone binding activity in mouse serum. (A) Mouse serum samples from male (m) and female (f) mice of the indicated *cbg* genotypes were incubated with [^{3}H]corticosterone in the absence (filled bars) or the presence (open bars) of excess unlabeled corticosterone, and the amount of [^{3}H]corticosterone bound to serum proteins was determined in triplicates as detailed in Materials and Methods. (B) [^{3}H]corticosterone (25 pmol) was injected intravenously into wild-type and CBG-deficient male mice, and the amount of radioactivity remaining in the circulation was determined by blood sampling at the indicated time points. Identical results were obtained by intraperitoneal application of the tracer.

Steroid binding assay. Binding of corticosterone to mouse serum samples was carried out as described previously (10). Briefly, 3 pmol of [^{3}H]corticosterone was incubated in a 300-μl sample volume with 32 μl of serum that had been preincubated for 30 min at room temperature with dextran-coated charcoal (DCC) to remove prebound steroids. Where indicated, the reaction buffer also included 300 pmol unlabeled corticosterone as a competitor. The sample mixture was incubated for 60 min at room temperature. Thereafter, free steroids were extracted by DCC treatment for 10 min at 4°C followed by centrifugation, and the amount of protein-bound radioactivity was determined.

Steroid and endocrine determinations. Blood sampling for steroid or endocrine measurements was performed by retro-orbital bleeding of anesthetized mice at 9 a.m.; urine samples were collected from mice placed in metabolic cages overnight (16 h).

Free corticosterone was separated from the bound fraction in serum by a method based on ultracentrifugation using Centrifree micropartition devices (YM membranes, 30,000 molecular weight cutoff; catalogue no. 4104; Millipore, Amicon). Serum (200 to 300 μl) was added to the ultrafiltration chambers and centrifuged at 2,000 × *g* for 30 min. The ultrafiltrate and noncentrifuged serum were assayed for free and total corticosterone, respectively, using a commercial radioimmunoassay (RIA) (catalog no. 07-120102; ICN). Levels of adrenocorticotrophic hormone (ACTH) were determined using an anti-human ACTH radioimmunoassay (07-106101; MP Biomedicals, Eschwege, Germany); mouse leptin and insulin concentrations were determined by enzyme immunoassay (Assay Designs, Inc.) and RIA (BioTrend, Cologne, Germany), respectively. Electrolytes were measured with a standard clinical chemistry analyzer.

Transcriptional analyses. Quantitative RT-PCR was performed on total RNA samples from wild-type and CBG-deficient animals using TaqMan technology (Applied Biosystems). The following oligonucleotides were used for detection of GAPDH: forward, 5′-GGC AAA TTC AAC GGC ACA GT-3′; reverse, 5′-AGA TGG TGA TGG GCT TCC C-3′; probe, 6-FAM-5′-AAG GCC GGA GAA TGG GAA GCT TGT CAT C-3′-TAMRA. TaqMan gene expression probes (Applied Biosystems) were used for the detection of phosphoenolpyruvate carboxykinase 1 (Mm00440636_m1) and tyrosine aminotransferase (Mm00455392_m1). Gene expression profiling was performed on total RNA samples extracted from livers of three wild-type and three CBG-deficient male mice using the mouse genome 430 2.0 probe array (Affymetrix) according to the manufacturer's protocols.

Sepsis experiment. Mice were injected i.p. with 40 (cytokine determination) or 200 (survival study) μg of endotoxin (lipopolysaccharide [LPS]; *Salmonella enterica* serovar Minnesota, purified by trichloroacetic acid extraction; Sigma) per g of body weight. For cytokine determination, the animals were sacrificed 6 h following LPS injection, and plasma, i.p. macrophages, and lung tissue were collected. Protein extracts were generated from the samples using the BioPlex lysis kit (Bio-Rad) and assayed for various cytokines (18-Plex mouse cytokine panel; Bio-Rad) according to the manufacturer's recommendation. Alternatively, lung specimens were fixed in 4% paraformaldehyde and subjected to routine paraffin sectioning and staining (hematoxylin/eosin and Giemsa) before determination of mononuclear cell counts.

RESULTS

The murine *cbg* gene is located on mouse chromosome 12E. It comprises five exons, with the complete CBG-encoding sequence spanning exons 2 through 5. To inactivate the gene in mice, we used homologous recombination in murine embryonic stem cells to insert a neomycin resistance cassette into exon 2 (Fig. 1A). The targeted disruption of the *cbg* gene was introduced into the germ line of mice, and animals homozygous for the null allele were identified by a PCR-based genotyping approach (Fig. 1B). Mice carrying two *cbg*-null alleles ($cbg^{-/-}$) were produced with normal Mendelian distribution (not shown). The animals were viable and fertile and did not exhibit any discernible anomalies upon external inspection. Successful ablation of CBG gene expression was confirmed by global gene expression profiling using whole mouse genome arrays that indicated 9.5-fold-reduced relative *cbg* mRNA expression levels in $cbg^{-/-}$ mice (113.94 ± 38.32 [mean ± standard deviation]) compared with $cbg^{+/+}$ mice (1,087.64 ± 234.93; $P = 0.001$) (probe set 1448506_at). Furthermore, Western blot analysis using a polyclonal antiserum raised against the full-length mouse protein indicated the complete absence of CBG immunoreactivity in plasma samples of $cbg^{-/-}$ compared with $cbg^{+/+}$ animals (Fig. 1C).

In blood, approximately 90% of circulating corticosteroids are bound to CBG, whereas the rest is either associated with albumin (7%) or present in the free fraction (3%) (4). Binding to CBG determines plasma transport as well as metabolic clearance rates for the steroid (3). Specific and saturable binding of [^{3}H]corticosterone to CBG could be detected in serum samples of wild-type male and female mice, with the latter exhibiting significantly larger binding capacity, which is in line with higher CBG levels in females (Fig. 2A) (7). In contrast, mice genetically deficient for CBG lacked any detectable cortico-

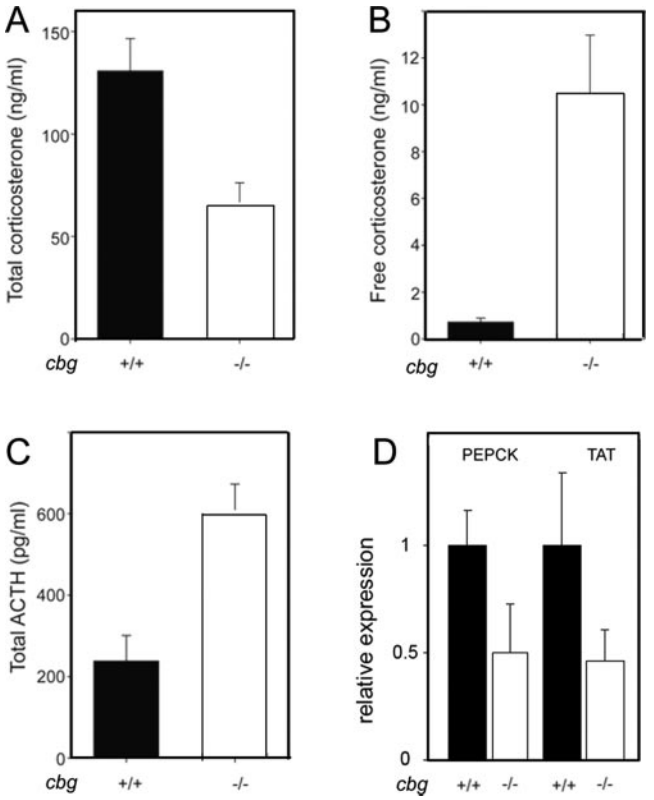

FIG. 3. Steroid hormone and glucocorticoid target gene expression levels in mice. (A to C) Levels of total (A; $P = 0.002$) and free (B; $P = 0.001$) corticosterone and ACTH (C; $P = 0.001$) were determined in serum samples from CBG-deficient male mice and their wild-type controls. Values are means (plus standard errors of the means) for 12 to 20 mice in each group. (D) Quantitative RT-PCR of mRNA levels of phosphoenolpyruvate carboxykinase 1 (PEPCK; $P = 0.19$) and tyrosine aminotransferase (TAT; $P = 0.12$) in livers from wild-type and CBG-deficient male mice fed ad libitum.

sterone binding activity in serum, demonstrating the unique role of CBG in corticosterone transport (Fig. 2A). Absence of the carrier significantly accelerated the plasma turnover of [^{3}H]corticosterone injected intravenously (Fig. 2B) or intraperitoneally (data not shown) into the animals. Within 5 min after application, fourfold-lower levels of the tracer were detected in the circulation of $cbg^{-/-}$ animals than in control animals (Fig. 2B). The same qualitative differences were seen between $cbg^{+/-}$ (4.9% ± 1.0% of injected tracer in the circulation after 5 min) and $cbg^{-/-}$ littermates (2.3% ± 0.5%; $P < 0.001$), excluding genetic background effects as the underlying cause of the dissimilarity. In both mouse lines, the major fraction of corticosterone accumulated in liver (~20%) and intestine (~9%) after 30 min (data not shown), in agreement with a predominant role of these organs in catabolism of corticosteroids in rodents (16).

To determine the effect of CBG deficiency on corticosteroid metabolism, we measured the concentration of total and free corticosterone, the major circulating glucocorticoid in mice (16). In blood samples taken from anesthetized animals, total corticosterone levels were reduced by 50% in $cbg^{-/-}$ animals (Fig. 3A) but the free fraction of the hormone was increased 10-fold (Fig. 3B), consistent with loss of the physiological carrier. Because the mode of blood sampling may affect circulating levels of stress hormones, we also performed measurement in samples taken from nonanesthetized animals (sacrificed by cervical dislocation; blood sampling by puncture of the heart). Although the overall levels of hormones were lower under this experimental condition, the qualitative differences in hormone levels were confirmed, with a significant reduction in total (9.6 ± 2.4 ng/ml versus 55.5 ± 9.43 ng/ml; $P = 0.0004$) and an increase in free corticosterone levels (2.98 ± 0.9 ng/ml versus 0.41 ± 0.24 ng/ml; $P = 0.02$) in $cbg^{-/-}$ compared with

TABLE 1. Endocrine and cardiovascular parameters in wild-type and CBG-deficient mice fed ad libitum or fasting overnight[a]

Parameter	Fed mice				Fasting mice			
	+/+[b]	*n*	−/−	*n*	+/+	*n*	−/−	*n*
Serum								
Insulin (ng/ml)	0.61 ± 0.08	8	0.81 ± 0.14	6	0.20 ± 0.05	7	0.24 ± 0.06	7
Leptin (ng/ml)	3.0 ± 0.2	28	3.9 ± 0.3	29	2.3 ± 0.6	9	3.1 ± 0.8	11
Glucose (mmol/liter)	9.0 ± 1.4	15	9.6 ± 1.4	17	5.1 ± 0.7	13	5.5 ± 1.1	11
Creatinine (μmol/liter)	10.9 ± 2.6	15	12.0 ± 6.0	17	10.3 ± 3.2	13	12.1 ± 4.9	11
Na^+ (μmol/liter)	149.9 ± 4.8	15	150.5 ± 2.7	17	149.4 ± 3.5	12	154.0 ± 2.0	11
Cl^- (mmol/liter)	121.5 ± 20.6	15	108.9 ± 4.0	17	109.0 ± 2.2	12	113.8 ± 3.3	11
K^+ (mmol/liter)	5.7 ± 0.8	15	5.6 ± 0.7	17	6.0 ± 0.6	12	6.5 ± 0.7	11
Ca^{2+} (mmol/liter)	2.5 ± 0.1	15	2.6 ± 0.1	17	2.5 ± 0.1	12	2.5 ± 0.2	11
Urine								
Glucose (mmol/liter)	2.9 ± 1.7	10	2.7 ± 1.5	12	0.4 ± 0.1	10	0.7 ± 0,4	10
Creatinine (μmol/liter)	2,434 ± 365	10	3,022 ± 1110	12	1,199 ± 167	10	2,300 ± 866	10
Na^+ (mmol/liter)	99 ± 22	10	121 ± 45	12	74 ± 13	10	85 ± 23	10
Cl^- (mmol/liter)	141 ± 38	10	185 ± 64	12	59 ± 13	10	67 ± 30	10
K^+ (mmol/liter)	318 ± 220	10	341 ± 238	12	96 ± 23	10	175 ± 144	10
Ca^{2+} (mmol/liter)	1.9 ± 0.7	10	2.7 ± 1.5	12	1.7 ± 1.4	10	1.1 ± 0.5	10
Cardiovascular								
HR (beats/min)	618 ± 5	5	621 ± 17	4	ND		ND	
Activity (counts/min)	4.8 ± 0.4	5	2.7 ± 0.4[d]	4	ND		ND	
Activity[c] (counts/min)	6.0 ± 0.7	3	3.6 ± 0.5[d]	3	ND		ND	

[a] HR, heart rate; *n*, number of animals; ND, not determined. Values are means ± standard errors of the means.
[b] Cardiovascular data in this column are for +/+ and +/− mice.
[c] Data obtained with $cbg^{+/-}$ and $cbg^{-/-}$ littermates from heterozygous breeding (6 weeks of age).
[d] $P < 0.001$; Mann-Whitney test.

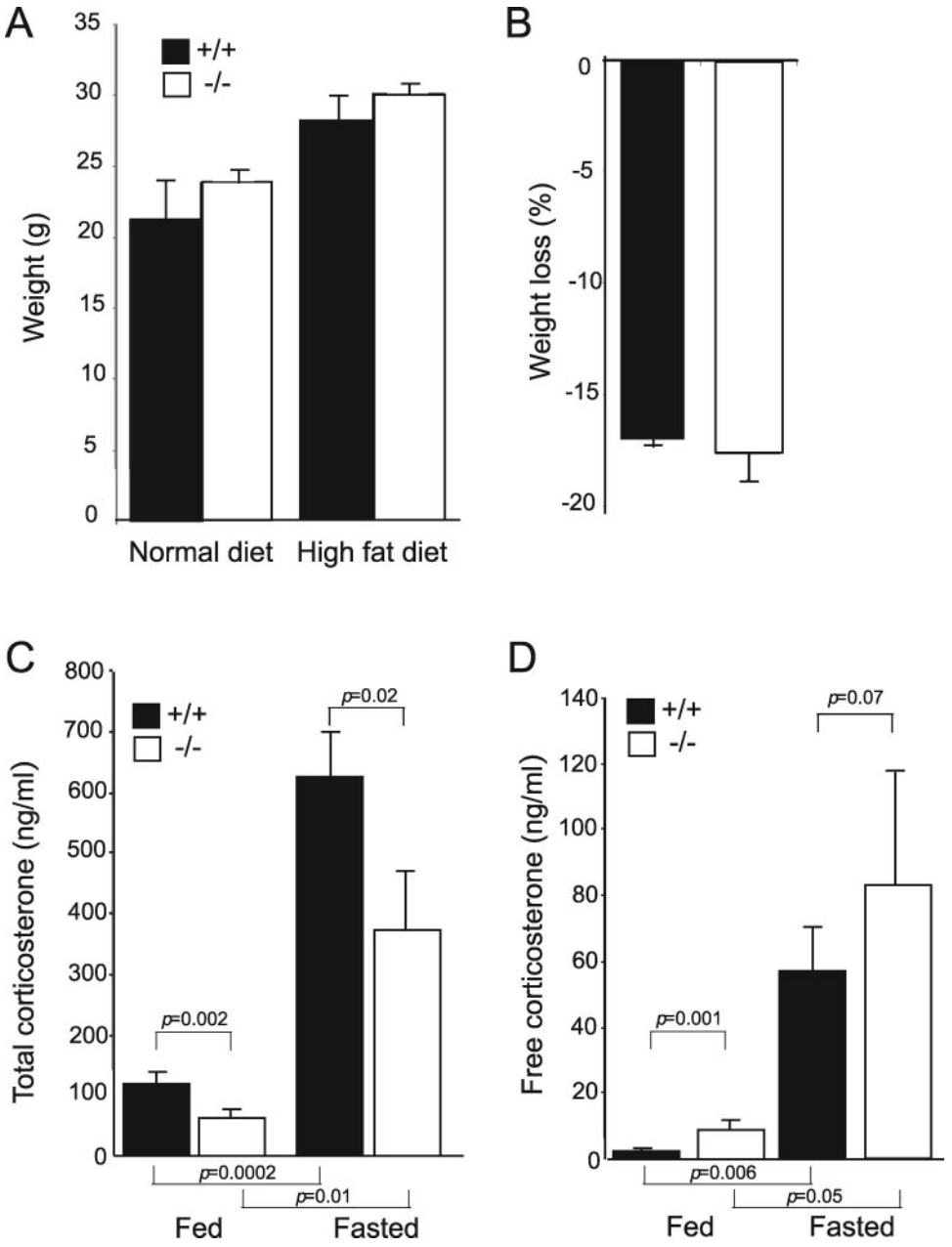

FIG. 4. Body weight and corticosterone levels in wild-type and CBG-deficient mice. (A) Body weights of 12-week-old wild-type and $cbg^{-/-}$ male animals (5 to 10 animals per group) fed a normal or fat-enriched Western-type diet for 6 weeks. (B) Percent weight loss induced in wild-type and $cbg^{-/-}$ male animals by 36 h of food deprivation. (C, D) Levels of total (C) and free (D) corticosterone in fed or starved wild-type and $cbg^{-/-}$ male animals (10 to 12 animals per group).

$cbg^{+/+}$ animals. Surprisingly, the significant increase in the free fraction of corticosterone did not result in a decrease in ACTH that is subject to negative feedback regulation by the steroid. Rather, the plasma concentration of ACTH was increased by almost 300% in knockout animals (599.3 ± 73.7 pg/ml versus 238.5 ± 62.3 pg/ml; $P = 0.0011$) (Fig. 3C), indicating a relative deficiency in sensing higher levels of free corticosterone in this mouse model. An inability to respond to the elevated levels of free corticosterone was further confirmed by determination of the expression levels of corticosteroid target genes in the liver using quantitative RT-PCR. Expression of tyrosine aminotransferase and phosphoenolpyruvate carboxykinase showed a trend towards lower rather than higher levels in the CBG-deficient mouse despite a 10-fold increase in the free hormone fraction (Fig. 3D).

Next, we evaluated the consequences of CBG deficiency for endocrine and metabolic parameters in our mouse model. No discernible differences in electrolyte (Na^+, Ca^{2+}, K^+, and Cl^-) and metabolite (creatinine, glucose, insulin, and leptin) concentrations in serum and urine were detected in mice fed ad libitum or starved overnight (Table 1). However, CBG-defi-

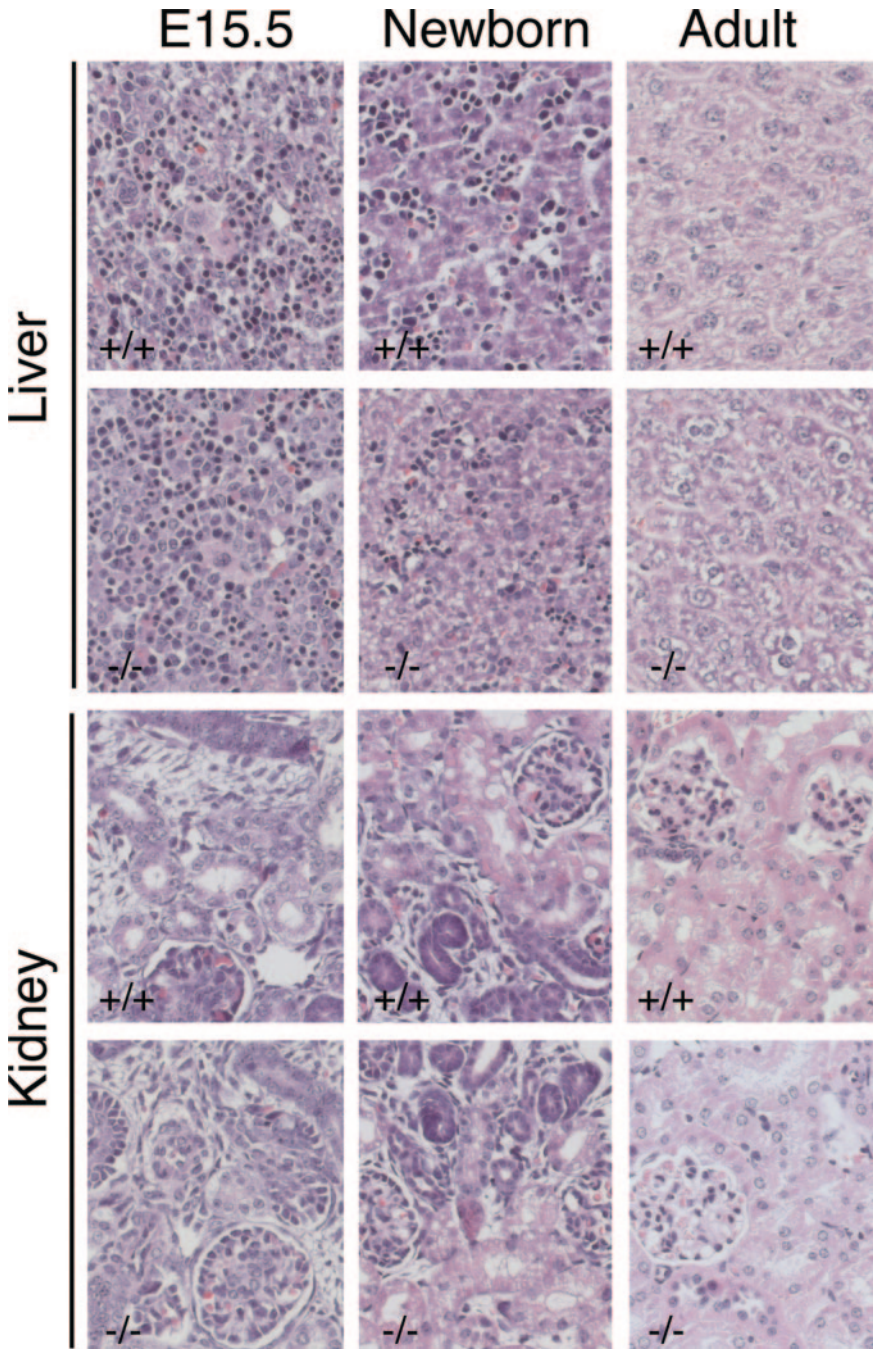

FIG. 5. Histological appearance of liver and kidney in wild-type and CBG-deficient mice. Comparative histological analysis of liver and kidney tissues from wild-type and *cbg*$^{-/-}$ embryos (E15.5), newborns, and adults.

cient mice exhibited a significant decrease in activity levels compared with control animals (Table 1; $P < 0.001$), while the heart rate was unchanged (Table 1). This phenotype was confirmed in *cbg*$^{+/-}$ and *cbg*$^{-/-}$ littermates from heterozygous breeding, excluding genetic background effects (Table 1). Fatigue syndrome in CBG$^{-/-}$ mice is a phenotype shared with patients suffering from familial corticosteroid binding globulin deficiency (35), demonstrating the relevance of this mouse model to the condition in humans.

To explore the consequence of CBG deficiency for metabolic homeostasis, we measured the body weights of 12-week-old animals either fed a normal chow (4% fat) or a Western-type diet enriched in crude fat (26%). No obvious phenotypic anomalies were seen between *cbg*$^{-/-}$ animals and age-matched controls (Fig. 4A; $P = 0.24$ for normal chow; $P = 0.08$ for high-fat diet). Also, weight loss induced by starving the animals for 36 h was similar in wild-type and knockout animals (Fig. 4B; $P = 0.71$). Normal metabolic stress response in CBG$^{-/-}$ animals was further confirmed by liver gene expression profiling using whole mouse genome arrays, which did not identify

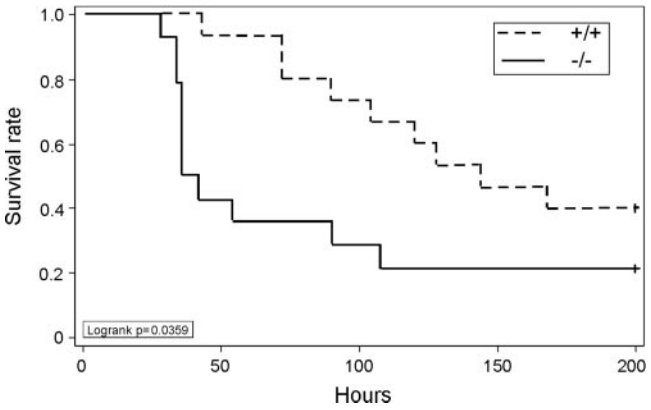

FIG. 6. Survival rates of mice subjected to intraperitoneal injection of LPS. CBG-deficient animals exhibited significantly decreased survival ($P = 0.035$) following i.p. injection of 200 μg/g of LPS compared to control mice. Each group had 14 (−/−) or 15 (+/+) mice.

any genes that are obviously differentially regulated upon starvation compared to wild-type animals (data not shown). Consistent with this notion, the stress-induced increases in total (Fig. 4C) and free (Fig. 4D) plasma corticosterone levels were similar in the CBG-deficient and control lines, suggesting that this stress response may not be influenced by the presence or absence of the carrier protein.

Glucocorticoids play a major role in organ development, in particular in maturation of the lung. This function is confirmed by the perinatal lethality in glucocorticoid receptor knockout mice due to lung atelectasis (6). In addition, corticosteroid-induced expression of CBG in mouse liver around embryonic day 15 (E15) to E16 (5, 27, 38) and in mouse kidney around birth (26) suggested distinct roles for the carrier in maturation of these organs in rodents. These functions were proposed to involve regulation of systemic glucocorticoid metabolism or local delivery of steroids via membrane receptors identified in embryonic tissues (5). Normal perinatal survival of the $cbg^{-/-}$ animals already indicated unimpaired maturation of the lung. Furthermore, histological examination showed no discernible difference in tissue architecture of livers and kidneys of E15.5 embryos, neonates, or adult mice lacking CBG compared with their respective controls (Fig. 5). Thus, normal development of lungs, kidneys, and livers in $cbg^{-/-}$ mice does not support a critical role for the carrier in development of these organs. Furthermore, other tissues, such as thymus and adrenal gland, that may be influenced by alterations in glucocorticoid metabolism appeared normal upon macroscopic inspection (data not shown).

The adrenocortical response is a key element of the normal defense repertoire against stress. Many of these stress-related activities of corticosteroids are likely influenced by CBG. In this study, we focused on the role of CBG in the regulation of inflammatory processes known to be modulated by corticosteroids. In particular, corticosteroids repress expression and action of cytokines released by activated neutrophils to balance inflammatory reactions and to prevent tissue damage by excessive cytokine production (1, 13). To test this concept, we evaluated the response of CBG-deficient mice to sepsis induced by i.p. injection of LPS. When injected with lethal doses of LPS, *cbg*-null animals exhibited a significantly increased susceptibility to acute septic shock compared to controls, as shown by a dramatic decline in survival rates within the first 48 h following LPS injection (Fig. 6). Again, this defect could be

TABLE 2. Inflammatory response in wild-type and CBG-deficient mice injected with 40 μg/g endotoxin[a]

Mouse group	Parameter	Value for mice with genotype:		*P*
		$cbg^{+/+}$	$cbg^{-/-}$	
LPS-injected	Plasma			
	G-CSF (pg/ml)	8,018.3 ± 2,227.8	26,466.5 ± 10,687.7	0.008
	IL-1β (pg/ml)	96 ± 75.9	99.9 ± 19.8	0.955
	IL-6 (pg/ml)	15,292.0 ± 3,739.8	17,619.6 ± 1,277.9	0.163
	RANTES (pg/ml)	786.5 ± 290.0	937.9 ± 280.0	0.360
	Lung			
	G-CSF (pg/mg)	3.6 ± 4.0	10.6 ± 12.4	0.232
	IL-1β (pg/mg)	221.5 ± 56.7	342.6 ± 58.4	0.003
	IL-6 (pg/mg)	74.4 ± 27.8	56.4 ± 27.5	0.246
	RANTES (pg/mg)	268.32 ± 136.5	743.08 ± 174.2	0.066
	Mononuclear cell count	106.6 ± 8.5	150.4 ± 17.1	0.03
	Total corticosterone (ng/ml)	399.4 ± 44.9	228.7 ± 29.9	0.005
	Free corticosterone (ng/ml)	49.4 ± 8.5	74.8 ± 8.3	0.058
Noninjected	Lung			
	G-CSF	ND	ND	
	IL-1β (pg/mg)	4.15 ± 0.50	3.96 ± 0.75	0.732
	IL-6 (pg/mg)	5.92 ± 1.84	2.55 ± 0.86	0.164
	RANTES (pg/mg)	45.86 ± 21.0	114.73 ± 42.3	0.133

[a] Values are means ± standard errors of the means for six animals in each group. Statistical evaluation was performed by Student's *t* test. ND, not detectable.

reproduced in offspring from heterozygous breeding, with five of five $cbg^{-/-}$ animals dying within the first 48 h after LPS injection compared to only two of five $cbg^{+/-}$ control littermates. Decreased survival of the mice correlated with a tendency toward increased levels of granulocyte colony-stimulating factor (G-CSF), interleukin 1β (IL-1β), IL-6, and RANTES; the increases reached statistical significance for G-CSF in plasma and IL-1β in lung (Table 2). Also, the number of infiltrated mononuclear cells in the lung was significantly elevated (Table 2). In contrast, cytokine levels in untreated animals were identical in both genotypes (Table 2). Remarkably, the increase in circulating free corticosterone as a consequence of inflammatory response was even slightly higher in knockouts (78.8 ng/ml) than wild types (49.4 ng/ml; $P = 0.058$). Thus, enhanced sensitivity to septic shock in $cbg^{-/-}$ animals was not due to inadequate levels of biologically active free steroids. Rather, the finding suggests the absence of an activity inherent to the CBG protein, such as local delivery of bound steroids to neutrophils.

DISCUSSION

Studies on the function of steroid hormones in vivo have primarily focused on the evaluation of circulating levels of the hormones and their correlation to biological activities. However, there is increasing evidence that factors other than systemic hormone concentrations contribute to and modify the cellular and organismal response to steroid hormones. Most importantly, an emerging role for steroid binding proteins in hormonal action received major attention. Rather than acting as mere cargo transporters for lipophilic hormones, carrier proteins appear to have specific roles in the regulation of bioavailability, local delivery, and cellular signal transduction of steroid hormones (2, 9, 19, 20, 22). To gain a better understanding of the role of CBG in glucocorticoid metabolism, we have generated a mouse model genetically deficient for this carrier. *cbg*-null mice are viable and fertile but exhibit distinct defects in glucocorticoid action and adrenocortical stress response consistent with a modulatory role of the protein in hormonal activity. Thus, $cbg^{-/-}$ animals display increased activity of the pituitary axis of hormonal control (ACTH), normal levels of gluconeogenetic enzymes in the liver, and fatigue syndrome, as well as an aggravated response to septic shock, albeit at approximately 10-fold-elevated levels of free steroids, indicating an overall inability of the animals to appropriately sense and respond to the free corticosterone. These findings are in line with the proposed function of CBG in mediating some of the activities ascribed to glucocorticoids in the body.

Foremost, a role for CBG as a plasma carrier for glucocorticoids is appreciated. In this scenario, CBG serves as a reservoir that stores steroid hormones in the plasma for release if needed (2). The exclusive role of CBG in plasma transport of glucocorticoids is supported by our finding that $cbg^{-/-}$ mice are devoid of any detectable plasma corticosterone binding activity (Fig. 2A). As a consequence, the plasma half-life of injected [^{3}H]corticosterone in these animals is significantly shortened compared to that in control animals (Fig. 2B). Because the plasma turnover of corticosterone is rapid even in wild-type animals, with up to 40% of the tracer accumulating in the liver within 10 min after injection (16), it is impossible to determine whether enhanced clearance of the tracer in $cbg^{-/-}$ animals was caused by accelerated shunt to the liver (or other tissues) or simply by adherence of the lipophilic substance to the vasculature. However, similar findings in mice genetically deficient for the vitamin D binding protein, the plasma carrier for vitamin D metabolites, indicated that faster plasma clearance of the hormones in the absence of high-affinity binding proteins is due to transfer of the steroids to the liver for metabolic inactivation and to excretion into the urine (25). Consistent with this model, gene expression profiling using microarrays demonstrated up-regulation of several drug-metabolizing enzymes in the livers of CBG-deficient mice, including cytochrome p450 2c39 (9.3-fold), hydroxysteroid 17-beta dehydrogenase 9 (4.2-fold), and carboxylesterase 1 (4.4-fold) (data not shown).

As well as acting as a transporter for glucocorticoids, CBG is believed to regulate the amount of free hormone that is available for diffusion into target tissues (free hormone hypothesis) (17). Consistent with this function, various stress conditions (such as tail shock or social stress) that require appropriate glucocorticoid action have been shown to reduce the levels of CBG expression as a possible means of increasing the free fraction of the hormone (8, 21, 32). This hypothesis is further supported by findings in patients genetically deficient for the carrier protein. In these individuals, absence of the carrier results in a fivefold increase in the percentage of the free hormone fraction. However, total hormone levels are lower than in healthy subjects, so that the overall concentration of the circulating free hormone remains within the normal range (35). Thus, the overall effect of CBG deficiency on normal body function is modest, with some families exhibiting fatigue and hypotension (35) while other families do not (4).

Low activity levels are a feature also shared by mice genetically deficient for CBG. In contrast to the condition in humans, however, the overall levels of circulating free steroids are significantly increased in the mouse model. Surprisingly, higher levels of the free steroid are not accompanied by phenotypic features normally seen in conditions of excess glucocorticoid signaling. Thus, in patients with Cushing syndrome (caused by, for example, ectopic ACTH-producing tumors or adrenal carcinomas), high levels of glucocorticoids result in a complex phenotype of excess central fat accumulation, muscle atrophy, hypertension, and alopecia (18). Some of these defects are seen in mice with nervous system-specific inactivation of the glucocorticoid receptor gene that lack feedback regulation of the hypothalamus-pituitary-adrenal axis (36) or in animals overexpressing the corticotropin-releasing factor (33). None of these phenotypes are shared by CBG-deficient animals. Rather, elevated levels of ACTH and normal to reduced levels of liver-specific target genes are features reminiscent of models that exhibit reduced glucocorticoid signaling (35).

Impaired activity of glucocorticoids is also suggested by another anomaly of CBG-deficient animals—their sensitivity to septic shock. Glucocorticoids inhibit the expression and activity of most cytokines (1). This mechanism is an important integral part of the host defense to balance between a necessary increase in cytokine action following infections and the need to control tissue damage caused by excess inflammatory response (29). Aggravated sensitivity of $cbg^{-/-}$ mice to LPS-induced septic shock, accompanied by significant increases in cytokine concentrations and mononuclear cell infiltration in

the lung (Fig. 6; Table 2), suggests poor control of cytokine reactions in this mouse model. These defects cannot be accounted for by inappropriate levels of free circulating glucocorticoids that are above the normal range (Table 2).

Taken together, studies in mice lacking the physiological carrier for glucocorticoids demonstrated some phenotypes indicative of a less-than-normal response to the steroid hormone during physiological and stress-induced body functions. Because the plasma levels of free corticosterones are higher in CBG-deficient mice than in control mice, unavailability of the circulating bioactive free hormone cannot be considered responsible for these defects. What functions may be inherent to the carrier protein responsible for such phenotypes? Many hypotheses point towards a local role of carriers such as CBG in mediating the action of steroid hormones. Such activities may involve interaction with cell surface receptors in target tissues that mediate endocytic uptake of carrier/steroid complexes or direct cell surface signaling. Potential clearance receptors for CBG have been identified in liver, endometrium, placenta, prostate, spleen, and kidney tissues (14, 15, 30, 34), while the existence of CBG signaling receptors in breast cancer cells has been documented (19). Finally, compelling experimental evidence points towards a role of CBG in local action of bound glucocorticoids via cleavage through neutrophil elastase (12, 23). The impaired response to septic shock seen in CBG-deficient mice is indeed in favor of such a model, although reduced signaling from surface receptors or endocytic uptake of the steroid/carrier complex cannot be excluded. At present, the exact mode of action of CBG in glucocorticoid metabolism, apart from its role in plasma steroid transport, is still unresolved, but the availability of a mouse model genetically deficient for the carrier will greatly aid in addressing these important issues.

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REFERENCES

1. **Brattsand, R., and M. Linden.** 1996. Cytokine modulation by glucocorticoids: mechanisms and actions in cellular studies. Aliment. Pharmacol. Ther. **10**(Suppl. 2)**:**81–90, 91–92.
2. **Breuner, C. W., and M. Orchinik.** 2002. Plasma binding proteins as mediators of corticosteroid action in vertebrates. J. Endocrinol. **175:**99–112.
3. **Bright, G. M.** 1995. Corticosteroid-binding globulin influences kinetic parameters of plasma cortisol transport and clearance. J. Clin. Endocrinol. Metab. **80:**770–775.
4. **Brunner, E., J. Baima, T. C. Vieira, J. G. Vieira, and J. Abucham.** 2003. Hereditary corticosteroid-binding globulin deficiency due to a missense mutation (Asp367Asn, CBG Lyon) in a Brazilian kindred. Clin. Endocrinol. (Oxford) **58:**756–762.
5. **Challis, J. R., E. T. Berdusco, T. M. Jeffray, K. Yang, and G. L. Hammond.** 1995. Corticosteroid-binding globulin (CBG) in fetal development. J. Steroid Biochem. Mol. Biol. **53:**523–527.
6. **Cole, T. J., J. A. Blendy, A. P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, and G. Schutz.** 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. **9:**1608–1621.
7. **Fernandez-Real, J. M., M. Pugeat, M. Grasa, M. Broch, J. Vendrell, J. Brun, and W. Ricart.** 2002. Serum corticosteroid-binding globulin concentration and insulin resistance syndrome: a population study. J. Clin. Endocrinol. Metab. **87:**4686–4690.
8. **Fleshner, M., T. Deak, R. L. Spencer, M. L. Laudenslager, L. R. Watkins, and S. F. Maier.** 1995. A long-term increase in basal levels of corticosterone and a decrease in corticosteroid-binding globulin after acute stressor exposure. Endocrinology **136:**5336–5342.
9. **Hammes, A., T. K. Andreassen, R. Spoelgen, J. Raila, N. Hubner, H. Schulz, J. Metzger, F. J. Schweigert, P. B. Luppa, A. Nykjaer, and T. E. Willnow.** 2005. Role of endocytosis in cellular uptake of sex steroids. Cell **122:**751–762.
10. **Hammond, G. L., and P. L. Lahteenmaki.** 1983. A versatile method for the determination of serum cortisol binding globulin and sex hormone binding globulin binding capacities. Clin. Chim. Acta **132:**101–110.
11. **Hammond, G. L., C. L. Smith, I. S. Goping, D. A. Underhill, M. J. Harley, J. Reventos, N. A. Musto, G. L. Gunsalus, and C. W. Bardin.** 1987. Primary structure of human corticosteroid binding globulin, deduced from hepatic and pulmonary cDNAs, exhibits homology with serine protease inhibitors. Proc. Natl. Acad. Sci. USA **84:**5153–5157.
12. **Hammond, G. L., C. L. Smith, N. A. Paterson, and W. J. Sibbald.** 1990. A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. J. Clin. Endocrinol. Metab. **71:**34–39.
13. **Hartung, T.** 1998. Anti-inflammatory effects of granulocyte colony-stimulating factor. Curr. Opin. Hematol. **5:**221–225.
14. **Hryb, D. J., M. S. Khan, N. A. Romas, and W. Rosner.** 1986. Specific binding of human corticosteroid-binding globulin to cell membranes. Proc. Natl. Acad. Sci. USA **83:**3253–3256.
15. **Maitra, U. S., M. S. Khan, and W. Rosner.** 1993. Corticosteroid-binding globulin receptor of the rat hepatic membrane: solubilization, partial characterization, and the effect of steroids on binding. Endocrinology **133:**1817–1822.
16. **Marandici, A., and C. Monder.** 1984. The fate of corticosterone and 11-deoxycorticosterone in C57BL/6 and BALB/c strains of mice: distribution and oxidative metabolism. J. Steroid Biochem. **21:**579–583.
17. **Mendel, C. M.** 1989. The free hormone hypothesis: a physiologically based mathematical model. Endocr. Rev. **10:**232–274.
18. **Miller, W. L., and J. B. Tyrrel.** 1995. The adrenal cortex, p. 555–711. *In* P. Felig, J. D. Baxter, and L. A. Frohman (ed.), Endocrinology and metabolism. McGraw-Hill, New York, N.Y.
19. **Nakhla, A. M., M. S. Khan, and W. Rosner.** 1988. Induction of adenylate cyclase in a mammary carcinoma cell line by human corticosteroid-binding globulin. Biochem. Biophys. Res. Commun. **153:**1012–1018.
20. **Nakhla, A. M., J. Leonard, D. J. Hryb, and W. Rosner.** 1999. Sex hormone-binding globulin receptor signal transduction proceeds via a G protein. Steroids **64:**213–216.
21. **Neufeld, J. H., L. Breen, and R. Hauger.** 1994. Extreme posture elevates corticosterone in a forced ambulation model of chronic stress in rats. Pharmacol. Biochem. Behav. **47:**233–240.
22. **Nykjaer, A., D. Dragun, D. Walther, H. Vorum, C. Jacobsen, J. Herz, F. Melsen, E. I. Christensen, and T. E. Willnow.** 1999. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell **96:**507–515.
23. **Pemberton, P. A., P. E. Stein, M. B. Pepys, J. M. Potter, and R. W. Carrell.** 1988. Hormone binding globulins undergo serpin conformational change in inflammation. Nature **336:**257–258.
24. **Rosner, W.** 1990. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: recent advances. Endocr. Rev. **11:**80–91.
25. **Safadi, F. F., P. Thornton, H. Magiera, B. W. Hollis, M. Gentile, J. G. Haddad, S. A. Liebhaber, and N. E. Cooke.** 1999. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. J. Clin. Investig. **103:**239–251.
26. **Scrocchi, L. A., S. A. Hearn, V. K. Han, and G. L. Hammond.** 1993. Corticosteroid-binding globulin biosynthesis in the mouse liver and kidney during postnatal development. Endocrinology **132:**910–916.
27. **Scrocchi, L. A., M. Orava, C. L. Smith, V. K. Han, and G. L. Hammond.** 1993. Spatial and temporal distribution of corticosteroid-binding globulin and its messenger ribonucleic acid in embryonic and fetal mice. Endocrinology **132:**903–909.
28. **Siiteri, P. K., J. T. Murai, G. L. Hammond, J. A. Nisker, W. J. Raymoure, and R. W. Kuhn.** 1982. The serum transport of steroid hormones. Recent Prog. Hormone Res. **38:**457–510.
29. **Simon, H. U.** 2003. Neutrophil apoptosis pathways and their modifications in inflammation. Immunol. Rev. **193:**101–110.
30. **Singer, C. J., M. S. Khan, and W. Rosner.** 1988. Characteristics of the binding of corticosteroid-binding globulin to rat cell membranes. Endocrinology **122:**89–96.
31. **Smith, C. L., S. G. Power, and G. L. Hammond.** 1992. A Leu—-His substitution at residue 93 in human corticosteroid binding globulin results in reduced affinity for cortisol. J. Steroid Biochem. Mol. Biol. **42:**671–676.
32. **Spencer, R. L., A. H. Miller, H. Moday, B. S. McEwen, R. J. Blanchard, D. C. Blanchard, and R. R. Sakai.** 1996. Chronic social stress produces reductions in available splenic type II corticosteroid receptor binding and plasma corticosteroid binding globulin levels. Psychoneuroendocrinology **21:**95–109.
33. **Stenzel-Poore, M. P., V. A. Cameron, J. Vaughan, P. E. Sawchenko, and W. Vale.** 1992. Development of Cushing's syndrome in corticotropin-releasing factor transgenic mice. Endocrinology **130:**3378–3386.

34. **Strel'chyonok, O. A., and G. V. Avvakumov.** 1991. Interaction of human CBG with cell membranes. J. Steroid Biochem. Mol. Biol. **40:**795–803.
35. **Torpy, D. J., A. W. Bachmann, J. E. Grice, S. P. Fitzgerald, P. J. Phillips, J. A. Whitworth, and R. V. Jackson.** 2001. Familial corticosteroid-binding globulin deficiency due to a novel null mutation: association with fatigue and relative hypotension. J. Clin. Endocrinol. Metab. **86:**3692–3700.
36. **Tronche, F., C. Kellendonk, O. Kretz, P. Gass, K. Anlag, P. C. Orban, R. Bock, R. Klein, and G. Schutz.** 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. **23:**99–103.
37. **Van Baelen, H., R. Brepoels, and P. De Moor.** 1982. Transcortin Leuven: a variant of human corticosteroid-binding globulin with decreased cortisol-binding affinity. J. Biol. Chem. **257:**3397–3400.
38. **Zhao, X. F., L. A. Scrocchi, and G. L. Hammond.** 1997. Glucocorticoids induce corticosteroid-binding globulin biosynthesis by immature mouse liver and kidney. J. Steroid Biochem. Mol. Biol. **60:**163–169.