Differential Effects of Estrogen on Corticosteroid-Binding Globulin Forms Suggests Reduced Cleavage in Pregnancy

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Corticosteroid-binding globulin (CBG) is secreted as high-affinity CBG (haCBG), which may be cleaved by tissue proteases to low-affinity CBG (laCBG), releasing free cortisol. Pregnancy and the estrogen-based combined oral contraceptive pill (COCP) increase CBG concentrations twofold to threefold. The relative effects of these two hyperestrogenic states on the CBG affinity forms are unknown. We performed an observational study in 30 pregnant women, 27 COCP takers and 23 controls. We analyzed circulating total CBG, haCBG, laCBG, and free and total cortisol concentrations. In pregnancy, total CBG and haCBG were increased compared to controls (both $P < 0.0001$); however, laCBG concentrations were similar. In COCP takers, total CBG and haCBG were increased [802 ± 41 *vs* compared to controls (both $P < 0.0001$)], but laCBG was also increased ($P = 0.03$). Pregnancy and use of COCP were associated with a comparable rise in haCBG, but laCBG was lower in pregnancy ($P < 0.0001$). These results were consistent with an estrogen-mediated increase in CBG synthesis in both hyperestrogenemic states but with reduced CBG cleavage in pregnancy relative to the COCP, perhaps due to pregnancy-induced CBG glycosylation. Speculatively, increased circulating haCBG concentrations in pregnancy may provide an increased reservoir of CBG-bound cortisol to prepare for the risk of puerperal infection or allow for cortisol binding in the face of competition from increased circulating progesterone concentrations.

Pregnancy is associated with profound immune and endocrine changes necessary for allograft tolerance and fetal maturation, including activation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in sustained physiologic hypercortisolism. Total plasma cortisol, either bound to corticosteroid-binding globulin (CBG) or albumin, as well as free-cortisol concentrations are elevated twofold to threefold in pregnancy [1–7]. The mechanisms for this are not fully resolved but include drive from placental corticotropin-releasing hormone under positive feedback from maternal cortisol, hyper-responsiveness of the adrenal cortex to adrenocorticotropic hormone, and increased adrenocorticotropic hormone secretion [8]. Total plasma CBG concentrations in pregnancy are also elevated twofold to threefold [1–5, 7, 9], as they are in

Abbreviations: CBG, corticosteroid-binding globulin; COCP, combined oral contraceptive pill; haCBG, high-affinity corticosteroid-binding globulin; HPA, hypothalamic-pituitary-adrenal; laCBG, low-affinity corticosteroid-binding globulin.

women taking combined oral contraceptive pills (COCPs), which result in supraphysiologic estrogen concentrations [3, 4, 10, 11].

CBG binds 80% to 90% of circulating cortisol; a further 10% is bound to albumin and approximately 5% is free. Free-cortisol concentrations increase exponentially when high total cortisol concentrations saturate CBG; low-affinity binding to albumin may then increase. CBG is able to target the delivery of free cortisol to inflamed tissues. This occurs via neutrophil elastase-mediated cleavage of the exposed reactive center loop of high cortisol-binding affinity CBG (haCBG; $K_a = 1.4 \times 10^9\,\mathrm{M}^{-1}$), resulting in the formation of 10-fold lower affinity CBG (laCBG; $K_a = 1.6 \times 10^8\,\mathrm{M}^{-1}$) and favoring release of free cortisol at inflammatory sites [12]. We have recently shown that the two forms coexist in the circulation [13]. Patients with sepsis have very low haCBG concentrations [14]. This agrees with *in vitro* data showing that CBG cleavage can be induced by neutrophil elastase [12]. At the tissue level, CBG cleavage increases the interstitial free-cortisol concentration threefold to fourfold, potentially enhancing cortisol's immunomodulatory effects [15–17].

CBG is heavily glycosylated, with five of six consensus sites typically occupied by biantennary and triantennary *N*-linked oligosaccharides in a 3:2 molar ratio [18, 19]. Branching increases significantly during pregnancy, producing pregnancy-specific CBG [20–23]. Glycosylation prolongs CBG half-life [24], alters cortisol-binding affinity, increases thermosensitivity [15], and may impair CBG-receptor interactions, particularly with syncytiotrophoblasts [25, 26].

The effect of pregnancy on CBG affinity forms is unknown. We hypothesized that elevated haCBG concentrations may be present in pregnancy. This may serve as a mechanism to prepare for puerperal sepsis, a leading cause of maternal and fetal morbidity and mortality [27], or allow for CBG binding competition from high progesterone concentrations. Hence, we studied CBG affinity-form concentrations in pregnancy, COCP takers, and nonpregnant women (control subjects).

1. Subjects and Methods

A. Study Participants

We performed a prospective observational study in pregnant women, women taking the COCP, and healthy control subjects at two tertiary centers in Adelaide, Australia, between 2013 and 2015. The protocol was approved by the Women's & Children's Health Network and the Royal Adelaide Hospital Human Research Ethics Committees. Pregnant women were recruited from prenatal outpatient clinics or inpatient wards after admission for delivery; women taking the COCP and healthy control subjects were recruited by local advertising. All provided written informed consent. Inclusion criteria were as follows: pregnant women, women taking an estrogen-based oral contraceptive pill for ≥3 months, or healthy control subjects. Exclusion criteria included active labor, concurrent infection or other acute illness, and use of immunosuppressive drugs, including betamethasone to improve fetal lung maturity. A single blood sample was collected at the time of enrolment. Median times (range) for sample collection were as follows: pregnancy, 10:30 AM (8:30 to 11:40 AM; n = 13); COCP takers, 2:19 PM (8:30 AM to 4:45 PM); and control subjects, 10:40 AM (8:20 AM to 4:15 PM). For women taking the COCP, samples were taken during the active phase of their pill cycle. Medical and demographic data were also recorded.

B. Laboratory Methods

EDTA plasma from pregnant women and serum from COCP takers and control subjects were stored at −70°C until analyzed. Total cortisol level was measured by electrochemiluminescence immunoassay on a Roche e601 analyzer (Roche Diagnostics, Castle Hill, NSW, Australia). Cortisol samples from control subjects and pregnant women were analyzed by the Elecsys Cortisol I assay (Roche Diagnostics). Cortisol samples from women taking the COCP were analyzed after the switch to the second-generation Elecsys Cortisol II assay (Roche Diagnostics). Accordingly, a conversion factor was applied to COCP cortisol samples, using a

Passing-Bablock fit equation (*i.e.*, $0.9 + 0.78x$). This equation was derived from internal validation analysis of 67 samples (range, 2.7 to 1175 nmol/L) and is similar to published data [28]. The fraction of free cortisol was determined using a temperature-controlled ultrafiltration/ligand-binding method [14] validated against equilibrium dialysis. Free-cortisol level was calculated from total cortisol level and free-cortisol fractions. The purpose of total and free-cortisol measurement was to assess a change in the free fraction in relation to changes in haCBG concentration, not to provide an assessment of cortisolemia.

Measurement of total and haCBG concentrations via two-site noncompetitive direct enzyme-linked immunosorbent assays was performed as previously validated [13, 29]. Briefly, paired microtiter plates were coated with a polyclonal CBG antibody. After addition of a 1:1000 sample in assay buffer or serially diluted recombinant CBG standard (catalog no. 10998-H08H; Sino Biological Inc. Beijing, China), the plates were treated with either the in-house monoclonal antibody RRID:AB_2632404 or RRID:AB_26632405 to measure total CBG and haCBG concentrations, respectively. Commercial peroxidase-conjugated anti-mouse secondary antibodies were then applied to the respective plates before substrate was added. Absorbance was read at 450 nm and concentrations determined by interpolation from standard curves. The level of laCBG was calculated indirectly [30]. Assays were performed in parallel with samples from two control subjects to ensure validity. Intra-assay coefficients of variation were 5.0% and 2.7% for total and haCBG, respectively.

C. Statistical Analyses

Data were analyzed using GraphPad Prism version 6 for Mac OS X (GraphPad Software, San Diego, CA). Results are presented as mean ± standard error of the mean unless otherwise stated. The D'Agostino-Pearson test was applied to confirm the normal distribution of CBG data. Comparison among three or more groups was performed by one-way analysis of variance with *post hoc* analysis carried out by Tukey multiple-comparison test. Multiplicity-adjusted P values are reported. Correlations between continuous variables were assessed with the Pearson correlation coefficient. $P < 0.05$ was taken as statistically significant.

2. Results

Thirty pregnant women, 27 COCP takers, and 23 healthy control subjects, were enrolled; their baseline characteristics are detailed in Table 1. Women taking a COCP were younger than women in other groups ($P < 0.05$). Four pregnant women were in the second trimester, whereas 26 were in the third trimester. Twenty-one women (78%) taking the COCP were receiving 25 to 35 μg of ethinylestradiol per day. Of the remaining six women, four received 20 μg and two received 50 μg of ethinylestradiol per day.

A. CBG in Pregnancy

Total CBG concentrations were greater in pregnant women than control subjects (877 ± 27 *vs* 466 ± 13 nmol/L; $P < 0.0001$; Fig. 1). haCBG concentrations were also greater (727 ± 27 *vs* 262 ± 13 nmol/L; $P < 0.0001$); however, laCBG concentrations did not differ (151 ± 22 *vs* 203 ± 14 nmol/L; $P = 0.24$). The percentage of haCBG to total CBG was significantly higher in pregnant women than in control subjects (83.3% ± 2.3% *vs* 56.7% ± 2.7%; $P < 0.0001$).

B. CBG and the COCP

Women taking the COCP had the highest total CBG concentrations (1093 ± 44 nmol/L; $P < 0.0001$ *vs* pregnant and control groups; Fig. 1). haCBG concentrations in women taking the COCP were higher than in control subjects (802 ± 41 nmol/L; $P < 0.0001$) but similar to that in pregnant women ($P = 0.18$). The COCP group also had higher concentrations of laCBG than both pregnant women and control subjects (292 ± 27 *vs* 151 ± 22, $P < 0.0001$; and 203 ± 14 nmol/L, $P = 0.03$, respectively). Percentage of haCBG to total CBG was higher in COCP

Table 1. Baseline Characteristics

	Pregnancy (n = 30)	COCP (n = 27)	Healthy Control Subjects (n = 23)
Age, y	31.9 (4.9)	24.0 (6.0)[a]	36.0 (8.8)
Gravity	2.4 (1.5)		
Parity	1.2 (1.2)		
Mode of delivery[b]			
NVD	12		
LSCS	9		
Gestational age at enrolment, wk	34.2 (4.5)		
Weight at birth, g[c]	3329 (488)		

Data given as mean (standard deviation) unless otherwise indicated.
Abbreviations: LSCS, lower segment cesarean section; NVD, normal vaginal delivery.
[a]$P < 0.05$ when compared with control subjects.
[b]Data missing for nine patients.
[c]Data missing for 11 patients.

takers compared with that in control subjects (73.1% ± 2.1%; $P < 0.0001$) but lower compared with pregnant women ($P = 0.006$).

C. Cortisol in Pregnancy

Total cortisol and free-cortisol concentrations were increased in pregnancy compared with those in control subjects ($P < 0.0001$ and $P = 0.017$, respectively; Table 2). The fraction of free cortisol was also increased in pregnant women ($P = 0.0003$).

There was no correlation between CBG affinity-form concentrations and maternal age or gestational age. There was a trend toward higher total cortisol and free-cortisol levels with later gestational age ($P = 0.052$, $r = 0.36$; and $P = 0.071$, $r = 0.33$, respectively). Total CBG level correlated with total cortisol ($P = 0.005$; $r = 0.5$) and free-cortisol levels ($P = 0.014$; $r = 0.440$), and with the free-cortisol fraction ($P = 0.018$; $r = -0.43$).

D. Cortisol and the COCP

Compared with control subjects, women taking the COCP had higher total cortisol, free cortisol, and fraction of free-cortisol levels ($P = 0.0004$, $P < 0.0001$, and $P < 0.0001$, respectively; Table 2). Total cortisol concentrations were no different compared with those in pregnant women, but free-cortisol level and the fraction of free cortisol were higher in women taking the COCP ($P = 0.003$ and $P < 0.0001$, respectively).

Total CBG correlated with total cortisol ($P = 0.002$; $r = 0.58$) and free cortisol ($P = 0.008$; $r = 0.5$). haCBG correlated with total cortisol ($P = 0.021$; $r = 0.44$).

3. Discussion

It is known that total CBG concentrations are elevated in pregnancy [1–5, 7, 9]. We show that this is due to increased uncleaved haCBG. In contrast, COCP use was associated with a comparable rise in haCBG to that during pregnancy; however, cleaved laCBG was also increased. This differential effect on haCBG and laCBG concentrations in pregnancy and the COCP group were consistent with an estrogen-mediated increase in CBG synthesis in both pregnancy and in the COCP group, but with reduced CBG cleavage in pregnancy relative to the COCP group. This may be due to pregnancy-induced CBG glycosylation [20–23].

The threefold elevation in circulating haCBG-cortisol in pregnancy may have a preparatory role to help deal with the risk of puerperal infection. This may be advantageous in reducing sepsis progression, because we have shown that high haCBG concentrations are associated with better prognosis in the cascade of sepsis to septic shock to death in humans [14]. CBG also binds progesterone ($K_a = 0.26 \times 10^{-8}$), which increases 200- to 300-fold during pregnancy and
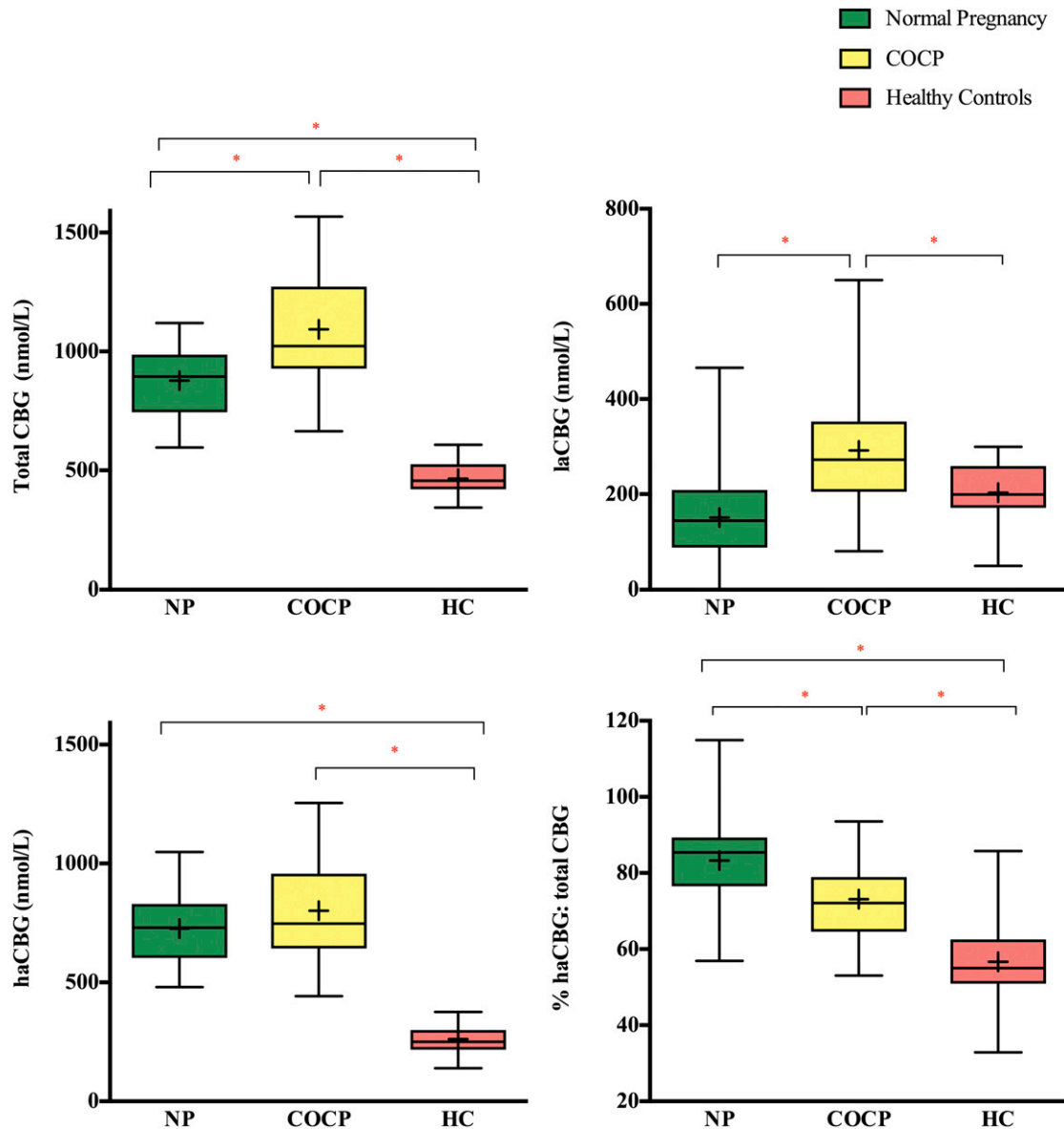
Figure 1. Total CBG and haCBG levels and percentage of haCBG to total CBG were higher in pregnant women (n = 30) than in control subjects (n = 23). Women taking the COCP (n = 27) had the highest total CBG, haCBG, and laCBG levels. *$P < 0.05$ between two groups. The line in each box marks the median; + indicates the mean. Each box extends from the 25th to 75th percentiles; whiskers extend from maximum to minimum. HC, healthy control subjects; NP, normal pregnancy.

can displace cortisol [2, 6, 31, 32]. Thus, higher levels of haCBG may be necessary to overcome binding competition. The cortisol-progesterone CBG interaction is particularly important at the materno-fetal interface. Here the progesterone-to-cortisol ratio is 75 times higher than in the maternal circulation; thus, intervillous CBG is heavily progesterone laden [33]. Elevated haCBG may also provide a buffer against the increased cortisol concentrations in pregnancy, protecting maternal tissues from excessive free-cortisol exposure.

Estrogen is likely to account for increased total CBG secretion in pregnancy and COCP takers, due to a direct effect on hepatocyte CBG production [34] and perhaps from other sites where CBG gene expression has been determined, including endometrium, ovary, fallopian tube, and placenta [35–38]. Estrogen may also account for sex differences in CBG concentrations: Women have 10% higher total CBG concentrations than men [39–41].

Table 2. Cortisol Levels

	Pregnancy (n = 30)	COCP (n = 27)	Healthy Control Subjects (n = 23)
Total cortisol, nmol/L	656 (58)[a]	621 (51)[a]	319 (34)
Free cortisol, nmol/L	33.8 (3.0)[a]	49.7 (4.1)[a]	20.0 (2.9)
Ratio of free to total cortisol	5.2 (0.1)[a]	8.0 (0.1)[a]	6.0 (0.0)

Data given as mean ± SEM.
[a]$P < 0.05$ when compared with control subjects.

The absence of a corresponding rise in laCBG concentration in pregnancy compared with COCP takers, despite a comparable increase in haCBG concentration, suggests reduced CBG cleavage in pregnancy. This was apparent despite a mild inflammatory state being present in pregnancy [42, 43]. We suggest reduced CBG cleavage in pregnancy may be due to pregnancy-specific glycosylation. The glycosylation site at Asp^{347} within the reactive center loop cleavage site is normally 80% glycosylated [25]. If specifically hyperglycosylated in pregnancy, it may reduce CBG cleavage.

CBG displays substantial carbohydrate microheterogeneity [34] and, during pregnancy, enhanced oligosaccharide branching is well recognized [20]. The classically described pregnancy-specific variant, containing only triantennary oligosaccharide chains, constitutes 7% to 14% of circulating CBG in pregnancy [44]. Altered CBG glycosylation is thought to affect the interaction of CBG with specific membrane receptors [25, 26, 45]. Specifically, pregnancy-specific variant CBG has a significantly higher binding affinity for syncytiotrophoblast cell membranes than nonpregnant CBG, perhaps facilitating the delivery of maternal cortisol to the fetus or participating in the regulation of placental corticotropin-releasing hormone production [20, 44]. *In vitro* stimulation of HepG2 cells with estradiol causes an increase in acidic glycoforms of CBG, approximating changes seen in pregnancy [46]. However, reports of *in vivo* examination of glycoforms present in women exposed to exogenous estrogen, using mass spectrometric techniques [25], are not available.

The maternal HPA axis has an important impact on the fetal HPA axis, with consequent effects in adult life [47, 48]. A CBG mutation, CBG A51V, common in Han Chinese, results in a 30% reduction in circulating total CBG concentrations during pregnancy and is associated with a female-skewed sex ratio in offspring [49]. These observations suggest that CBG elevations in pregnancy are important and, when lacking, may affect fetal survival.

Free cortisol was elevated in both pregnant and COCP groups in this study. However, previous studies using the gold standard of 24-hour urine free-cortisol excretion found no increase in free-cortisol concentrations in women taking the COCP [4]. The single cortisol measurement used here was not intended to provide a measurement of overall cortisolemia in these patients but to determine whether a change in haCBG concentrations related to a change in the free-cortisol fraction, which it did not. We believe the elevated free-cortisol concentrations and free-cortisol fractions seen in young women taking the COCP were an artificial stress effect of venipuncture rather than a reflection of true hypercortisolemia. This effect has been well documented, with up to one-third of patients experiencing a cortisol response to venipuncture [50, 51]. A rest period after intravenous catheter placement may have avoided artifactual hypercortisolemia.

There are data suggesting that CBG can influence the fast actions of cortisol in the brain [52] and CBG has been found in human hypothalamus and cerebrospinal fluid [53, 54]. Hence, CBG may influence feedback in pregnancy; however, to our knowledge, there are no published data on this and further investigation is required.

In conclusion, the rise in total CBG concentrations during pregnancy was due to increased haCBG. The rise in haCBG concentrations in the COCP takers was comparable to that seen in pregnancy; however, laCBG concentration was also increased. These results are consistent with an estrogen-mediated increase in CBG synthesis in both pregnancy and in women taking the COCP, but with reduced CBG cleavage in pregnancy relative to that in women taking the

COCP. Increased circulating haCBG concentrations in pregnancy may provide an increased reservoir of CBG-bound cortisol in preparation for the risk of puerperal infection, or they may allow for cortisol binding in the face of competition from high circulating progesterone concentrations.

Acknowledgments

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