A Longitudinal Study of Plasma and Urinary Cortisol in Pregnancy and Postpartum

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Context: There is a paucity of longitudinal data on plasma and urinary cortisol levels during pregnancy using modern assays. Furthermore, conflicting data exist as to the effect of the low-dose oral contraceptive pill (OCP) on cortisol.

Design, Subjects, and Measurements: We conducted a prospective longitudinal study on morning plasma cortisol (total and free), corticosteroid-binding globulin (CBG), and 24-h urinary free cortisol (UFC) levels in 20 pregnant women during the first, second, and third trimesters and 2–3 months postpartum compared with 12 subjects on low-dose OCP and 15 nonpregnant subjects not taking the OCP (control group).

Results: A progressive rise in total plasma cortisol, CBG, and 24-h UFC was demonstrated during pregnancy, peaking during the third trimester (mean 3-fold rise compared with controls). Plasma free cortisol increased 1.6-fold by the third trimester. In the OCP group, total plasma cortisol and CBG were 2.9- and 2.6-fold elevated, respectively, whereas 24-h UFC and plasma free cortisol were not significantly different from controls. Compared with liquid chromatography-mass spectrometry, a commercial immunoassay underestimated mean total plasma cortisol concentrations by 30% during second and third trimesters and in OCP users and overestimated UFC levels by 30–35% during pregnancy.

Conclusions: Our study demonstrated elevations in total plasma cortisol and CBG concentrations during pregnancy and with low-dose OCP use. Pregnancy was also associated with significant increases in plasma free cortisol and UFC, suggesting that the rise in total plasma cortisol is contributed to by up-regulation of the maternal hypothalamic-pituitary-adrenal axis in addition to elevated CBG. **(*J Clin Endocrinol Metab* 96: 1533–1540, 2011)**

Normal human pregnancy dramatically affects the maternal hypothalamic-pituitary-adrenal (HPA) axis (1), resulting in progressive rises in CRH (2–4), ACTH (5, 6), and cortisol (6–12). It has been suggested that the increase in total plasma cortisol concentration is primarily due to the estrogen-stimulated increase in corticosteroid-binding globulin (CBG) concentrations (1, 8), whereas the increase in free cortisol reflects alterations in the regulation of the maternal HPA axis (13). Previous studies have demonstrated a 2- to 3-fold increase in cortisol levels during the second and third trimesters of pregnancy (6–12). However, there is a paucity of longitudinal studies that have evaluated both plasma and urinary cortisol levels in the same group of pregnant women from early pregnancy to postpartum using modern assays.

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Abbreviations: CBG, Corticosteroid-binding globulin; HPA, hypothalamic-pituitary-adrenal; LCMSMS, liquid chromatography-tandem mass spectrometry; OCP, oral contraceptive pill; UFC, urinary free cortisol.

TABLE 1. Clinical characteristics of control, pregnancy, and OCP groups

Characteristic	Control (n = 15)	Pregnancy (n = 20)	OCP (n = 12)
Age (yr)[a]	36.7 ± 1.7	33.3 ± 0.7	30.7 ± 1.3[b]
BMI (kg/m^2)[a]	26.5 ± 1.8	22.9 ± 0.9[c]	24.0 ± 1.2
Preexisting medical illness (n)	1 participant had hypertension controlled on medication	0	0
Singleton pregnancy (%)	N/A	100	N/A
Gestational age at which samples were collected (wk)[a]			
T1	N/A	12 ± 0.4	N/A
T2		21 ± 0.4	
T3		32 ± 0.4	
PP		11 ± 0.3	
Gestational age at delivery (wk)[a]	N/A	39.7 ± 0.4	N/A
Birth weight (kg)[a]	N/A	3.6 ± 0.1	N/A
Breast-feeding at the time of postpartum sample (%)	N/A	95	N/A

[a] Values are represented as mean ± SEM. N/A, Not applicable; PP, postpartum; T1, first trimester; T2, second trimester; T3, third trimester.

[b] $P < 0.05$ *vs.* control group.

[c] Body mass index (BMI) during the first trimester.

It has been long known that exogenous estrogens administered alone or in combination with progestin in the oral contraceptive pill (OCP) increase CBG (14, 15) and total plasma cortisol (11, 14, 16) concentrations. However, conflicting data exist regarding the effect of low-dose OCP containing 35 μg or less of ethinylestradiol or equivalent on cortisol. Some studies report similar total plasma cortisol concentrations compared with control subjects (12, 16), whereas other studies have found elevation in total cortisol concentrations (17, 18).

We conducted a prospective longitudinal study on plasma cortisol (total and free), CBG. and 24-h urinary free cortisol (UFC) in 20 pregnant women compared with 12 women using the low-dose OCP and 15 nonpregnant subjects not taking the OCP (control group). The main aims of the study were to assess cortisol levels in normal pregnancy from first trimester to postpartum and compare these with women using the low-dose OCP. A secondary aim of this study was to compare a commercially used automated immunoassay with liquid chromatography-tandem mass spectrometry (LCMSMS) in the measurement of total plasma cortisol and UFC in pregnancy. Our hypothesis was that there is up-regulation of the HPA axis across pregnancy and that the increase in total cortisol is not solely due to elevated CBG concentrations.

Subjects and Methods

Subjects and study design

This study was conducted at St. Vincent's Hospital (Melbourne, Australia) between 2006 and 2009. The study protocol was approved by the St. Vincent's Hospital Human Research Ethics Committee, and informed consent was obtained from all subjects.

Three groups of subjects were recruited by advertisement from the general community and were studied as outpatients: 1) control group, 2) pregnancy group, and 3) OCP group. The control group consisted of 15 premenopausal women (age range 18–45 yr) who were not pregnant and not taking exogenous estrogens. The pregnancy group consisted of 20 healthy pregnant women who were followed longitudinally from first trimester to postpartum; these women had uncomplicated pregnancies and delivered at term. The OCP group consisted of 12 women (age range 18–45 yr) who had been taking the low-dose combined OCP, containing no more than 35 μg ethinylestradiol for at least 3 months. The clinical characteristics of the three groups are shown in Table 1. Exclusion criteria for all three groups were preexisting Cushing's syndrome, adrenal insufficiency, psychiatric disorders, alcoholism, creatinine clearance less than 60 ml/min, and the use of glucocorticoid medication. We excluded women who developed pregnancy-related complications, such as gestational hypertension (n = 1), gestational diabetes (n = 1), and preeclampsia (n = 1).

Each subject in the control and OCP groups had one blood test between 0800 and 0900 h and completed one 24-h urine collection. Samples were collected from the control group during the follicular phase from d 7–14 and from the OCP group during the active pill-dosing phase from d 7–21. Samples were collected from the pregnancy group during the following four stages: 1) first trimester from 8–14 wk, 2) second trimester from 18–24 wk, 3) third trimester from 30–36 wk, and 4) 2–3 months postpartum. Eighteen pregnant subjects (90%) had a blood test between 0800 and 0900 h and completed one 24-h urine collection at each of the four stages. One pregnant subject (5%) had blood tests only and declined urine collections. One pregnant subject (5%) withdrew from the study after the second stage. All the available data from the pregnancy group were included for analysis.

Plasma was separated within 1 h of collection and was stored at −70 C until assay for total cortisol, free cortisol, CBG, and creatinine concentrations. The 24-h urine volumes were measured, and urine was stored at −20 C until assay for urine cortisol concentration (nanomoles per liter), excretion rate (nanomoles per day), cortisone, and creatinine. Samples from individual

pregnant subjects were measured in the same assay to eliminate interassay variation.

Assays

Total plasma cortisol was measured by two methods: an in-house LCMSMS assay and a commercially available immunoassay (ADVIA Centaur; Siemens Healthcare Diagnostics, Deerfield, IL). The preparation of sample for cortisol measurement by LCMSMS involved the following steps: 50 μl of 1 μmol/liter d4-cortisol in 50% methanol was added to all samples and calibration standards before extraction with *tert*-butylmethyl-ether and vigorously vortexed 10 times for 10 sec each. After separation of the phases by centrifugation for 5 min at 13,000 × *g*, the organic phase was transferred to a fresh tube and evaporated at room temperature. The dried residue was redissolved in 150 μl 50% methanol in water and centrifuged for 5 min at 13,000 × *g* to remove insoluble material. The measurement of cortisol by LCMSMS involved the following steps: 50 μl of reconstituted sample was injected onto a 50- × 2-mm Phenomenex Hypersil BDS C8 3-μm HPLC column with a C8 guard column. HPLC was performed with an Agilent 1200 instrument and mobile phase of 50:50 methanol/formic acid (0.2%) at 0.3 ml/min with a column temperature of 30 C. An API 3200 Q Trap mass spectrometer (Applied Biosystems, Scoresby, Australia) was used with electrospray in positive mode for detection of mass transitions mass to charge ratio of 363–121 for cortisol and 367–121 for d4-cortisol. Quantitation was carried out from peak area ratios using Analyst version 1.4 software. The imprecision of the method (coefficient of variation) was 6.2% at a cortisol concentration of 70 nmol/liter, 3.4% at 360 nmol/liter, and 4.6% at 1000 nmol/liter. The intra- and interassay coefficients of variation for ADVIA Centaur immunoassay were less than 4 and 6%, respectively, and the analytical sensitivity was 5.5 nmol/liter. To evaluate the possibility of interference in this immunoassay, five samples from third trimester and OCP group were assayed before and after heat treatment at 60 C for 60 min to inactivate cortisol-CBG binding as previously described (19).

Plasma CBG was measured by a monoclonal ELISA as previously described (20).

Urine cortisol concentration (nanomoles per liter) was measured by two methods: LCMSMS as described above and a commercially available immunoassay (Immulite 2000; Siemens) after dichloromethane extraction. The intraassay coefficient of variation for the immunoassay was less than 8%. UFC excretion rate (nanomoles per day) was calculated by multiplying urine cortisol concentration by 24-h urine volume. Urine cortisone was measured by LCMSMS as described above.

Plasma free cortisol was isolated at 37 C using a commercial equilibrium dialysis kit [Pierce (Rockford, IL) rapid equilibrium dialysis device inserts (catalog item 89810)], and measured by LCMSMS as described above. Equilibrium dialysis involved the following steps: 200 μl plasma was dialyzed against 350 μl PBS (pH 7.4) for 6 h at 37 C with orbital shaking at 100 rpm, and 100 μl retained sample and 200 μl dialysate were removed for analysis. Plasma free cortisol was calculated using the following equation: free cortisol = total cortisol × dialysate cortisol/retentate cortisol.

Statistical analysis

Results are presented as the mean ± SEM. For statistical purposes, the value corresponding to the limit of detection of assays was used for undetectable concentrations. The unpaired Student's *t* test was used for comparisons between two groups. ANOVA was used for comparisons of three or more groups with *post hoc* analysis carried out by Tukey's multiple-comparison test. Statistical significance was taken as $P < 0.05$. Statistical analyses were performed using Prism version 4.0 (GraphPad, San Diego, CA).

Results

Total plasma cortisol measured by LCMSMS

A progressive rise in morning total plasma cortisol was demonstrated during pregnancy ($P < 0.0001$ by ANOVA, Table 2 and Fig. 1A), peaking during the third trimester, and the differences between trimesters were statistically significant ($P < 0.05$). The mean total cortisol concentrations measured by LCMSMS were 1.6-, 2.4-, and 2.9-fold elevated during the first, second, and third trimesters, re-

TABLE 2. Total plasma cortisol, CBG, plasma free cortisol, and 24-h UFC levels (mean ± SEM) in the control, pregnancy, and OCP groups

Group	Total plasma cortisol by LCMSMS (nmol/liter)	CBG (nmol/liter)	Plasma free cortisol (nmol/liter)	24-h UFC excretion by LCMSMS (nmol/d)	24-h urine concentration by LCMSMS (nmol/liter)	24-h urine volume (liters/d)
Control group	364 ± 28	375 ± 19	12.2 ± 1.6	78 ± 12	46 ± 9	1.97 ± 0.2
Pregnancy group						
T1	577 ± 30[a]	700 ± 43[a]	14.5 ± 1.2	135 ± 10[a]	81 ± 9[a]	1.99 ± 0.2
T2	878 ± 28[a]	920 ± 43[a]	17.2 ± 1.4[a]	187 ± 13[a]	87 ± 8[a]	2.36 ± 0.2
T3	1043 ± 41[a]	1107 ± 52[a]	19.6 ± 1.5[a]	242 ± 15[a]	121 ± 13[a]	2.27 ± 0.2
PP	486 ± 38[a]	657 ± 43[a]	12.3 ± 2.0	75 ± 9	43 ± 5	2.09 ± 0.2
OCP group	1048 ± 61[a]	973 ± 55[a]	16.8 ± 1.5	114 ± 15	66 ± 11	1.93 ± 0.2

Results are expressed in Système International (SI) units. To convert plasma cortisol from the SI unit (nanomoles per liter) to the metric unit (micrograms per deciliter), divide by the conversion factor 27.59. To convert CBG from the SI units (nanomoles per liter) to the metric unit (milligrams per liter), divide by 19.18. To convert UFC from the SI unit (nanomoles per day) to the metric unit (micrograms per 24 h), divide by 2.76. PP, Postpartum; T1, first trimester; T2, second trimester; T3, third trimester.

[a] $P < 0.05$ *vs.* control group.

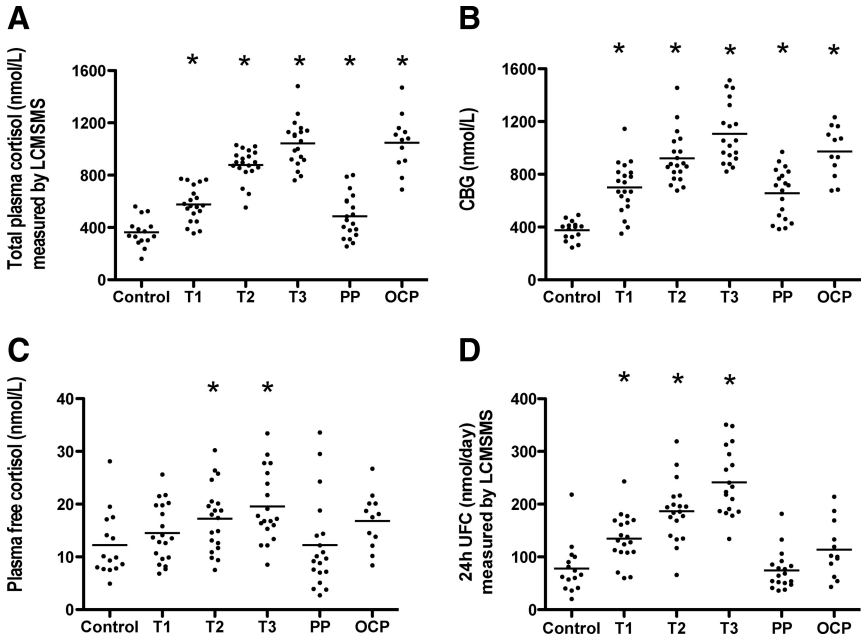

FIG. 1. Total plasma cortisol (nanomoles per liter) (A), CBG (nanomoles per liter) (B), plasma free cortisol (nanomoles per liter) (C), and 24-h UFC (nanomoles per day) (D) levels in the control group, first trimester (T1), second trimester (T2), third trimester (T3), 2–3 months postpartum (PP), and OCP group. *, $P < 0.05$ *vs.* control group. To convert from the SI unit to the metric unit, divide by the conversion factor 27.59 for plasma cortisol, 19.18 for CBG, and 2.76 for 24-h UFC.

spectively, compared with the control group. Postpartum, the mean total cortisol concentration was lower compared with values obtained during pregnancy but higher compared with the control group ($P = 0.02$). In the OCP group, the mean total cortisol concentration was 2.9-fold elevated compared with the controls ($P < 0.0001$).

Total plasma cortisol measured by immunoassay

The mean total plasma cortisol concentrations by immunoassay were not significantly different from those by LCMSMS in the control group ($P = 0.37$) or during the postpartum period ($P = 0.15$). However, compared with LCMSMS, total plasma cortisol by immunoassay was 15% lower in the first trimester ($P = 0.02$) and 30% lower in the second ($P < 0.0001$) and third ($P < 0.0001$) trimesters and in the OCP group ($P = 0.0003$, Fig. 2A). Table 3 shows total plasma cortisol concentrations by immunoassay before and after the heat treatment compared with LCMSMS in a representative sample of five subjects in each group. After heat treatment, cortisol concentrations by immunoassay were similar to those obtained by LCMSMS (third trimester, $P = 0.8$; OCP, $P = 0.9$).

CBG

A progressive rise in CBG was demonstrated during pregnancy ($P < 0.0001$ by ANOVA, Table 2 and Fig. 1B), and the differences were significant between the trimesters. The mean CBG concentrations were 1.9-, 2.5-, and 3.0-fold elevated during the first, second, and third trimesters, respectively, compared with the control group. CBG concentrations remain elevated 2–3 months postpartum and 1.8-fold higher compared with the control group ($P < 0.0001$). In the OCP group, the mean CBG concentration was 2.6-fold elevated compared with the control group.

Plasma free cortisol

Plasma free cortisol concentrations increased during pregnancy ($P = 0.0054$ by ANOVA, Table 2 and Fig. 1C). The mean concentrations were 1.2-, 1.4-, and 1.6-fold elevated during the first, second, and third trimesters, respectively, compared with the control group. There was a wide spread of results within the groups, and in *post hoc* analysis, the between-group differences reached significance only between the controls and third trimester. Postpartum, plasma free cortisol concentrations were similar to controls ($P = 0.996$). There was a trend toward higher plasma free cortisol concentrations in the OCP group that bordered on statistical significance ($P = 0.05$).

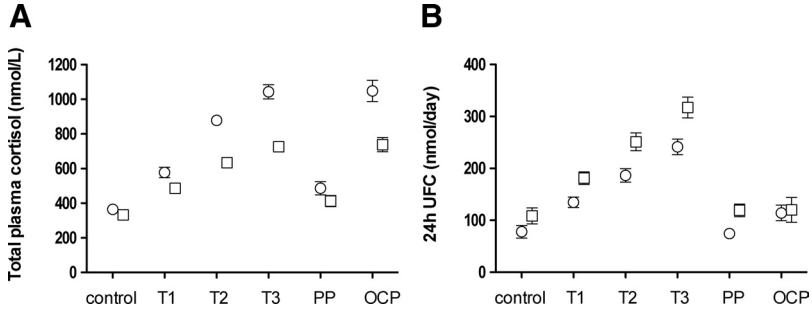

FIG. 2. Mean (± SEM) total plasma cortisol concentrations (nanomoles per liter) (A) and 24-h UFC excretion (nanomoles per day) (B) measured by LCMSMS (○) and immunoassay (□). To convert from the SI unit to the metric unit, divide by the conversion factor 27.59 for plasma cortisol and 2.76 for 24-h UFC. PP, Postpartum; T1, first trimester; T2, second trimester; T3, third trimester.

UFC and cortisone measured by LCMSMS

UFC excretion rates (nanomoles per day) and concentrations (nanomoles per liter) increased across the gestation ($P < 0.0001$ and $P < 0.0001$, respectively, by ANOVA, Table 2 and Fig 1D), reaching peak values

TABLE 3. Total plasma cortisol concentrations before and after treatment with heat measured by ADVIA Centaur immunoassay and total plasma cortisol concentration measured by LCMSMS

Participant	Total plasma cortisol (nmol/liter) before heat treatment by immunoassay	Total plasma cortisol (nmol/liter) after heat treatment by immunoassay	Total plasma cortisol (nmol/liter) by LCMSMS
Third trimester			
1	876	1256	1270
2	690	1173	1130
3	638	1346	1200
4	551	1149	1110
5	641	970	1100
Mean	680	1179	1162
OCP group			
1	570	934	1110
2	536	953	911
3	653	1155	1070
4	908	1313	1470
5	563	1017	898
Mean	646	1074	1092

during the third trimester. The mean 24-h UFC excretion rates were 1.7-, 2.4-, and 3.1-fold elevated during the first, second, and third trimesters, respectively, compared with the control group. The mean 24-h UFC postpartum and in the OCP group were not significantly different from controls ($P = 0.8$ and $P = 0.08$, respectively). There were no differences in 24-h urine volumes ($P = 0.6$ by ANOVA) or creatinine excretion ($P = 0.2$ by ANOVA) either across the gestation or compared with the control group. Urinary cortisone excretion increased during pregnancy to peak during the third trimester (475 nmol/d), whereas levels were similar between OCP (220 nmol/d) and control (207 nmol/d) groups ($P=0.76$).

UFC measured by immunoassay

Compared with LCMSMS, the mean UFC excretion rates measured by immunoassay were 30–35% higher in the first ($P = 0.007$), second ($P = 0.005$), and third ($P = 0.005$) trimesters and 60% higher during the postpartum period ($P = 0.006$, Fig. 2B). The UFC excretion by immunoassay was not significantly different from LCMSMS in the OCP group ($P = 0.8$) or the controls ($P = 0.13$, Fig. 2B).

Discussion

We conducted a prospective longitudinal study measuring cortisol and CBG levels during pregnancy and postpartum compared with nonpregnant subjects and women taking the OCP. The major findings are 1) maternal total plasma cortisol, CBG, plasma free cortisol, and 24-h UFC increased in normal pregnancy to reach peak levels during the third trimester; 2) total plasma cortisol and CBG remained elevated 2–3 months postpartum compared with the control group, whereas UFC and plasma free cortisol returned to baseline; 3) women taking the OCP had higher total plasma cortisol and CBG but similar 24-h UFC and plasma free cortisol levels compared with the control group; 4) compared with LCMSMS, a commercial immunoassay underestimated mean total plasma cortisol concentrations during pregnancy and in OCP users that corrected after heat treatment; and 5) immunoassay yielded higher UFC than LCMSMS during pregnancy.

Our study demonstrated a statistically significant increase in plasma total cortisol by the late first trimester (mean 12 wk gestation) compared with control subjects, consistent with a previous cross-sectional study that demonstrated a rise in total cortisol above nonpregnant values after the 11th week of gestation (8). Previous studies have reported a 2- to 3-fold increase in total plasma cortisol concentrations during second and third trimesters (6–10), similar to our findings. Two reported a plateau in total plasma cortisol concentrations between second and third trimesters (6, 7), whereas larger longitudinal studies (9, 10), including the present study, have provided more convincing data demonstrating a progressive rise in total plasma cortisol concentrations with advancing gestation. Our study also confirms a progressive rise in plasma CBG concentrations during pregnancy in parallel to the rise in total plasma cortisol, consistent with previous reports (9, 10).

In addition to increases in CBG and total plasma cortisol, our study also demonstrated significant increases in urinary and plasma free cortisol levels indicating up-regulation of the HPA axis during pregnancy. Previous data on maternal free cortisol levels were largely obtained from cross-sectional studies (8, 11, 13, 21), with few exceptions (7, 9). Ho *et al.* (9) reported a progressive rise in plasma

free cortisol by 1.8-fold from 16–36 wk gestation in healthy pregnant women. Cousins *et al.* (7) reported a 2.8-fold increase in the 24-h urinary free corticoid levels by RIA in eight women during the second and third trimesters compared with their postpartum results. Our study followed 19 women from the first trimester and found a progressive increase in 24-h UFC excretion with advancing gestation, reaching a 3.1-fold increase in the third trimester. We measured urine volumes and creatinine excretion to demonstrate adequacy of urine collections that was not documented in previous studies on UFC during pregnancy (7, 11). Furthermore, we demonstrated that increases in 24-h UFC excretion rates were not related to changes in urine volumes that were similar across the gestation, consistent with previous literature on osmoregulation during pregnancy (22). We found a proportionally greater increase in UFC than in plasma free cortisol during pregnancy. One possible explanation for this is that metabolic clearance of cortisol is increased in pregnancy. Thus, to achieve an increase in plasma free cortisol concentration, a proportionally greater increase in cortisol production is required.

There are a number of mechanisms that have been proposed to explain the rise in free cortisol during pregnancy. Previous studies have demonstrated increases in plasma CRH and ACTH concentrations during second and third trimesters (2, 6), possibly as a result of CRH and ACTH production by the placenta (23, 24), which is autonomous and not subject to normal glucocorticoid feedback control (1, 6). In contrast to the negative feedback effects of cortisol on hypothalamic CRH, cortisol stimulates placental CRH release, resulting in a positive feedback loop that is terminated by delivery (25). In addition, the adrenal glands have increased responsiveness to ACTH during pregnancy compared with nonpregnant women, as demonstrated by a greater rise in the unbound cortisol in response to synthetic ACTH, which increased as pregnancy advanced (13). Another possible explanation for the elevation in free cortisol is that pregnancy represents a state of refractoriness to cortisol action, resulting in resetting of the HPA axis at a higher level (13).

The increase in free cortisol during pregnancy has important clinical consequences. During the final weeks of pregnancy, a substantial fall in plasma CBG with corresponding rise in plasma free cortisol occurs (9), which was not assessed in our study because the third-trimester samples were collected before 36 wk gestation. Physiologically, the rise in free cortisol in late pregnancy may be important for preparing the mother for the metabolic demands of pregnancy and labor and for growth and organ maturation in the fetus (9, 26). By term, 25% of circulating fetal cortisol is of maternal origin (27) despite the action of placental 11β-hydroxysteroid dehydrogenase type 2 enzyme, which inactivates cortisol to cortisone (28). In the investigation of possible cortisol excess during pregnancy, the differentiation between physiological hypercortisolism and Cushing's syndrome during pregnancy can be difficult, highlighting the importance of using trimester-specific reference ranges (26).

During the postpartum period, the HPA axis gradually recovers from its activated state during pregnancy (29). Previous studies have shown that CRH and ACTH decrease within 2 h of delivery, and cortisol concentrations normalize within 1 wk (30). There are inconsistent data on the rate of normalization of CBG concentrations postpartum, with some studies reporting normal CBG concentrations by 3–6 wk postpartum (12, 31), whereas another study found that it took about 3 months or longer for CBG to fall within the nonpregnant range (32). Our study found that compared with nonpregnant control subjects, total plasma cortisol and CBG concentrations remain elevated 2–3 months postpartum, which was not explained by use of exogenous estrogens because the majority of participants (95%) were breastfeeding at the time of postpartum sample and not taking the combined OCP. One possible explanation for the raised CBG concentrations postpartum is the presence of pregnancy-specific CBG, an acidic glycoform containing only triantennary oligosaccharides, which accounts for 7–14% of total CBG concentration at term (33). Previous data suggest that either the half-life of the pregnancy-specific CBG in the circulation is significantly longer than that of normal CBG (about 5 d) (34) or its biosynthesis continues for some period of time after delivery (33). Despite an increase in CBG, the finding of normal urinary and plasma free cortisol concentrations in our study indicate that there is no decrease in tissue exposure to free cortisol 2–3 months postpartum compared with the control state.

Previous studies using combined OCP containing at least 50 μg ethinylestradiol (or equivalent) have demonstrated 2- to 3-fold elevation in CBG (14) and total plasma cortisol concentrations (11, 14, 16). However, there are conflicting data regarding the effect of low-dose OCP containing no more than 35 μg ethinylestradiol (12, 16–18). In our study, we found an increase in total plasma cortisol and CBG in the OCP group similar in magnitude to pregnant women in the third trimester. Similar to results of one previous study (16), we found normal urinary and plasma free cortisol levels, indicating that low-dose OCP has no stimulatory effect on the HPA axis. This is consistent with the observation that OCP users do not exhibit clinical features of Cushing's syndrome, even though their concentrations of total plasma cortisol can be as high as those in patients with Cushing's syndrome (35).

Measurement of cortisol by structurally based assays such as LCMSMS is considered to be the gold standard because it is not as subject to interference as some immunoassays. Compared with mass spectrometry, immunoassays overestimate UFC concentrations by 1.5- to 2.0-fold (36), particularly in unextracted samples that contain cross-reacting cortisol metabolites. Although UFC was measured in our study after extraction, we found higher UFC levels by immunoassay compared with LCMSMS in the pregnancy group, which raises the possibility of interference of immunoassay by higher concentrations of cortisol metabolites. We found increased urinary cortisone levels during pregnancy compared with the control subjects. Other metabolites such as dihydro- and tetrahydrocortisol were not assessed and warrant further investigation in future studies. In contrast to the UFC data, measurement by an automated immunoassay underestimated total plasma cortisol concentrations during the second and third trimesters of pregnancy and in the OCP group. High CBG levels during pregnancy and in OCP users may interfere with antibody binding to cortisol, thus resulting in falsely low plasma cortisol concentrations by immunoassay. CBG is heat sensitive, and heat treatment ablated this interference, similar to a previous report (19). However, heat inactivation may also affect other proteins, and the interference in plasma cortisol immunoassay may be due to other substances that have not been specifically identified.

Strengths of the study include its prospective design, sample size, and recruitment of participants from early pregnancy. This is the largest study to date that has assessed both plasma and urinary cortisol levels in a cohort of pregnant women across the gestation and postpartum using modern assays. To obtain cortisol data from normal, uncomplicated pregnancies, we recruited healthy women with no preexisting illnesses and excluded those who developed pregnancy-related complications, such as preeclampsia or gestational hypertension, which may be associated with HPA overactivity from immune activation or, conversely, HPA underactivity from placental dysfunction (9). The control and OCP groups provided robust data for comparison. We collected plasma samples within a specified time between 0800 and 0900 h to minimize the effect of the expected diurnal fall in plasma cortisol. Plasma free cortisol was measured using equilibrium dialysis at 37 C to mitigate the effect of temperature on protein-binding equilibrium (37). Samples were taken from women taking the OCP during the active dosing phase because total plasma cortisol and CBG concentrations decrease during the pill-free (placebo) interval (18).

Although the mean age of the control group was higher than the OCP group, there was no difference compared with the pregnancy group. However, there are no data to suggest that cortisol concentrations change with age either in pregnant or nonpregnant individuals (9, 38).

Conclusion

Our study demonstrated a rise in plasma free cortisol during pregnancy, indicating up-regulation of the maternal HPA axis. Pregnancy was also associated with greater increases in UFC, reflecting the increases in both cortisol production and clearance during pregnancy. Total plasma cortisol and CBG increased during pregnancy and with low-dose OCP use. Given our findings, we recommend that pregnancy gestation-specific reference ranges for plasma cortisol and UFC need to be developed, and the use of exogenous oral estrogens must be considered in the interpretation of total plasma cortisol concentrations in nonpregnant women.

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