

ORIGINAL ARTICLE

Salivary cortisol determined by enzyme immunoassay is preferable to serum total cortisol for assessment of dynamic hypothalamic–pituitary–adrenal axis activity

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Summary

Objective The aim of this study was to determine whether salivary cortisol measured by a simple enzyme immunoassay (EIA) could be used as a surrogate for serum total cortisol in response to rapid changes and across a wide range of concentrations.

Design Comparisons of matched salivary and serum samples in response to dynamic hypothalamic–pituitary–adrenal (HPA) axis testing.

Subjects Healthy women ($n = 10$; three taking oral oestrogens) and men ($n = 2$), aged 23–65 years, were recruited from the community.

Measurements Paired saliva and serum samples were obtained during three protocols: 10 min of exercise at 90% of maximal heart rate ($n = 8$), intravenous administration of corticotrophin-releasing hormone (CRH; $n = 4$), and dexamethasone suppression ($n = 7$). Cortisol was measured in saliva using a commercial high-sensitivity EIA and total cortisol was measured in serum with a commercial radioimmunoassay (RIA).

Results The time course of the salivary cortisol response to both the exercise and CRH tests paralleled that of total serum cortisol. Salivary cortisol demonstrated a significantly greater relative increase in response to the exercise and CRH stimuli ($697 \pm 826\%$ vs. $209 \pm 150\%$, $P = 0.04$ saliva vs. serum). A disproportionately larger increase in free cortisol, compared with total, would be expected when the binding capacity of cortisol-binding globulin (CBG) is exceeded. In response to dexamethasone suppression, relative decreases in cortisol were not significantly different between the two media ($-47 \pm 56\%$ vs. $-84 \pm 8\%$, $P = 0.13$ saliva vs. serum). Although a significant linear correlation was found for all paired salivary and serum total cortisol samples ($n = 183$ pairs, $r = 0.60$, $P < 0.001$), an exponential model provided a better fit ($r = 0.81$, $P < 0.001$). The linear correlations were strengthened when data from subjects on oral oestrogens ($n = 52$ pairs, $r = 0.75$, $P < 0.001$) were separated from those not taking oestrogens ($n = 131$ pairs, $r = 0.67$, $P < 0.001$).

Conclusions Salivary cortisol measured with a simple EIA can be used in place of serum total cortisol in physiological research protocols. Evidence that salivary measures represent the biologically active, free fraction of cortisol includes: (1) the greater relative increase in salivary cortisol in response to tests that raise the absolute cortisol concentration above the saturation point of CBG; (2) the strong exponential relationship between cortisol assessed in the two media; and (3) the improved linear correlations when subjects known to have increased CBG were analysed separately. Thus, an advantage of measuring salivary cortisol rather than total serum cortisol is that it eliminates the need to account for within-subject changes or between-subject differences in CBG.

(Received 25 February 2005; returned for revision 15 March 2005; finally revised 17 June 2005; accepted 17 June 2005)

Introduction

Cortisol secreted from the adrenal glands is dispersed to all water spaces of the body and can be detected in urine, serum or saliva. The obvious drawback of urinary measures is the inability to assess rapid changes in cortisol levels. Serum measures are often used in clinical and research settings, but the stress of venipuncture itself can increase cortisol.¹ Furthermore, it is not practical to perform serial blood collections throughout normal daily activities. Conversely, salivary samples have the advantage of being easily collected and salivary cortisol is thought to represent only the bioactive fraction, that is, cortisol not bound to cortisol-binding globulin (CBG) or other proteins. With the recent focus on the links between physiological dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis and components of the metabolic syndrome,^{2–4} clinical researchers in this area would benefit from the ability to use a commercially available methodology to easily assess free cortisol levels.

Several researchers have compared salivary cortisol measured by radioimmunoassay (RIA) with serum total cortisol and concluded that saliva is a reliable medium for assessing this hormone under basal and stimulated conditions as well as in response to exogenous glucocorticoid administration.^{5–11} In an attempt to eliminate the

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complexities of dealing with radioactivity, one group adapted a serum cortisol enzyme immunoassay (EIA) kit for use with saliva.¹² Unfortunately, this technique is not practical because it requires a 20-h incubation period. Raff *et al.*¹³ found that basal salivary cortisol values measured by RIA were strongly correlated with a high-sensitivity, commercially available EIA methodology developed exclusively for use with salivary samples ($r = 0.98$, $P < 0.001$). However, we are not aware of any published studies comparing salivary cortisol assessed by this simpler EIA technique with paired samples of serum total cortisol, across a wide range of concentrations, or when cortisol levels are rapidly changing.

The primary aim of the present study was to determine the relationship between salivary cortisol assessed with a commercial EIA kit and serum total cortisol measured at moderate levels (in the late afternoon), during rapidly changing increases and decreases (during and after vigorous treadmill exercise or after corticotrophin-releasing hormone (CRH) administration), and at very low levels (after dexamethasone suppression). A secondary aim was to confirm that salivary cortisol measured by the EIA technique represents the free fraction of serum cortisol.

Materials and methods

Subjects

Twelve healthy volunteers (10 women, two men) aged 44 ± 16 years (range 23–65 years) participated in the study. Among the women, four were premenopausal (two were taking oral oestrogen-containing contraceptives) and six were postmenopausal (one taking oral oestrogen hormone therapy). Subjects were excluded if they had an abnormal exercise stress test or used oral glucocorticoids. All of the subjects provided written informed consent to participate in the study, which was approved by the Colorado Multiple Institutional Review Board.

Exercise protocol

Eight subjects (six women, two men) participated in the exercise protocol, which was designed to provide several time-matched salivary and serum cortisol samples. The test was conducted in the late afternoon when cortisol levels were expected to be moderate and declining. After obtaining basal samples, a 10-min exercise bout at 90% of maximal heart rate (HR_{max}) was used to stimulate a rapid rise in cortisol. Subjects were then monitored for a further 2 h to collect samples while cortisol levels decreased.

Subjects reported to the General Clinical Research Center (GCRC) at 1530 h and rested supine for 20 min. An intravenous catheter was placed in a forearm vein (time point = 0) for serial blood sampling. Subjects rested while blood and saliva were simultaneously collected at 0, 5, 10, 15, 30, 45 and 60 min. Subjects then performed a 5-min warm-up on the treadmill before exercising at 90% of HR_{max} for 10 min. For this exercise bout, the speed and grade of the treadmill were adjusted to achieve and maintain 90% HR_{max} (average percentage of HR_{max} measured during the last 5 min of the exercise bout was $93 \pm 4.5\%$). Blood and saliva samples were collected at the onset, midpoint, and conclusion of exercise (time points = 70, 75

and 80 min) and during a brief exercise recovery period (time points 85 and 90) during which HR and blood pressure responses were monitored. Subjects then returned to quiet supine rest while serum and saliva samples were obtained every 10 min for 40 min and then every 15 min for another 60 min. In total, there were 20 time-matched blood and saliva samples collected over 190 min. For both media, the average of the 45- and 60-min time points was considered the basal pre-exercise value and the average of the 100- and 110-min time points served as the peak exercise value.

Dexamethasone suppression test (DST) protocol

Seven of the subjects (five women, two men) who performed the exercise protocol participated in the DST protocol. Subjects ingested 1 mg of dexamethasone at 2300 h and then presented to the GCRC the following morning at 0800 h in the fasted state. Serum and saliva samples for cortisol were collected 15 and 30 min after placement of an intravenous catheter. The average of these two values was used to calculate the maximal cortisol suppression. Because the timing of the morning acrophase can be strongly influenced by environmental factors,¹⁴ we chose to express the percentage decrease in cortisol after the DST relative to the late afternoon, basal, pre-exercise value. This reference point also ensured that the dynamic change in response to the DST would represent cortisol concentrations with absolute values lower than the binding capacity of cortisol.

Corticotrophin-releasing hormone (CRH) protocol

Four postmenopausal women participated in the CRH protocol. Subjects presented to the GCRC at 0700 h in the fasted state and rested quietly in bed throughout the study. Baseline serum and saliva samples for cortisol were obtained 15 and 30 min after the placement of an intravenous catheter (time points –15 and 0). Subjects then received an intravenous dose of 1 µg/kg body weight of CRH (corticotrophin ovine triflutate, or Acthrel, Ferring Pharmaceuticals). Serum and salivary samples for cortisol were obtained 15, 30, 60, 90 and 120 min after CRH administration. The averages of the cortisol values for the –15- and 0-min time points and the 60- and 90-min time points represented the basal and peak values, respectively.

Salivary cortisol

Subjects provided at least 1 ml of saliva per sample by spitting into a collection tube. No water was consumed within 5 min prior to any sample collection and no food was consumed during any of the experiments. Because salivary cortisol concentration is independent of flow rate¹⁵ and Trident Original Flavor® sugarless gum does not interfere with the salivary assay (Salimetrics LLC, State College, PA, USA), subjects were permitted to chew this brand of gum if needed to stimulate saliva flow. Subjects were instructed not to brush their teeth within 30 min of the protocol and to refrain from wearing lipstick on the test day.

Saliva samples were frozen at -20°C to precipitate mucins, and then thawed, centrifuged at $1500\text{ g} \times 15\text{ min}$, and the supernatant was collected and stored at -80°C . All samples for an individual were assayed together in duplicate using a commercial high-sensitivity

salivary cortisol EIA (Salimetrics LLC) according to the manufacturer's instructions. The lower limit of detection for the assay was 0.19 nmol/l. Samples exceeding 50 nmol/l, the upper limit of the standard curve, were re-analysed after dilution. The intra- and interassay coefficients of variation (CVs) were both < 6%.

Serum total cortisol

For each sample, 1.5 ml of blood was collected via venous catheter into a serum separator tube. Sera were stored at -80°C until analysed in duplicate by commercial RIA (Diagnostic Products Corporation, Los Angeles, CA, USA) according to the manufacturer's instructions. The lower limit of detection for the assay was 10 nmol/l. The intra- and interassay CVs were less than 6% and 10%, respectively.

Cortisol-binding globulin (CBG)

This assay was performed on samples from two representative subjects in the exercise protocol who were suspected of having different levels of CBG because only one of the two women was taking oral oestrogens. Five samples from each subject that spanned the range of observed total cortisol values were analysed for CBG to ensure stability in the protein concentration during the exercise stimulus. CBG was determined by RIA (Labor Diagnostika Nord, Nordhorn, Germany) according to the manufacturer's instructions. All samples were run in duplicate in a single assay (intra-assay CV < 5%). Serum free cortisol was calculated using a standard formula that accounts for the concentration-dependent binding characteristics of cortisol.¹⁶

Statistical analysis

Data were analysed with SPSS Version 11.0 for Windows. Linear and exponential curve fitting was performed to examine the association between cortisol measured in saliva and serum. Paired, two-tailed *t*-tests were used to compare the relative cortisol changes in both media in response to the dynamic stimuli. All data are presented as mean \pm standard deviation unless otherwise specified. Statistical significance was defined as an alpha level ≤ 0.05 .

Results

The salivary cortisol and serum total cortisol response patterns for the exercise and CRH stimuli are depicted in Fig. 1a and 1b, respectively. As predicted for the exercise protocol, mean cortisol concentrations were moderate and slowly declining for the first 70 min. The exercise-induced increase in cortisol was apparent in both serum and saliva 10 min after initiation of exercise. The general pattern of response was a rapid rise, peaking at 100 min in serum and 110 min in saliva, followed by a gradual decrease that was similar in both media. Likewise, the patterns of serum and salivary cortisol response to the CRH stimulus paralleled one another, with the peak occurring at 90 min in serum and 60 min in saliva. Despite the large inter-individual variability in the magnitude of response to both HPA axis stimuli, as demonstrated by the standard error bars in Fig. 1, the average time course of the cortisol response was similar in both media.

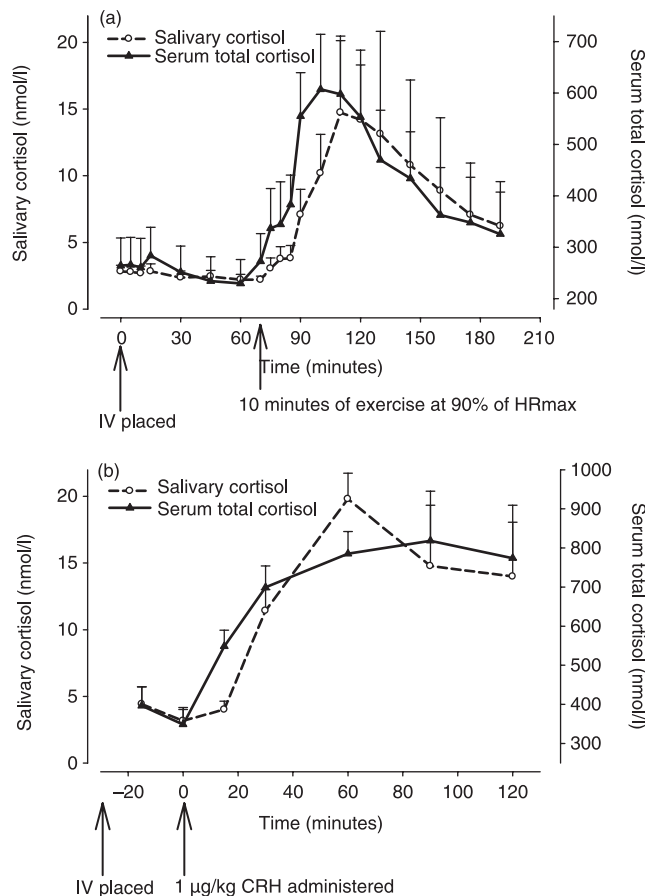


Fig. 1 The time course and response pattern (mean \pm standard error) in salivary cortisol (\circ ; broken line) and serum total cortisol (\blacktriangle ; solid line): (a) for the exercise protocol ($n = 8$) and (b) after administration of corticotrophin releasing hormone (CRH; $n = 4$).

The relative increase in salivary cortisol in response to both the exercise and CRH stimuli was significantly larger than the relative increase in serum total cortisol ($697 \pm 826\%$ vs. $209 \pm 150\%$, $P = 0.04$ saliva vs. serum). The relative decreases in cortisol from the moderate pre-exercise levels to the very low dexamethasone-suppressed levels were not significantly different between the two media ($-47 \pm 56\%$ vs. $-84 \pm 8\%$, $P = 0.13$ saliva vs. serum).

The linear correlation of salivary cortisol with serum total cortisol for all subjects was significant, but moderate ($n = 183$ pairs, $r = 0.60$, $P < 0.001$). The association between the cortisol values from the two media was stronger when fit to an exponential model ($r = 0.81$, $P < 0.001$), supporting the concept that free cortisol (i.e. measured in saliva) increases more rapidly once the binding capacity of CBG is exceeded. In addition, the linear correlations were strengthened when subjects on oral oestrogens ($n = 52$ pairs, $r = 0.75$, $P < 0.001$), which are known to raise CBG and total serum cortisol, were analysed separately from those not taking oestrogens ($n = 131$ pairs, $r = 0.67$, $P < 0.001$). Using the equations generated by the exponential regression lines illustrated in Fig. 2a, for a given salivary cortisol value of 10 nmol/l, the expected serum total cortisol value would be 826 nmol/l for an individual on oral oestrogens compared with 530 nmol/l for an individual not on oral oestrogens. Similarly, the

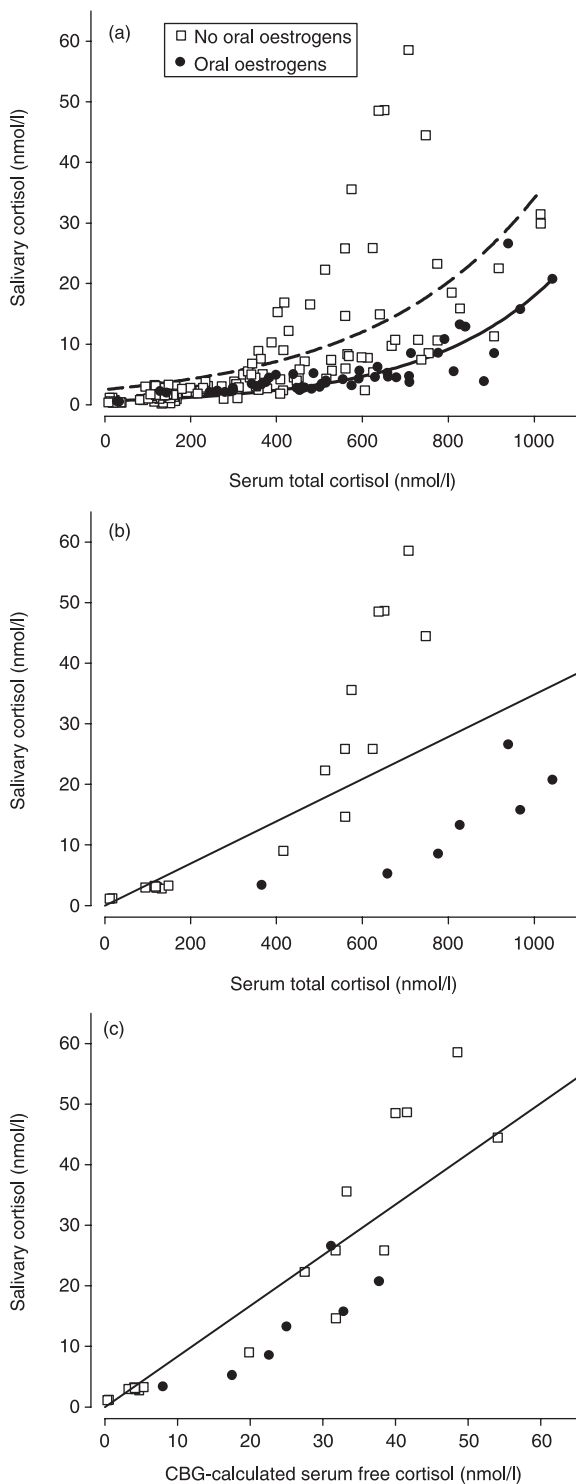


Fig. 2 (a) The 183 paired serum total cortisol and salivary cortisol samples from 12 subjects, nine not on oral oestrogens (\square ; broken line) and three on oral oestrogens (\bullet ; solid line); the exponential regression equations ($y = ab^x$) were $y = (2.513)(1.0026)^x$ and $y = (0.622)(1.0034)^x$ for those not on and those on oral oestrogens, respectively. (b) The 26 paired serum total cortisol and salivary cortisol samples for two representative subjects, one on (\bullet) and one not on (\square) oral oestrogens. (c) Comparison of the CBG-calculated serum free cortisol with salivary cortisol for the two individuals from (b). Linear regression lines are shown in (b) and (c) ($r = 0.59$ and $r = 0.89$, respectively, both $P < 0.001$).

exponential regression lines would predict that for a given total serum cortisol value of 700 nmol/l, the expected salivary cortisol value would be 6.6 nmol/l for an individual on oral oestrogens compared with 15.6 nmol/l for an individual not on oral oestrogens. Figure 2b depicts the linear correlation between salivary cortisol and total serum cortisol for two representative subjects ($n = 26$ pairs, $r = 0.59$, $P = 0.001$), one on and one not on oral oestrogens. Within-subject CBG levels remained stable during the exercise stimulus; average values were 1.77 ± 0.09 and $0.97 \pm 0.13 \mu\text{M}$ for the subjects on and not on oral oestrogens, respectively. The strongest linear correlation was found when salivary cortisol was plotted against the CBG-calculated serum free cortisol ($n = 26$ pairs, $r = 0.89$, $P < 0.001$, Fig. 2c).

Discussion

The primary intent of this study was to determine the relationship between salivary cortisol and serum total cortisol, measured by commercial EIA and RIA, respectively, over a wide range of values and while cortisol concentrations were changing rapidly. Participants in this study provided frequent saliva and serum samples while at rest and exercising at 90% of HRmax, during recovery from exercise, after dexamethasone suppression, and in response to CRH. The time course of response for all of these conditions was similar in both media. However, the magnitude of the relative change in salivary cortisol was significantly greater than that of serum total cortisol after the exercise and CRH stimuli, but not after dexamethasone suppression. When coupled with the better fit of an exponential ($r = 0.81$) rather than a linear ($r = 0.60$) model for the relationship between salivary cortisol and serum total cortisol, these findings support the concept that salivary cortisol represents the biologically active free fraction of cortisol and that the free cortisol fraction increases exponentially after CBG is saturated.

Previously published data on salivary cortisol analysed by the Salimetrics EIA are limited to studies that assessed diurnal rhythms^{17–22} or used a mental stress stimulus that did not result in cortisol elevations above the binding capacity of CBG.²³ Additionally, none of these studies presented data on paired salivary and serum samples. Therefore, to our knowledge, our results are the first to provide a direct comparison of salivary cortisol with simultaneously obtained serum cortisol samples and to document the fact that this EIA methodology is appropriate for assessing dynamic changes in HPA axis activity when cortisol concentrations change rapidly, as during exercise, and when cortisol concentrations routinely exceed the binding capacity of CBG, as after CRH administration.

Measurement of salivary cortisol has advantages over serum measures: the stress of venipuncture is avoided, samples can be collected during free-living conditions, and only the bioactive fraction of the hormone is measured. Furthermore, the methodology used to assess salivary cortisol in the present study, a commercially available EIA kit, is easy to perform compared with the more time-consuming and expensive methodologies required for direct determination of the free cortisol fraction in serum, such as equilibrium dialysis or ultrafiltration.^{9,15} The EIA kit avoids the need to use radioactivity, and salivary cortisol values obtained with this method correlate well ($r = 0.98$) with a standard commercial RIA.¹³ Our data expand the applicability of this EIA for salivary cortisol by demonstrating a

strong exponential relationship with serum total cortisol and a strong linear relationship with CBG-calculated serum free cortisol.

Our finding that the significant association between salivary cortisol and serum total cortisol was best described by an exponential model is in agreement with others. Using fluoroimmunoassay, Port⁷ determined that there was an exponential relationship ($r = 0.86$, $P < 0.001$) between 50 paired salivary and fingerstick cortisol values obtained from six men undergoing a cycling VO_2max test. However, O'Connor and Corrigan⁸ found strong linear relations ($r = 0.60$ – 0.93 , $P < 0.01$) between RIA-determined cortisol from saliva and serum venipuncture samples in eight men performing 30 min of cycling at 75% of VO_2max . Although methodologies for obtaining and analysing samples differed in these studies, the most likely explanation for this discrepancy has to do with the variation in relative intensity of the exercise stimulus. The peak serum total cortisol value after the submaximal exercise bout used by O'Connor and Corrigan was only about 500 nmol/l, compared with about 800 nmol/l after the maximal exercise bout used by Port. The exponential relationship between paired salivary and serum total cortisol documented by Port and by the present study is probably attributed to the fact that 40% and 32% of the serum values, respectively, exceeded 500 nmol/l, the average CBG saturation point for individuals not on oral oestrogens.²⁴ Similarly, in another study in which there was a significant linear correlation between salivary cortisol and serum total cortisol values, the authors noted that the slope of the regression line increased when the serum total cortisol concentration exceeded 500 nmol/l.¹¹

When dynamic tests of the HPA axis do not include absolute total cortisol values above 500 nmol/l, as in the current study after dexamethasone suppression, as well as in others using this test⁹ or insulin tolerance testing,¹⁵ the relative changes in salivary cortisol and serum total cortisol measures were not significantly different. We observed a significantly larger relative change in salivary cortisol compared with serum total cortisol for our dynamic HPA axis tests that generated cortisol peaks > 500 nmol/l. When RIA methodology has been used for salivary cortisol determination, the same pattern of a disproportionate increase in salivary cortisol compared with serum total cortisol has been documented in response to stimuli such as CRH/AVP or ACTH.^{5,9,15,25} Importantly, our study included subjects taking oral oestrogens, a condition known to increase CBG.¹⁶ When differences in cortisol binding capacity were accounted for in two subjects, one on and one not on oral oestrogens, the highest linear correlation was observed ($r = 0.89$ for the relationship between salivary cortisol and CBG-calculated serum free cortisol). Others have documented strong linear relations ($r = 0.89$ – 0.97) between salivary cortisol determined by RIA and measured serum free cortisol.^{5,9,15}

Although the aim of our study was to address the applicability of the EIA technique to physiological research rather than clinical diagnosis, it is interesting to note that one of the two women on oral contraceptives (OCs) in our study had a serum total cortisol of 138 nmol/l in response to the DST, which is at the clinically accepted cut-off for nonsuppression (< 140 nmol/l). However, this subject's salivary cortisol DST value (2.0 nmol/l) was well within the accepted cut-off for suppression (< 5 nmol/l).²⁶ Ansseau *et al.*²⁷ found that two of 14 OC users would be considered nonsuppressors in response to a 1 mg DST based on serum total cortisol but not based on serum free cortisol values. Tiller *et al.*²⁸ found a significantly higher cut-off

value to define nonsuppression in OC users vs. healthy controls. In combination, these data suggest the need for caution when interpreting serum total cortisol results from clinical tests performed in women taking OCs. Others have reported that salivary measures can be effective for diagnosing pathophysiological alterations in HPA axis activity.^{10,26,29,30} Thus, salivary measures may provide greater diagnostic accuracy by decreasing false-positive test results in women taking oral oestrogens. Scott *et al.*³¹ demonstrated that although CBG levels were significantly elevated in both OC users and pregnant women compared with normally menstruating women, only the pregnant women had significantly higher diurnal salivary cortisol values. Therefore, increases in CBG may or may not result in increases in the physiologically relevant free cortisol fraction.

In summary, we have demonstrated that salivary cortisol measured with a simple, commercially available EIA kit can be used instead of serum total cortisol for the assessment of dynamic HPA axis activity. Similar to salivary cortisol assessed by RIA or fluorimetric immunoassay, salivary cortisol assessed by EIA reflects the unbound, active cortisol fraction. This finding has important implications for cross-sectional studies of HPA axis activity in populations with varying CBG levels (e.g. women on and not on oral oestrogens) as well as for intervention studies in which CBG levels would be expected to change (e.g. weight loss). Therefore, assessment of salivary cortisol should be considered over serum total cortisol because more physiologically relevant data are obtained, particularly when the cortisol response to an HPA axis stimulus exceeds the saturation point of CBG.

Acknowledgements

We thank the nursing, bionutrition, core laboratory, information systems and administrative staff of the General Clinical Research Center for their assistance in conducting this study. We also thank the members of our research group who carried out the day-to-day activities for the project, including M. Goldstein and S. Howar. Finally, we thank the subjects who volunteered to participate in the study for their time and effort. This research was supported by awards from the National Institutes of Health, including R01 AG18198 (W.M.K.), R01 MH37373 (M.L.L.), F32 AG05899 (W.S.G.), T32 AG00279 (J.S.L.), M01 RR00051 (General Clinical Research Center) and P30 DK48520 (Clinical Nutrition Research Unit). W.S.G. was supported in part by a North American Menopause Society/Wyeth-Ayerst Clinical Research Fellowship and a Hartford/Jahnigen Center of Excellence career award.

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