# Increased Salivary Cortisol Reliably Induced by a Protein-Rich Midday Meal

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**Objective:** This study was conducted to determine whether an increase in salivary free cortisol would be reliably elicited by a midday meal, thus providing a convenient physiological challenge to the hypothalamic-pituitary-adrenal (HPA) axis, and whether this cortisol release depended on the protein content of the meal. **Method:** In healthy men, free cortisol was measured in saliva samples taken before and after two identical protein-rich midday meals (39% energy as protein) and compared with a day on which no meal was eaten. Next, in healthy women in a nonclinical setting, salivary cortisol was measured before and after a protein-rich meal (32% energy as protein) on one day and a low-protein meal (5% energy as protein) on another day. Measures of mood, appetite, and psychological well-being were also taken. **Results:** An acute meal-dependent increase in salivary cortisol occurred, which was reliable over 2 test days. This increase in cortisol depended on the proportion of protein in the meal, increasing after the high-protein but not the low-protein meal. The extent of this increase in cortisol correlated significantly with poor psychological well-being in women. Some postmeal improvement of mood (positive affect) was associated with the high- but not the low-protein meal. **Conclusions:** The cortisol response to meals may have implications for the effects of meal composition on mood, cognitive function, and food choice. The measurement of free cortisol in saliva provides a psychologically stress-free and reliable technique to assess the cortisol response to a standard protein-rich meal, ie, a physiological challenge to the HPA axis in men and women that could be investigated in naturalistic settings outside the laboratory. **Key words:** cortisol, hypothalamic-pituitary-adrenal axis, protein, nutrition, appetite, saliva.

HPA = hypothalamic-pituitary-adrenal; BMI = body mass index; CBG = corticosteroid-binding globulin; GHQ-12 = 12-item General Health Questionnaire; DEBQ = Dutch Eating Behavior Questionnaire; AUC = area under the curve; ANOVA = analysis of variance; TRP = tryptophan; LNAA = large neutral amino acids; 5-HT = 5-hydroxytryptamine; PANAS = Positive and Negative Affect Schedule.

## INTRODUCTION

The diversity of health implications now associated with control and consequences of the release of cortisol, together with its sensitivity to psychological stress, has given this major human adrenal glucocorticoid hormone much importance in behavioral medicine (1). Release of cortisol in response to various challenges provides an index of the functioning of the HPA axis. In relation to health, dysfunction of this neuroendocrine axis has been implicated in particular in dysphoric disorders, such as major depression (2), whereas hypercortisolemia in Cushing's syndrome is accompanied by physical symptoms, such as accumulation of abdominal adipose tissue, together with muscular atrophy of the limbs, providing powerful evidence of the well-established metabolic and nutritional consequences of chronic hypercortisolemia (3).

In addition to pathological and pharmacological challenges to the HPA axis, use of techniques such as insulin and glucose clamps have provided evidence that normal physiological variations in cortisol in humans have a significant direct influence on macronutrient metabolism (4–6). In this respect, cortisol seems to increase lipolysis and proteolysis, as well as increasing gluconeogenesis, thereby raising the contribution of protein and fat to energy substrate supply, while protecting glycogen stores. Indeed, the ability of cortisol to increase plasma free fatty acid levels may underlie the emerging link between cortisol and abdominal obesity, together with its associated metabolic syndrome (7).

In line with such observations, there is much evidence from animal studies that HPA axis function can profoundly influence expression of appetite and regulation of body weight (8), whereas HPA axis activity itself can be modified by changes in feeding patterns (9). It is somewhat surprising, therefore, that relatively little attention has been given to studying the relationship between food intake and cortisol in human beings (10, 11). Nevertheless, it has been observed clinically that plasma cortisol can show a midday increase at a time when levels are generally declining, and this observation led to more systematic studies, which demonstrated an association between meals and the midday increase in plasma cortisol (12). Furthermore, it

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was apparent that, although increases in plasma cortisol could occur in some subjects after breakfast, the midday meal, or an evening meal, the greatest effect occurred after the midday meal (13).

It was also shown that postmeal plasma cortisol levels could be affected by the proportions of macronutrients consumed: that is, meals containing 20% to 40% protein (as % total energy) produced a greater plasma cortisol response than meals with high carbohydrate or fat levels (14–16). By comparison, meals containing 10% protein resulted in weaker secretion of plasma cortisol (14), and protein-free glucose or fat loads did not stimulate cortisol release (15). Furthermore, mean plasma cortisol levels may be altered by chronic changes in macronutrient intake: thus, Anderson et al. (17) found that mean cortisol levels were higher in men after a high-protein/low-carbohydrate diet for 10 days compared with a low-protein/high-carbohydrate diet. However, it should be noted that the high-protein diet in that study also increased CBG, so there is some doubt as to whether substantial changes in free unbound cortisol had occurred.

The implications of these findings should be of much interest to behavioral medicine: understanding the association between food intake, macronutrient composition, and cortisol release could provide useful insight into links between the HPA axis, appetite, food choice, and nutritional and psychological health. This is emphasized by the recent finding that cortisol secretion elicited by stress or smoking is abolished by mild food deprivation in men (18). Meal- and macronutrient-dependent effects on cortisol release might also help explain reported effects of meal composition on subsequent mood and cognitive performance (19, 20), because, eg, cortisol is known profoundly to affect limbic neural function (21, 22).

An important point is that a meal-induced increase in cortisol could provide an inexpensive, innocuous challenge to test HPA axis function that could be repeated easily and administered outside of the clinic, ie, in less stressful environments, and perhaps used in large-scale population surveys. However, it would be necessary to establish the parameters influencing this response, and to demonstrate its reliability. Yet, data from the above studies of effects of meals often showed notable interindividual variation in plasma cortisol that was not explained. Although it is known that genetic factors are likely to be important in determining perhaps 50% of variation in both baseline and stress-response levels of cortisol (1, 23), other potentially important factors were often not taken into account in the above human studies. For instance, neither smoking status nor sex were addressed as possible sources of variance, although they are now known to influence cortisol release (24, 25).

A difficulty with interpretation of most of the above studies, which hinders additional investigation of nongenetic influences on cortisol release, is that only total plasma cortisol was reported. Approximately 90% to 95% of plasma cortisol is bound to CBG, albumin, and erythrocyte membranes (26), whereas only the free fraction is thought to be physiologically active. Moreover, the invasive nature of blood sampling can itself be a stimulus for cortisol release (27), as well as being likely to interfere with normal appetite. By contrast, cortisol in saliva is a valid measure of free cortisol levels and is easily sampled repeatedly without distress (1, 26).

Therefore, we investigated whether an increase in cortisol after a protein-rich meal could be measured in saliva, and to what extent such a response was repeatable within individuals. In a second experiment, we determined whether the proportion of protein in the meal was a critical variable, as had been suggested by earlier studies of changes in plasma cortisol, by comparing salivary cortisol levels after a high- and low-protein meal within the same individuals. This comparison also allowed for testing of the effects of nutrients on postprandial mood.

## EXPERIMENT 1

### Method

*Subjects and Briefing.* Ten healthy nonsmoking men were recruited from among staff of the Bethlem and Maudsley NHS Trust Hospitals, London and Kent, UK, by poster advertisements asking for volunteers to take part in research on the effects of the midday meal on cortisol in saliva, for which payment of UK £30 would be given. During an initial briefing, volunteers were asked to complete three questionnaires: the 12-item GHQ-12 (28) to screen for lack of psychological well-being including clinically relevant anxiety or depression; the Dietary Restraint scale of the DEBQ (29) to screen for a high level of concern about dieting to prevent weight gain; and the Perceived Stress Scale (30), to be used as a potential covariate of the cortisol response. At this time, weight and height of volunteers were measured, and BMI [weight (kg)/height ($m^2$)] was calculated.

The following criteria were used to exclude potential subjects: GHQ-12 score (0, 0, 1, 1, scoring) $> 7$; DEBQ-R score $> 3$; BMI $> 30$; also, diabetes or other metabolic disorder. In addition, volunteers were asked to report any psychiatric history and recent or current drug use, which were exclusion criteria; however, they had been screened by the Occupational Health Service for their employer. No volunteers had to be excluded on these criteria. All subjects gave their informed consent, and the study was approved by the local Ethical Committee on Research.

*Design.* This experiment assessed the impact of a protein-rich midday meal on release of free cortisol as measured in saliva. The aim was to compare the cortisol response with the same meal on two occasions (Meal A and Meal B) for each subject with the response on a day on which no meal was eaten (Figure 1). Thus, each subject attended the ward on three occasions; the sequence of meal (Meal A)

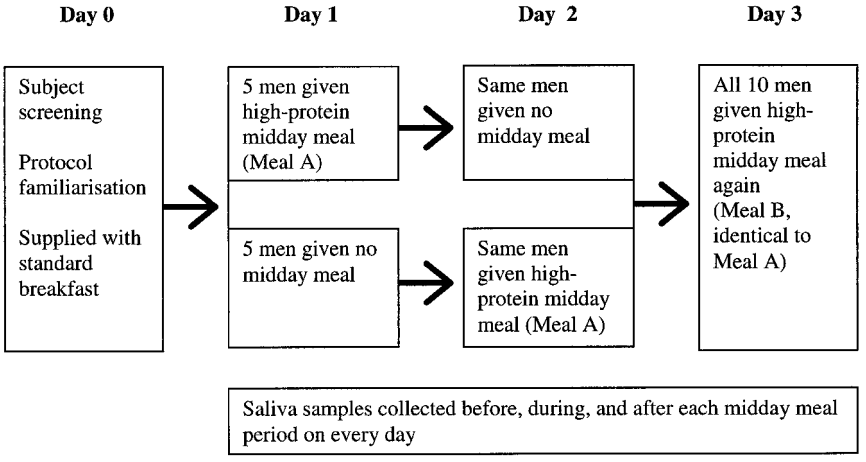


Fig. 1. Design of Experiment 1 showing sequence of high-protein midday meals versus no meal. *Note:* Days 2 and 3 did not immediately follow the respective preceding days (1 or 2).

or no meal was randomized over the first and second visits, whereas on the third visit, subjects always received the meal (Meal B). Fifteen saliva samples were given at intervals each day between 9:00 AM and 5:00 PM. Questionnaires assessed premeal and postmeal mood, appetite and pleasantness of the meal, as well as stress, excitement, and exertion levels throughout the day.

*Procedure.* The experimental meal manipulations in this study were performed by a research nurse (Lucia Poon) in a dedicated research area on a ward in the Affective Disorders Unit of the Bethlem Royal Hospital, Beckenham, Kent, UK. Subjects attended the ward on each experimental day between 11:45 AM and 2:15 PM, and saliva sampling outside these times was performed wherever subjects continued their normal activities for that day. During briefing the day before their first experimental day, subjects were shown the room in which they were to have the meal, and the procedure was explained to them. Each subject was asked whether the foods to be eaten would be acceptable, and was advised to bring some light reading or work to occupy the 2.5 hours during which they would be on the ward.

Subjects were given the following instructions: On the evening before a saliva sampling day, do not drink alcohol after 6:00 PM; on saliva sampling days, before and during the sampling period, consume only the supplied food and drink, or water (eg, do not drink tea, coffee, chocolate, or cola); avoid exercise likely to cause breathlessness.

*Saliva Sampling.* Subjects were instructed in the saliva sampling technique using Salivette tubes (Sarstedt, Leicester, UK): These centrifugable tubes are specifically designed for saliva sampling, and contain a cotton roll, which is placed in the mouth without handling, and rolled around on the tongue for 1 minute before ejection back into the tube. Each subject was given a wristwatch with an hourly alarm to aid compliance to the saliva sampling schedule when away from the ward, and sufficient Salivette tubes, each labeled with an appropriate sampling time. Subjects were instructed to return saliva samples and an accompanying record sheet to the ward that evening, or to refrigerate the samples until their return could be arranged.

The record sheet (one for each experimental day) consisted of a table in which every row represented a saliva sample, and columns indicated time of sampling, tube code, and boxes for subjects to indicate, from 0 = none to 5 = extreme, their level of stress, excitement, and exertion experienced since the last sample. These ratings could then be used to screen for possible confounding stimulation of the HPA axis.

Saliva sampling was performed hourly from 9:00 AM to 12:00 noon, then every 15 minutes from 12:30 PM to 2:00 PM, with the meal being presented and eaten between 12:30 PM and 1:00 PM. On arrival at the ward (11:45 AM), subjects handed in morning saliva samples and then were asked to rest in a quiet room. On leaving the ward, additional saliva samples were given at 2:30 PM, 3:00 PM, 4:00 PM, and 5:00 PM.

*Mood and Appetite Questionnaires.* Self-report measures of mood and appetite were taken on each experimental day before and after the meal (or at equivalent times when no meal was eaten). These allowed moods and appetitive states to be assessed for any significant variation between experimental days. The questions also measured the extent to which the experimental meal was regarded by each subject as palatable and representative of a typical midday meal.

Shortly before the mealtime (12:20 PM), subjects completed a brief mood questionnaire consisting of seven 100-mm line scales anchored by the following pairs of semantic differential adjectives: upset/content, dreamy/attentive, excited/calm, happy/sad, relaxed/tense, energetic/tired, vulnerable/safe. Subjects were asked to draw a mark across each line to indicate how they were feeling at the moment. They completed this questionnaire again at 2:00 PM (1 hour after finishing the meal, on meal days).

Just before and just after the meal (12:30 PM and 1:00 PM), subjects completed scales in response to the following questions: "How hungry/full do you feel right now?" (100-mm line anchored by "not at all/extremely"); "How hungry/full do you feel compared to just before/after your typical lunch?" (5-point scale, "much less–much more"); "How pleasant does the meal look to you/did you find the meal?" (9-point scale, "extremely unpleasant–extremely pleasant"). One hour after the meal (2:00 PM), subjects completed three of these questions again, ie, "How hungry/full do you feel right now?", "How pleasant did you find the meal?"

*Breakfast and Midday Meal Compositions.* The day before each experimental day, every subject was supplied with a prepacked standardized breakfast to take home (identical for each subject and on each day): They were instructed to consume all of the items between 7:00 AM and 8:00 AM, and to note the time of finishing eating on the record sheet. The breakfast menu was a typical light English breakfast, consisting of cereal (bran flakes, raisins, semi-skimmed milk), French toast, sunflower margarine, marmalade, and orange juice, although no tea or coffee was permitted (for macronutrient composition, see Table 1).

The midday meal menu was designed to be high in protein (Table

**TABLE 1. Macronutrient Composition of the Meals**

| | Breakfast (550 kcal) | | | Midday Meal (550 kcal) | | |
|---|---|---|---|---|---|---|
| | Protein | Carbohydrate | Fat | Protein | Carbohydrate | Fat |
| Grams | 13 | 101 | 12 | 53 | 56 | 14 |
| % Energy | 10 | 70 | 20 | 39 | 39 | 22 |

1), reasonably palatable and easy to prepare. It consisted of a salad of tuna steak (tinned in brine), mixed pulses (J. Sainsbury PLC, canned, drained, and containing soya, chick-pea, pinto, black-eye, red kidney, and aduki beans), fresh whole tomato, half-fat cottage cheese with vegetable pieces, French toast, sunflower margarine, and a dessert of fat-free fromage frais with a fruit sauce. Pilot work with midmeal saliva sampling suggested that the procedure did not disrupt appetite appreciably: For this sample and that immediately after the meal, subjects were asked to rinse their mouths with water and wait for 3 minutes before inserting the cotton roll, to minimize contamination of the sample with food.

*Salivary Cortisol Assay*. Cortisol in saliva was quantified by means of the "Magic cortisol" radioimmunoassay (RIA) kit (Ciba-Corning, Halstead, Essex, England), based on an established method (31) but slightly modified in the following way: 1) the lowest and highest standards were 0.7 and 96.6 nmol/liter, respectively; 2) the incubation time was shortened to 30 minutes at 37°C and the precipitate was washed once with phosphate buffered saline (0.3 ml) containing 1% (vol/vol) Tween 20. In addition, the internal walls of the RIA tubes were wiped carefully with a piece of tissue paper before counting for improved precision. All aliquots of standards and specimens were dispensed to the RIA tubes with the GENESIS Robotic Sample Processor (TOCAN, UK). The percent cross-reactivity of the antiserum (manufacturer's data) was: cortisol, 100%; prednisone, 26%; 11-deoxycortisol, 8%; tetrahydrocortisol, 1%; and less than 1% for corticosterone, 11-deoxycorticosterone, cortisone, estradiol, estriol, testosterone, progesterone, and dexamethasone. The interassay precision (percent coefficient of variation) was 4.2 and 3.1 at 4.0 and 10.7 nmol/liter, respectively ($N = 8$), and the minimum detectable concentration of cortisol was 0.4 nmol/liter.

*Data Analysis*. The effect of the midday meal on salivary cortisol was analyzed by calculating the AUC (trapezoid method) for the change in cortisol (Δ-cortisol) from the premeal baseline sample (12:30 PM) until 2:00 PM, after which it was apparent that cortisol had returned to unstimulated levels (the 12:00 noon sample was not used in calculating the baseline inasmuch as cortisol levels tended to decline additionally at 12:30 PM). These AUCs were analyzed for an effect of midday meal by one-way repeated measures ANOVA, with meal type as the within-subjects factor. After a significant ANOVA, AUCs for Δ-cortisol on each meal day were compared with the AUC on the No Meal day by paired *t* tests. Also, Δ-cortisol levels were compared at each time point from 12:45 PM to 2:00 PM between pairs of days by paired *t* tests, with significance at .05 corrected for the number of comparisons made by the Bonferroni method: thus, the No Meal day was compared separately with Meal A and Meal B, and these two meal days were compared with each other. Reliability (degree of agreement between meals) of the meal-induced cortisol response was assessed using the mean difference and limits of agreement for AUCs for Δ-cortisol after Meals A and B (32), and Pearson's correlation.

## Results

*Subject Characteristics*. The men were aged between 18 and 51 years [mean (SD) = 32.0 (8.3) years]. Their body mass indices ranged from 20.0 to 28.1 [mean (SD) = 24.6 (2.4)], indicating a range from low/normal to somewhat overweight, but non-obese. Subjects were low on dietary restraint [a selection requirement: mean (SD) restraint score = 1.85 (0.6)]. The group mean score on the Perceived Stress Scale [mean (SD) = 20.3 (5.8)] was very similar to that given for a large stratified random sample (33). Given the maximum score of 26 for this group, these subjects did not seem to be unduly stressed. Likewise, the maximum score for the GHQ-12 was 1 (in three subjects), implying good psychological well-being throughout the group.

*Midday Meal Effects on Salivary Cortisol*. As predicted, both protein-rich meals increased cortisol concentration in saliva acutely, as assessed from the start of the meal (12:30 PM) to 1 hour after finishing eating (2:00 PM) [Figure 2: repeated-measures ANOVA on AUC of Δ-cortisol, overall meal effect, $F(2,18) = 5.07$, $p < .02$, $\epsilon^2 = 0.36$, observed power = 0.75; paired *t* tests on AUCs for Meal A vs. no meal, $t(9) = 1.86$, $p < .05$, 1-tail, and for Meal B vs. no meal, $t(9) = 2.66$, $p < .025$, 1-tail]. When no meal was eaten, average salivary cortisol concentrations declined steadily, as would be expected from the typical circadian rhythm for basal cortisol levels (Figure 2). By time point comparison, the increases in cortisol from 12:30 PM (premeal) after Meals A and B were significantly greater than after No Meal at 1:30 PM (Figure 2: Bonferroni *t* tests). The postmeal increase in cortisol did not differ between Meal A and Meal B when any time points from 12:45 PM to 2:00 PM were compared, nor when AUCs for Δ-cortisol were compared [$t(9) = 1.80$, not significant (NS); mean difference (arbitrary AUC units) = −55.9; limits of agreement (± 2 SD of difference) = −252.0 to 140.3; $r = .81$, $p < .01$], implying a reliable and linearly correlated response on repetition.

*Hunger, Fullness, Pleasantness, and Mood Ratings*. Baseline hunger and fullness ratings did not differ significantly between any days (data not shown). On average, subjects rated the meal as seeming quite pleasant [mean (SD) = 1.5 (1.9), 1.3 (1.8) for Meals A and B, respectively], and this pleasantness rating did not change significantly after eating the meals, although the mean ratings increased (data not shown).

There was no evidence here that the variation in meal-induced increases in cortisol across subjects was related to any changes in hunger or fullness ratings, or to ratings by which subjects compared these states with those typically experienced (ie, no significant

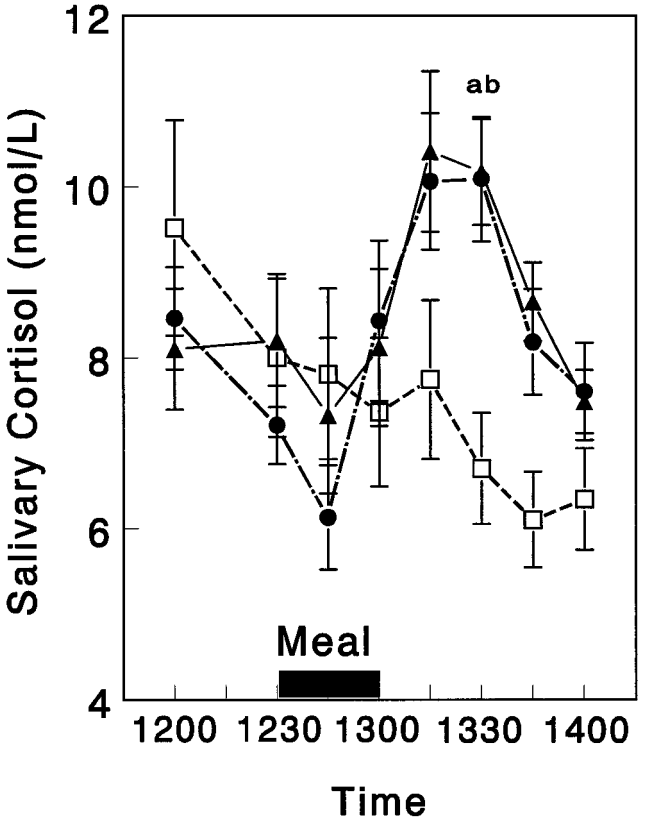


Fig. 2. Effects of two identical high-protein midday meals on salivary cortisol levels, compared with a day on which no meal was served (Meal A ▲; Meal B ●; No Meal □). Data are expressed as mean ± SE ($N = 10$). ab, Δ-cortisol at this time differed significantly from No Meal for Meal A and for Meal B: Bonferroni $t$ tests, $t(9) = 3.44$, $p < .05$; $t(9) = 4.78$, $p < .01$, respectively. For unit conversion, μg/dl = nmol/liter × .03625. (Note that time is given in the European or military form.)

correlations with either AUCs or absolute peak Δ-cortisol and rated scores or their differences; data not shown). However, this design may not have been sufficiently powerful to detect such relationships. Premeal mood ratings did not differ significantly among any days. Some differences in postprandial mood changes were found between the meal and no meal days, as would be expected (data not shown).

## EXPERIMENT 2

This second experiment had four main aims: 1) to test the hypothesis that the meal-stimulated increase in cortisol is dependent on the proportion of protein in the meal; 2) to demonstrate this effect in women; 3) to demonstrate that the meal-dependent increase in cortisol can be reproduced outside of a clinical laboratory setting; and 4) to measure postmeal mood changes in circumstances in which nutrient effects on mood and neurotransmitter levels have been claimed, for instance, in comparison between protein-free and protein-rich nutrient loads (34).

This last aim required a meal containing as little protein as can reasonably be achieved in a midday meal menu of assorted foods. Our low-protein meal contained 5% protein by energy. This served two purposes: first, previous research (15) suggested that such a minimal amount of protein would probably not stimulate cortisol release, although this has not been shown for free cortisol; second, there is evidence that this low level of protein can result in an increase in the ratio of plasma tryptophan to large neutral amino acids (TRP/LNAA), which, in turn, allows increased transport of TRP across the blood-brain barrier (35). Some evidence suggests that this increased uptake of TRP, the precursor amino acid for serotonin (5-HT) synthesis, may increase synthesis of 5-HT in the brain, inasmuch as the enzyme tryptophan hydroxylase is not fully saturated (36, 37). Furthermore, it has been claimed that this increase in brain levels of 5-HT may underlie differences in mood after protein-free and protein-rich meals (34, 38). Evidence also suggests that a very low protein meal is most likely to increase the TRP/LNAA ratio if no food has been consumed for several hours, such as after overnight deprivation (37, 39). Therefore, in this experiment, subjects did not eat breakfast before the experimental midday meals. Many of the procedures were similar to Experiment 1; thus only differences in the methods will be reported.

### Method

*Subjects and Briefing.* The subjects were 10 healthy female medical college students: They were recruited through personal contact and were not paid. Exclusion criteria were similar to those in Experiment 1; again, the GHQ-12 and DEBQ-Restraint scales were administered, but the PSS was not used. In this study, dietary restraint was measured, but not used as a strict exclusion criterion, because that would present difficulties for recruitment of young women. In the event, one subject who initially took part displayed extremely high salivary cortisol levels both at baseline and after the meals (up to 170 nmol/liter), which approximated 3.0 SD above the mean, and exceeded the limits of reliability of the cortisol assay; therefore, this subject was excluded from additional consideration. It is possible that this extreme cortisol level was due to recent use of the illicit drug, 3,4-methylenedioxymethamphetamine (Ecstasy), which is known to stimulate the HPA axis (40).

Two of the volunteers reported currently using an oral contraceptive. The only other current medication reported (by different subjects) was paracetamol for one subject and anti-asthma medication for another. Although it had been intended to avoid recruiting smokers, two of the women admitted to smoking one or two cigarettes a day, particularly during social occasions (although not on experimental days). Date of onset of menstruation and cycle length were recorded. The possible impact of within-subject change in menstrual cycle was limited by testing both meals on a given subject within 5 days. Height and weight were obtained by self-report. Each

subject gave her signed consent, and the study was approved by the local Ethical Committee on Research.

*Design*. This experiment was designed to compare the change in salivary cortisol after high- and low-protein midday meals that were otherwise equicaloric. The high-protein meal was designed to contain at least 30% energy as protein, which was expected to be sufficient to stimulate release of cortisol. By contrast, as mentioned above, a meal containing 5% protein was not expected to stimulate cortisol release. The meals were also designed to be equicaloric, and to have similar menu structures, so that any confounding effects of energy absorption, as well as preferences and expectations, could be minimized. All subjects consumed both meals, thus acting as their own controls; subjects were pseudorandomly assigned to meal day sequence, so that half of the group received either the high- or low-protein meal first.

*Procedure*. Volunteers expressing interest in participating were first given an information pack that also contained the GHQ-12 and DEBQ-Restraint questionnaires, and other health questions, so that subjects could be excluded, if necessary, as described above. Details of the meal menus were also given, so that only subjects for whom those foods were acceptable were encouraged to volunteer. Subjects were informed of the intention to measure changes in moods and salivary cortisol and the possible association with protein consumption, but the details of the nutrient content of the meals were not discussed until completion of the experiment.

The study was performed in private accommodation in an informal atmosphere that contrasted with the ward setting of Experiment 1. Subjects arrived on the site at 11:45 AM, when the protocol was explained, and they were asked to practice using the Salivette saliva sampling cotton rolls and collection tubes.

At 12:00 noon, subjects gave the first proper saliva sample, and completed the mood questionnaires (see below). Subsequently, saliva sampling and mood questionnaires were completed at 12:30 PM (ie, immediately before eating the midday meal), 1:00 PM, 1:30 PM, and 2:30 PM. The midday meal was presented at 12:30 PM and half an hour was allowed for consumption. Also, at 2:30 PM, subjects completed 9-point scales to indicate 1) "How hungry do you feel right now?" and 2) "How pleasant did you find the meal?". Subjects left the site on completion of these final questionnaires. Saliva samples were refrigerated as they became available, for up to 4 days, before being analyzed using the radioimmunoassay procedure given for Experiment 1.

*Meal Composition*. The format of the menus for both meals was soup followed by stir-fried rice and vegetables. The high-protein meal had crab meat and sweet corn in the soup, and tuna and cottage cheese in the main course. The low-protein meal had pearl barley in the soup, and unsweetened apple juice as a drink (in addition to water being available). Both meals were equicaloric and provided an amount of energy quite typical for the midday meal (630 kcal). Nutrient compositions of the meals are given in Table 2: Proportions of fat were approximately the same for both meals (and quite high, unlike Experiment 1), whereas they differed substantially in percentage of contribution to energy from both protein and carbohydrate, as is necessary to equate energy levels between the high- and low-protein recipes.

**TABLE 2. Macronutrient Composition of the High- and Low-Protein Midday Meals**

| | Low-Protein Meal (630 kcal) | | | High-Protein Meal (630 kcal) | | |
|---|---|---|---|---|---|---|
| | Protein | Carbohydrate | Fat | Protein | Carbohydrate | Fat |
| Grams | 7.8 | 89.0 | 30.0 | 50.3 | 33.5 | 33.8 |
| % Energy | 5 | 52 | 43 | 32 | 20 | 48 |

*Mood Questionnaire*. Moods were assessed at each sampling time using the PANAS (41). The PANAS lists 20 adjectives, 10 describing positive moods, and 10 describing negative moods. Each adjective is rated on a 5-point scale (very slightly or not at all; a little; moderately; quite a bit; extremely). This questionnaire has been shown to be valid and reliable, and can be used to measure both state and trait positive and negative affect. Watson et al. (41) describe high positive affect as ". . . a state of high energy, full concentration, and pleasurable engagement, whereas low positive affect is characterized by sadness and lethargy." They describe negative affect as ". . . including anger, contempt, disgust, guilt, fear, and nervousness, with low negative affect being a state of calmness and serenity."

Positive and negative affect, as measured by the PANAS, are essentially uncorrelated dimensions. Negative, but not positive, affect has been found to correlate with depression and perceived stress (41).

*Data Analysis*. Area under curves for Δ-cortisol after high- and low-protein meals were compared by ANOVA and *t* tests as in Experiment 1. Changes in mood from baseline were assessed by comparing the difference from baseline to zero (baseline) by nonparametric Wilcoxon matched-pairs tests. Effects of meal type on mood were assessed by independent *t* tests. In addition, the variability in GHQ-12 scores in these subjects allowed those scores to be compared with the difference in Δ-cortisol response between the two meals by Pearson's product-moment correlations.

## Results

*Subject Characteristics*. The women were aged 20 to 25 years ($N = 9$, mean [SD] = 22.4 [1.7] years), and mean (SD) BMI was 20.6 (3.0). Mean (SD) DEBQ-Restraint score was 2.86 (1.24), indicating greater dietary restraint in these subjects than was present in the men in Experiment 1. There was no evidence that the current use of medication or occasional smoking systematically influenced salivary cortisol in these subjects (data not shown). All subjects were either just finishing menstruation, in the follicular phase or midcycle, and so it is unlikely that any variation in cortisol response to the meals would be due to differences in menstrual cycle phase, because it is the later luteal phase in which changes in energy and glucocorticoid homeostasis seem to occur (42).

*Effect of Meal Protein Content on Salivary Cortisol*. Salivary cortisol levels were significantly higher after the high-protein meal than after the low-protein meal, as expected [Figure 3: one-way repeated-measures ANOVA on AUCs for Δ-cortisol from the start of the meal to 2 hours later, $F(1,8) = 5.47$, $p < .05$]. Mean Δ-cortisol levels were greater after the high-protein than low-protein meal at 30 and 60 minutes from the start of eating (Figure 3), but no longer differed by 120 minutes after the start of the meal. Salivary cortisol levels tended to decrease after the low-protein meal, being significantly less than at the start of the meal (12:30 PM) by 1:00 PM (Figure 3), although the differ-

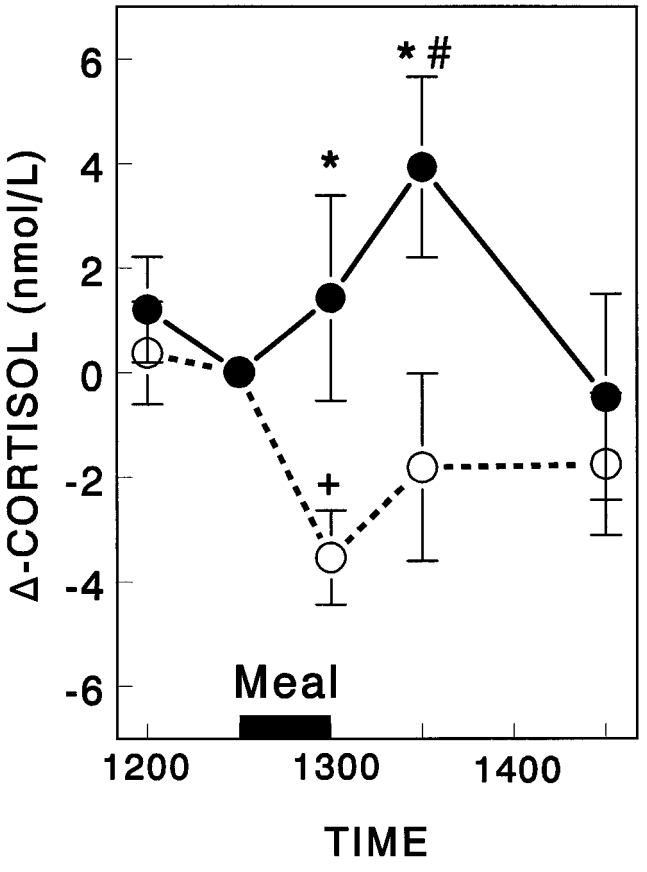


Fig. 3. Changes in salivary cortisol levels in women after eating a high-protein meal (●, 32% energy as protein; 630 kcal) and an equicaloric low-protein meal (○, 5% energy as protein). Data are expressed as mean ± SE ($N$ = 9). *Significantly greater than cortisol levels after the low-protein meal: at 30 minutes, $t(8) = 2.30$, at 60 minutes, $t(8) = 2.59$, $p < .025$, 1-tail. +Significantly less than baseline (12:30 PM) cortisol levels, $t(8) = 3.92$, $p < .01$. #Significantly greater than baseline cortisol levels, $t(8) = 2.27$, $p < .05$, 1-tail. (Note that time is give in European or military form.)

ence was not significant at 1:30 PM and 2:30 PM. By contrast, as predicted, cortisol increased above premeal baseline levels after the high-protein meal, reaching a peak 1 hour after the start of the meal (Figure 3). The two subjects using oral contraceptives did not differ from nonusers in their protein-dependent cortisol response (high-low protein difference in peak Δ-cortisol, users vs. nonusers: mean ± SD = 5.40 ± 4.95 vs. 4.71 ± 5.69 nmol/liter).

*Cortisol Response and Psychological Health*. Although the GHQ-12 scores of all subjects were below the exclusion criterion of 7 (maximum score = 6), there was greater variation in this group than for Experiment 1, with six of the women scoring above zero. An important question is whether the cortisol stimulation by protein-rich meals is at all sensitive to the subject's psychological health status: thus, the GHQ-12 scores were correlated with the AUCs for Δ-cortisol after the high-protein meal, and also the difference in AUCs for Δ-cortisol after the high- and low-protein meals. Significant correlations were indeed found for both of these measures of the cortisol response with GHQ-12 scores ($r = .77$, $p < .025$, and $r = .84$, $p < .005$, 2-tail, respectively). Furthermore, peak Δ-cortisol after the high-protein meal, and the difference in peak Δ-cortisol between meals, correlated with GHQ-12 scores ($r = .79$, $p < .025$, and $r = .86$, $p < .005$, respectively; Figure 4). These relationships were found despite the small and relatively healthy sample: that is, women with poorer psychological well-being showed larger increases in cortisol after the high-protein meal.

*Meal Effects on Mood*. Mean premeal positive affect (PANAS scores) seemed to be slightly lower in this group of women than that reported for a large sample of American students [this sample vs. Watson et al. (35), mean (SD) = 24.1 (4.3) vs. 29.7 (7.9), respectively]. Mean premeal negative affect in this sample was very similar to that found for the American sample [mean (SD) = 13.6 (3.2) vs. 14.8 (5.4), respectively].

Positive affect tended to decrease after the low-protein meal, being significantly less than the premeal baseline after 60 minutes (Figure 5: Wilcoxon matched-pairs test of affect change vs. zero, $z = 2.03$, $p < .05$), although not after 30 and 120 minutes. How-

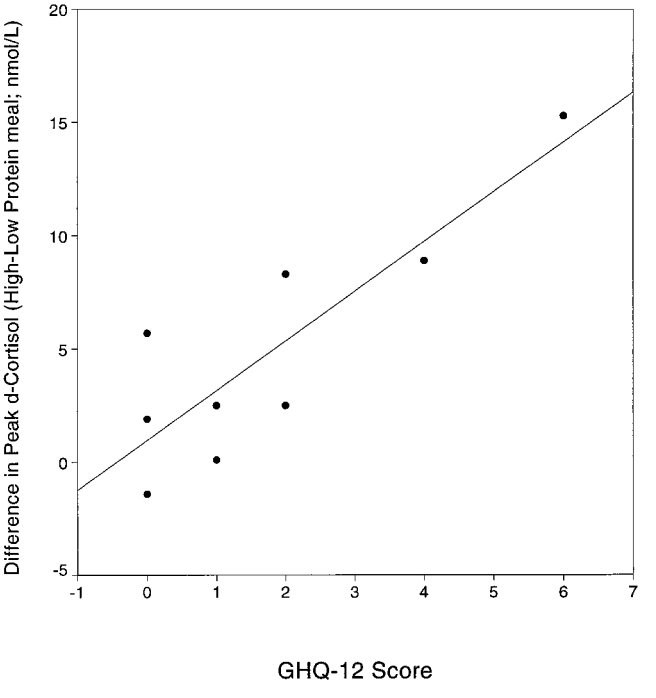


Fig. 4. Scattergram of GHQ-12 scores versus difference between meals in peak Δ-cortisol (nmol/L) (Experiment 2). The line is plotted by linear regression ($R^2 = .74$).

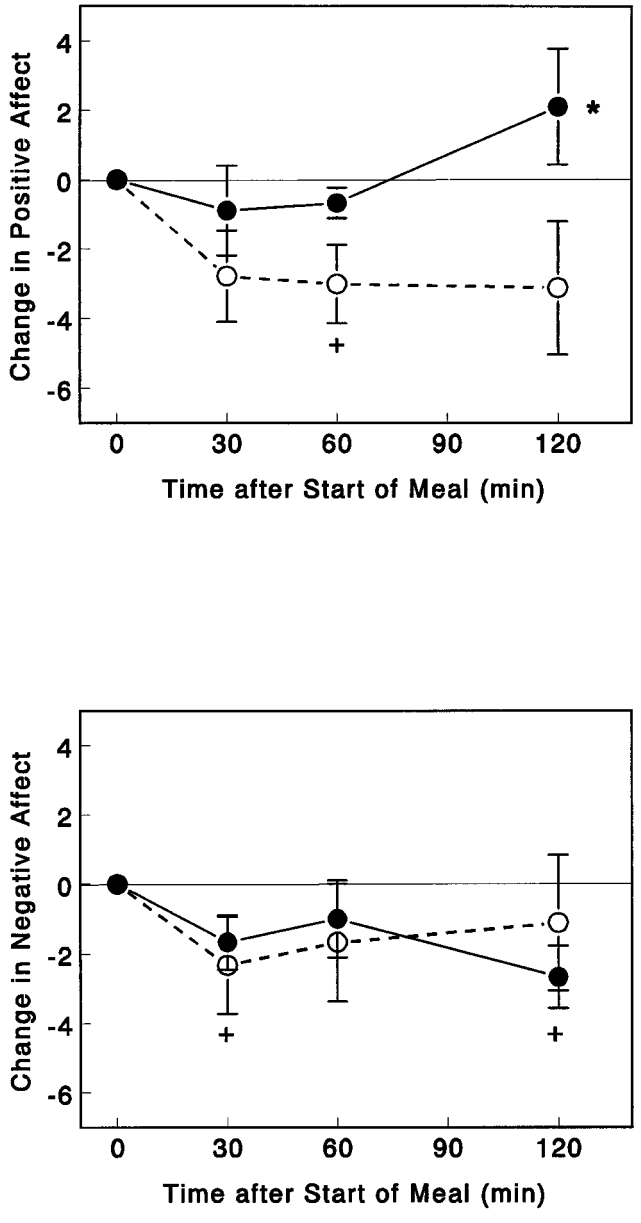


Fig. 5. Changes in positive (upper panel) and negative (lower panel) affect (PANAS scores, differences from premeal baseline) in women after eating a high-protein meal (●) and an equicaloric low-protein meal (○). Data are expressed as mean ± SE ($N = 9$). *Significantly greater than mean affect (at that time) after the low-protein meal. +Significantly different from baseline (zero at 1230 h). See text for statistical details.

ever, after the high-protein meal, positive affect did not decrease, but, in fact, began to increase by 120 minutes, when mean positive affect was significantly greater than that after the low-protein meal [Figure 5: $t(8) = 2.46$, $p < .05$]. The difference in positive affect after the two meals approached significance even by 60 minutes [$t(8) = 2.19$, $p = .06$].

Negative affect scores tended to decline after either meal, with no significant effect apparent for protein level (Figure 5). Significant reductions from premeal baselines occurred at 30 minutes (Wilcoxon matched pairs test vs. zero: low-protein meal, $z = 2.03$, $p < .05$; high-protein meal, $z = 1.94$, $p = .05$) and at 120 minutes (high-protein meal, $z = 2.17$, $p < .05$).

## DISCUSSION

These results demonstrate that salivary cortisol increases substantially (approximately one and one-half- to two-fold on average) and significantly after a protein-rich meal (30% to 40% energy as protein). The increase begins toward the end of a 30-minute meal period, and peaks at approximately 1 hour after the start of the meal: after approximately 2 hours, salivary cortisol levels have declined and are no longer significantly different from those seen either in the absence of a meal or after a low-protein meal. Also, the size of the cortisol response is consistent across two occasions within the same men.

Our results show that greater than 5% protein (as percent total energy) is required to detect a reliable increase in salivary cortisol. The minimum necessary proportion of protein for reliable stimulation of cortisol remains unknown, but results of studies measuring plasma cortisol suggest that at least 10% protein may be needed (14). It is also possible that it is the absolute amount of protein consumed, perhaps interacting with the particular pattern of amino acids, that is the critical stimulus: The results of this and other studies (14) would suggest that a meal intake of at least 20 g of protein may be necessary; moreover, the higher the intake of protein, the greater the secretion of cortisol is likely to be (14). The variation in carbohydrate and fat levels of the high-protein diets in Experiments 1 and 2 also suggests a unique contribution of protein.

This acute stimulation of cortisol release may be part of a homeostatic mechanism in response to a high influx of amino acids. In animals, glucocorticoid hormones are known to be important for optimal maintenance of nitrogen balance (43): thus, corticosterone deficiency in rats impaired both dietary protein absorption and retention of absorbed nitrogen (44), whereas corticosterone insufficiency inhibited protein consumption on refeeding food-deprived rats (10). There is no conflict here with the known counterregulatory action of cortisol. Although plasma insulin will have increased after any of these meals, cortisol is not inevitably inversely associated with insulin (6, 45). Rather, the ability of cortisol to increase gluconeogenesis and reduce glucose uptake should aid the removal and metabolism of plasma amino acids.

Our studies replicated previous findings that a midday meal can stimulate release of cortisol, but in addition this effect was shown here for salivary free cortisol, rather than the mainly protein-bound plasma cortisol. Furthermore, we have demonstrated that this meal-stimulated cortisol release is reliable, stress-free, and capable of being repeated outside a laboratory setting (ie, at home or at work). The size of the cortisol response was not related here to any overt appetitive effects, such as reported hunger, fullness, or pleasantness of the meals; however, larger subject numbers and more sensitive measures of appetite may be necessary to detect any such relationships.

Despite a relatively homogeneous subject sample, considerable variation in cortisol response was seen between subjects. Possibly, the meal-stimulated release of cortisol may depend on composition of the habitual diet. For instance, in subjects typically eating a protein-rich diet, adaptation of hepatic enzymes required for amino acid metabolism may reduce the need for a homeostatic hormonal response to a high-protein meal. By comparison, insulin and glucose responses to test meals differed, depending on whether subjects had previously been fed a high-carbohydrate (low in fat and protein) or low-carbohydrate (high in fat and protein) diet (46).

This meal-dependent challenge to the HPA axis could prove sensitive to psychological traits, inasmuch as GHQ-12 scores in the nine healthy young women correlated with the extent of the cortisol response to the high-protein meal, even though the variation in GHQ-12 scores was relatively small. This might reflect some impaired negative feedback by cortisol on the HPA axis, as is frequently seen in more substantial cases of dysphoric disorders (2). The size of the cortisol response might conceivably reflect a lack of adaptation to a protein-rich diet in women with poorer psychological well-being. For instance, appetite is sensitive to anxiety/depression (47), and stress may alter food choice in favor of fattier, more energy-dense foods (48). However, this correlation is not likely to be due to (short-term) undereating, because fasting during the day has been found to abolish rather than enhance the salivary cortisol response to stimulation of the HPA axis (18) (although we still found this meal-dependent increase after overnight deprivation until midday). In addition, acute psychological state before the meal could be important: In an earlier study (49), subjects undergoing a stressful task before a meal showed increased plasma cortisol during the task, which was then followed by a suppressed cortisol response to the meal. Yet, there was no indication here that subjects were unduly stressed before the meal.

The implication of substantial hormonal changes to meals of relatively unusual macronutrient composition provides a possible mechanism for several nutrient-dependent changes in mood and cognitive performance that have been reported, either postmeal (19, 50) or more chronically (51). Even though several hormones could be candidates for such a mechanism, cortisol is particularly appealing because of its known action on the central nervous system, especially the hippocampus, where cortisol-induced changes in receptor sensitivity may alter memory and attentional processes, as well as emotional reactivity (21, 22). The possibility that increased cortisol secretion might underlie the meal-dependent increase in arousal is strengthened by evidence that the degree of cortisol secretion elicited by cigarette smoking is positively correlated with change in arousal (52). Moreover, the change in salivary cortisol levels elicited here by protein-rich meals is comparable with that reported for smoking in men (18) and psychological stress in women (1).

In Experiment 2, the high-protein meal prevented the tendency for postprandial positive affect to decline, and by 2 hours after starting eating, positive affect was beginning to increase after this meal. To the extent that positive affect reflects greater arousal and ability to concentrate, this finding is in line with other evidence (34, 38) that women, in particular, were less sleepy and more attentive after a high-protein meal than after a high-carbohydrate meal. It has been speculated (34) that such changes may be mediated by reduced serotoninergic activity after a high-protein relative to a high-carbohydrate meal, due to changes in availability of the precursor amino acid, TRP (see above). However, although such differential serotoninergic function, or at least synthesis, may have been present in our design, the stimulation of cortisol release could also provide a mechanism for such psychological effects. A role for HPA axis function in such effects of meals is supported additionally by reports that more anxious subjects are less susceptible to such meal-dependent changes in mood or cognition (50). Also, it may be relevant that the mood measure affected here, ie, positive affect, shows a circadian rhythm akin to that of cortisol, albeit delayed by 2 or 3 hours (41), whereas the unaffected negative affect shows no such rhythm.

There is some evidence that maintaining a very high-protein diet may chronically stimulate the HPA axis (17) and increase release of vasoactive hormones (53). Therefore, it is worth noting that increased HPA activity and cortisol release have been linked to increased risk of insulin resistance, hypertriglyceridemia, and hypercholesterolemia (3, 7, 54), whereas high intake of animal protein can lead to hypercholesterol-

emia (55). Reducing the carbohydrate:protein ratio of diets chronically has also been associated with deterioration in mood (51, 56), although it has been pointed out that such diets may influence mood through poor acceptability of the foods by subjects (57).

In summary, the experiments reported here have shown that protein-rich meals can reliably increase free cortisol levels in most subjects, as measured by changes in salivary cortisol concentrations. This relatively simple manipulation may provide a useful and innocuous technique for assessing HPA axis function. The findings also raise interesting questions about the contribution of the HPA axis to modulation of psychological functioning by nutrient intake. Additional research could address whether acute postprandial changes in cortisol underlie differences in the satiating action of macronutrients (58, 59). Another important issue is whether there are subjective, perhaps even subcognitive, sequelae of this hormonal response that could influence the development of food preferences, and even food choice in particular circumstances or states. If such processes exist, then an obvious possibility is that such effects might be relevant to changes in food choice under stress.

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