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Consistent Sex Differences in Cortisol Responses to Psychological Stress

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In four independent studies, sex differences in cortisol responses to psychological stress were investigated in healthy adolescents and adults (total n = 153). Public speaking and mental arithmetic in front of an audience (Studies 1-3) reliably induced increases in free cortisol levels in both sexes with 2- to 4-fold increases above baseline levels. Mean cortisol responses were 1.5- to 2-fold higher in men compared with women. In Study 3, cortisol profiles were additionally investigated after human corticotropin-releasing hormone (h-CRH) and bicycle ergometry until exhaustion. Here, both sexes showed very similar adrenocortical responses. Furthermore, men showed elevated cortisol levels in anticipation of the psychological stress situation without actually having to perform the tasks (Study 4). Under this condition cortisol concentration was unchanged or decreased in women. From these data we conclude that the observed sex difference does not reflect an overall lower responsiveness of the female adrenal cortex. Although these studies do not provide conclusive data, we suggest sex differences in cognitive and/or emotional responses to distressing psychosocial situations which in turn may influence cortisol secretion.

Key words: cortisol; saliva; psychological stress; gender differences; corticotropin-releasing hormone; exercise

INTRODUCTION

The hypothalamus-pituitary-adrenal axis (HPA) serves vital physiological functions in the mammalian organism both under unstimulated conditions as well as during challenges such as physical and emotional stress (1). Besides impact on structures in the central nervous system mediated by corticotropin-releasing hormone (CRH), most peripheral and central effects of HPA activity are modulated by the major glucocorticoid hormone cortisol (in man) or corticosterone (in rodents), respectively. The presence of moderate cortisol levels throughout the day is mandatory for basic physiological processes like cardiovascular functions. On the other hand, vigorous glucocorticoid responses have been shown to counterbalance or prevent negative effects brought about by stressors (2). For example, in animal models of autoimmune disorders, there is preliminary evidence that a marked adrenocortical response to antigenic stimulation and a concomitant response to distressing stimuli is associated with a delayed or abrogated disease onset (3-6). A suppression of HPA function leads to exacerbation of symptoms in these models. Clear-cut sex differences in adrenocortical activity exist in rodents. Female rats show higher baseline corticosterone lev-
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ELs (7–10), larger corticosterone secretion after ACTH injections (7, 11), and enhanced responses to stressors like handling, ether, restraint, shocks, or high conflict situations (7, 9, 12–17). Due to their profound HPA responses, stressed female rats have been shown to be better protected against autoimmune disease compared with males of the same strain (6).

The enhanced adrenocortical activity in female rats correlates with sex steroid levels. Gonadectomy in females results in corticosterone profiles similar to males (8, 9, 17).

In humans the literature on sex differences in adrenocortical activity is more controversial and contradicts findings in rodents. While some studies reported higher baseline cortisol levels in men (18–22), no differences were found in other investigations (23–27). It should be noted that lower concentrations were found in females only during the follicular phase. Cortisol levels were comparable with men when measured in the luteal phase (19, 22). Similarly, cortisol responses to stimulation yielded heterogeneous results. Filip et al. (28) found higher cortisol responses to clomipramine in women, while men tended to show higher cortisol levels after stimulation with desipramine. Larger increases of cortisol in men were observed following 5-hydroxytryptophan administration (29), and no sex differences in cortisol responses were observed under physical stress (24, 25). Similarly, no sex differences in adrenocortical activity could be observed in studies exposing healthy subjects to mild psychosocial stress (30–33); reviewed in (34).

The absence of sex differences in cortisol responses in these studies could probably be ascribed to the methods employed. For instance, only marginal increases or even decreases in cortisol levels were induced in two studies (30, 33). In other investigations, the small number of samples (i.e., two) (31, 32) might have been inappropriate for unraveling cortisol response differences between sexes.

The reason for the small number of samples obtained in those studies might have been the unavailability of alternative laboratory techniques for cortisol determination. This situation has changed with the advent of more sensitive biochemical assays. Stress-free assessment of cortisol levels at frequent intervals is now available by cortisol measurement in saliva. This method has proven to be a useful and accurate substitute for plasma or urine assessment. Since salivary cortisol levels correlate highly with the unbound ('free') hormone concentration in plasma and were shown to be independent of flow rate or plasma-binding globulin levels, this technique appears to be the method of choice for a variety of applications (for reviews see: Refs. 35–38).

We recently devised a psychosocial stress protocol that induces significant adrenocortical activation in a laboratory setting (39). Combining this stress protocol with repeated cortisol measures in saliva, we investigated sex differences in adrenocortical responses in healthy adolescents and adults.

Methods

Subjects and General Experimental Outline

In four separate studies, a total of 153 apparently healthy subjects were investigated for cortisol responses to psychological stress. The groups consisted of 19 males and 31 females in Study 1, 23 males and 14 females in Study 2, 22 males versus 26 females in Study 3, and nine males and nine females in Study 4. Since one aim of Study 3 was the investigation of...
a possible genetic determination of adrenocortical responses to external stimulation, 13 monozygotic and 11 dizygotic twin pairs were tested in this study. The respective results on intra-twin resemblance are presented elsewhere (39).

The mean age of the volunteers was 22.6 years (Study 1, 19–33 years; Study 2, 19–29 years; Study 3, 15–33 years; Study 4, 20–29 years). The subjects in Studies 1, 2, and 4 were paid volunteers recruited among students of the University of Trier responding to a bulletin board message. In Study 3 the subject sample mainly consisted of non-academic individuals. They were invited to participate in a psychological study on endocrine responsiveness through announcement in a local newspaper or by direct contact (i.e., letter). They received no monetary incentive for participation.

All subjects were medication-free except for contraceptive drugs. Since cortisol levels appear to be unaltered with low estrogen-containing contraceptives (37), and previous studies in our laboratory did not indicate different cortisol responses (unpublished data), women on contraceptive medication were not excluded from participation. All participating women were post-pubertal, and they were sampled randomly through the menstrual cycle.

In Studies 1 and 4, experiments were performed both in the morning (10 A.M. to 1 P.M.) as well as in the late afternoon (4 P.M. to 6:30 P.M.). Studies 2 and 3 were conducted in the latter period only. While in Studies 1, 2, and 4, volunteers were tested only once (psychological stress), subjects in Study 3 participated either in two or three experimental sessions with different stimulation protocols (see below). The sequence of the tests in Study 3 was randomized, and each test was performed on a different day. With these exceptions all subjects received identical treatment in the four studies. In order to avoid interference with experimental effects, subjects were asked to refrain from smoking, physical exercise, meals, alcoholic beverages, and soft drinks with low pH at least 1 hour prior to testing.

Psychological Stress Test (All Three Studies)

After arrival at the laboratory, subjects rested for 10 minutes in room A. At time 0 minutes they were taken to a second room (room B) and introduced to the tasks they would have to perform soon. In this room three persons were already sitting behind a table with a video camera and a tape recorder installed. The investigator told subjects that they should take the role of job applicant who were invited to introduce themselves to the company’s staff managers. Publicly speaking for 5 minutes, they should try to convince the three members of a selection committee that they were the appropriate person for this particular position. The members of the selection committee were introduced as being specially trained to monitor nonverbal behavior. Furthermore, it was announced that a voice frequency analysis and an analysis of nonverbal behavior would be performed on the tape-recorded talk. The selecting committee was comprised of males and females in each study.

Following this introduction, the volunteers were taken back to room A and given 10 min for preparation of their talks. At time +10 minutes, they were taken to room B again to deliver their talk for 5 minutes. Immediately following, the subjects were asked to perform a second task. They had to serially subtract the number 13 from 1022 as quickly and accurately as possible. On every failure the subjects had to restart at 1022. At time +20 minutes, volunteers were taken back to the first room and allowed to rest for 50 minutes (Study 1 and 2), 90 minutes (Study 3), or 30 minutes (Study 4).

It should be noted that this stress protocol induced significant endocrine stress responses in the majority of subjects tested. In previous experiments we used mental arithmetic problems as the only task and found only mediocre response rates (40). We therefore chose to use a compound stressor with mental arithmetic and public speaking tasks. This protocol did not attempt to investigate possible differential stress responses to speaking versus arithmetic task: subjects always had to speak in front of an audience and solve the arithmetic problem.

CRH Test (Only Study 3)

Thirty-eight (males and females) out of 48 subjects volunteered in this test. At —30 minutes an indwelling catheter was inserted into the antecubital vein and kept open by physiological saline. At time 0 minutes they received a bolus injection of 100 µg synthetic human corticotropin-releasing hormone (Bisendorf Peptide, Wedemark, Germany). After injection the subjects rested for 90 minutes.
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Ergometry (only Study 3)

All 48 subjects participated in the bicycle ergometry test. After a 10-minute rest, they started working at 50 watts (women), or 75 watts (men) for the first 2 minutes. Thereafter, workload was increased by 25 watts every 2 minutes until exhaustion. Heart rate was continuously monitored and blood pressure readings were obtained at 5-minute intervals. Subjects were asked to perform physical exercise until exhaustion, thus the duration of workload differed interindividually from 8 to 22 minutes with a mean exercise time of 14 minutes.

Study 4

Nine male and nine female subjects entered the same experimental protocol for the psychological stress as volunteers in Studies 1 to 3; however, after preparation of their talks they were not taken back to room B. At this point they were informed that they were participating in a study on anticipatory stress responses and that they would not have to give their public speech.

Saliva Sampling and Cortisol Analysis

At regular intervals, saliva samples were obtained from the volunteers using the Salivette sampling device (Sarstedt, Römmelsdorf, Germany) as previously described (41). Briefly, the subjects gently chewed on the cotton swab to stimulate saliva flow. No additional saliva flow stimulant was used. This procedure allows for rapid (<1 minute) saliva sampling. Under psychological stress and physical stimulation, saliva was sampled at 10-minute intervals from −10 minutes (baseline) to +70 minutes (Studies 1 and 2), −10 minutes (baseline) to +90 minutes (Study 3), or −5 minutes (baseline) to +50 minutes (Study 4). In the CRH condition additional samples were obtained at −35 minutes, −30 minutes, and −20 minutes before drug administration to monitor a potential increase in cortisol due to catheterization. All samples were stored at −20°C until assayed.

Cortisol analyses were performed with a time-resolved fluorescence immunosassay (42). This assay has a lower detection limit of 8.6 pg/well (95% confidence interval). The intra- and interassay coefficients of variation were less than 7% and 9%, respectively.

Statistical Analysis

Analyses of variance (ANOVAs) with repeated measures were computed to reveal possible effects of the treatments (Time) as well as sex-specific response patterns (Time × Sex) with baseline cortisol levels serving as covariates. Taking into account the sphericity assumption, degrees of freedom were adjusted employing the Greenhouse-Geisser approach where appropriate. Possible baseline differences between sexes were tested using Student’s t tests for independent samples. Stimulation effects were tested with Student’s t tests for dependent measures comparing the baseline cortisol levels with the respective peak cortisol concentrations.

RESULTS

Figure 1 shows the mean cortisol levels of males and females in response to the psychological stress test. ANOVA results revealed a significant time effect with F values of 30.2, 24.9, 56.1 (p < 0.0001). While cortisol baseline levels were not significantly different between both sexes (t values: 0.6, 1.0, 0.8, not significant), males responded to the task of public speaking and mental arithmetic with larger hormone increases than women. The mean absolute increase was 7.1, 11.4, and 10.9 nmol/liter in males, and 4.3, 4.1, and 6.2 nmol/liter in females. This equaled a 3- to 3.5-fold elevation in men and a 1.5- to 2.5-fold increase in women. t-Test results showed highly significant increases from baseline levels for both sexes (Study 1: men t = 4.9; women t = 4.2; Study 2: men t = 8.7, women t = 3.9; Study 3: men t = 6.6, women t = 5.0; all p < 0.001). After Greenhouse-Geisser adjustment of degrees of freedom, the interaction Sex × Time was significant in Studies 2 and 3 (Study 2: F = 4.5, df = 2.2, 74.4; p = 0.014; Study 3: F = 3.7, df = 2.0, 91.3, p = 0.028) while it approached signifi-
Fig. 1. Salivary cortisol responses (means ± SEM) to public speaking (5 minutes) and mental arithmetic (5 minutes) in front of an audience in men and women observed in three independent studies (A, B, C). At time 0 minutes, subjects were introduced to the task, at +10 minutes the public speech was given.

The ANOVA results for Studies 1 and 2 may have been contaminated by unequal numbers of subjects in both groups. Since subjects were recruited from the same sample of university students and treated identically in both studies, we performed an additional ANOVA combining all subjects of Study 1 and Study 2 (n = 42 males, n = 45 females). Providing further statistical support for sex differences, there was
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a highly significant Sex × Time effect after Greenhouse-Geisser adjustment ($F = 7.1$, $df = 2.2$, $184.7$, $p = 0.001$).

Interestingly, within the same sex the peak cortisol levels were closely comparable in both stressor tasks. While in males cortisol levels rose to $14.7 \pm 1.1$ nmol/liter and $15.2 \pm 1.8$ nmol/liter, females peaked at $9.8 \pm 0.8$ nmol/liter, and $10.7 \pm 1.7$ nmol/liter, respectively. Moreover, in both studies peak cortisol levels were observed at +30 minutes following stimulation onset in both sexes.

In Study 3, both CRH and ergometry stimulation led to significant increases in cortisol levels in both sexes ($t$ values: men $6.3$ and $4.4$; women: $4.1$ and $4.3$; all $p < 0.001$; Figure 2). In contrast to the response to psychological stress, however, in neither test could significant differences between sexes be observed with respect to cortisol profiles (ANOVAs; all $F < 1$). Cortisol levels peaked at 50 minutes for males or 70 minutes for females after CRH administration; mean cortisol peaks in both sexes were observed at +40 minutes following ergometry stimulation. No sex difference could be detected in peak heart rates under ergometry exercise (men: $189.3 \pm 2.4$ beats per minute; women: $188.5 \pm 2.0$ beats per minute; $t = 0.3$, not significant).

Sex differences were also observed in anticipation of public speaking (Figure 3). Again, men showed increasing cortisol levels with peak values at 20 to 30 minutes, while in women cortisol levels declined over time. Due to the small number of subjects studied, this effect was only significant at an alpha-probability level of 9% after Greenhouse-Geisser adjustment ($F = 2.6$; $df = 1.9$, 30.4).

Fig. 2. Salivary cortisol responses (means ± SEM) after injection of $100 \mu g$ human corticotropin releasing hormone (CRH) and following bicycle ergometry until exhaustion (ERG) in both sexes.

Fig. 3. Salivary cortisol responses (means ± SEM) in anticipation of the stress of public speaking in front of an audience in male and female subjects.
DISCUSSION

Psychological stress of public speaking and mental arithmetic in front of an audience led to significant increases in salivary cortisol levels. In three independent studies (Studies 1–3), we observed similar peak adrenocortical responses that correspond to mean increases of free cortisol levels of 50% to 250% above individual baseline levels. Not only did the laboratory stressor reliably induce cortisol changes, we also found a consistent sex difference in adrenocortical responses. Like larger catecholamine and blood pressure responses observed in other studies (31, 32, 43), males showed higher cortisol levels when compared with age-matched females. This result can probably not be attributed to setting effects since different experimenters of both sexes conducted the studies.

The question arises whether the obtained sex difference reflects a biological or a psychological phenomenon. In search of biological variables, corticosteroid-binding globulin (CBG) could be considered a candidate for explaining the sex difference. It may be speculated that the reported effect is due to higher levels of binding protein in some of the women studied, e.g., as a result of estrogen-containing contraceptive medication (36). Upon stimulation, cortisol molecules released from the adrenal cortex may then more readily be bound to CBG resulting in lower free cortisol concentration measured in saliva compared with males. In a recent study on exercise-induced hormonal changes (44), cortisol responses of oral contraceptive users (n = 7) and control women (n = 8) were investigated. While women on oral contraceptives tended to have a slower onset of the cortisol response, both groups reached similar peak cortisol levels. Unfortunately, free cortisol levels were not measured in this study, thus leaving the question unanswered whether oral contraceptives may influence the biologically active hormone in response to stimulation. This issue deserves attention in future research.

Furthermore, it could be argued the female adrenocortex may generally secrete less cortisol. However, in agreement with data from other laboratories (23–26), we found almost identical cortisol response profiles in both sexes following pharmacological and physical stimulation. Administration of h-CRH as well as bicycle ergometry until exhaustion induced parallel increases in men and women with almost identical peak values. Thus, there is no strong evidence for an exclusively biological explanation of the findings described above.

Alternatively, differences in psychological variables may be considered responsible for the enhanced cortisol secretion observed in men. Several other studies have shown that merely the anticipation of a distressing event can be sufficient to induce cortisol elevation (45–47). In other words, cognitive and/or emotional processing of psychosocial stimuli can alter the activity of the HPA axis (48) with probably different patterns in the two sexes. Results from Study 4 provide preliminary evidence for this hypothesis. Women showed decreasing cortisol concentrations while anticipating the stress of speaking, whereas men again responded with increasing cortisol levels.

Perhaps, males tend to interpret the confrontation with a potentially detrimental situation in a different way than females do. As shown by other investigators, males may use other cognitive and/or emotional strategies to cope with such a situation (49–51). Whether this differ-
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ence in coping styles is responsible for the observed sex differences to the psychological stress of public speaking and mental arithmetic in front of an audience remains to be elucidated. The clinical significance of sex differences in cortisol responses in humans is yet unknown. Results from animal studies, however, suggested an involvement of glucocorticoid responses in the onset and course of certain diseases, e.g., autoimmune disorders (5, 6).

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