Overnight Urinary Cortisol and Cortisone Add New Insights into Adaptation to Training

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ABSTRACT

GOUARNE, C., C. GROUSSARD, A. GRATAS-DELAMARCHE, P. DELAMARCHE, and M. DUCLOS. Overnight Urinary Cortisol and Cortisone Add New Insights into Adaptation to Training. Med. Sci. Sports Exerc., Vol. 37, No. 7, pp. 1157–1167, 2005. Purpose: To examine the effects of training on the HPA axis using two new noninvasive tools: salivary cortisol response to awakening and overnight urinary cortisol and cortisone excretion, and on the sympathoadrenal system using overnight catecholamines excretion. To dissociate the effects of training to those of seasonal hormonal variations, endurance-trained men were compared with sedentary men. Methods: Nine untrained (UT) men and 10 triathletes were followed during a 10-month season. Clinical (total score of fatigue, total training load, and performances during the competition period) and hormonal parameters (overnight excretion of glucocorticoids and catecholamines, increment of saliva cortisol response to awakening) were measured. Results: Significant seasonal variations in overnight urinary glucocorticoids (decreased in June) and catecholamines (increased in June) concentrations and in saliva cortisol response to awakening were depicted in the two groups. Whereas urinary cortisol excretion was similar between both groups, overnight urinary cortisone excretion was significantly higher in triathletes compared with UT men (ANOVA: training effect: F2,45 = 9.50, P = 0.0003), suggesting that during a resting day there is a higher inactivation of cortisol into cortisone in highly trained men. Two triathletes developed an overtraining syndrome and presented an increased urinary cortisol/cortisone ratio (≥1) due to lower cortisone inactivation compared with the triathlete group. Conclusion: When not taken into account, seasonal variations may induce errors in the interpretation of hormonal variations with training. The increased intracellular inactivation of cortisol during the night in endurance-trained men uncovers subtle changes in HPA function during training. We show in this study the interest of noninvasive biological markers of the activity of the neuroendocrine system to monitor the repercussion of training load during longitudinal follow-up of athletes. Key Words: URINARY CORTISOL/CORTISONE RATIO, AWAKENING SALIVA CORTISOL, 11-HSD, URINARY CATECHOLAMINES, SEASONAL VARIATIONS, TRIATHLETES

Exercise represents a potent physiological stimulus upon the hypothalmo-pituitary adrenal (HPA) axis (7,8,9,16,28) and the sympathoadrenal system (30).

Glucocorticoids (GC) exert many beneficial actions in exercising humans increasing availability of metabolic substrates and protecting the organism from an overreaction of the immune system in the face of exercise-induced muscle damage (25). On the other hand, when an acute bout of endurance exercise is stopped, the hormonal profile is expected to converge toward anabolic processes. However, we and others have previously demonstrated that after a 2-h run, plasma cortisol levels remain significantly increased during almost 2 h after the end of the exercise (8,9,16,28). When training for a marathon race, subjects run an average of 120–180 km·wk−1. This implies daily sessions of prolonged and/or intense running and consequently prolonged phases of endogenous hypercortisolism (i.e., during exercise and during postimmediate exercise recovery). Given the antagonistic action of glucocorticoids on muscle anabolic processes as well as their immunosuppressive effects, this has led us to hypothesize that endurance-trained men might develop adaptive mechanisms in order to protect muscle and other GC sensitive tissues against this increased postexercise cortisol secretion. Indeed, the response to GC is regulated not only by the concentration of GC but also by the availability of cortisol and the sensitivity to GC of the target tissues. Changes in availability and/or sensitivity to GC may explain the discrepancy between repeated and prolonged exercise-induced HPA axis activation and the lack of metabolic consequences of such increased cortisol secretion.
In agreement with this hypothesis, in a previous study we demonstrated a plasticity of sensitivity of monocytes to GC in endurance-trained men (decreased in resting conditions and increased after an acute bout of endurance exercise) (9). Several mechanisms may explain these transient changes in sensitivity to GC. Upstream the classical hypothesis of variations in the amount of GC receptors (GR) (11), the extracellular and/or the intracellular cortisol availability could also be modified. Extracellular bioavailability depends on the free fraction of the hormone (free cortisol) and therefore depends mainly on the concentration of its binding protein: cortisol-binding globulin (CBG) (4). Intracellular bioavailability depends on tissue specific enzymes, 11β-hydroxysteroid dehydrogenases (11β-HSD), which interconvert hormonally active cortisol and inactive cortisone and have been shown to modulate cortisol action on target cells (27). It has been shown that the peripheral metabolism of cortisol can be assessed accurately from the urinary free cortisol/cortisone ratio, which is a good index of the measurement of whole body 11β-HSD activity (27). Using this new marker, we have shown for the first time that 24-h urinary cortisol/cortisone ratio was positively related to the total training load in a population of swimmers (1). However, there are no data on the interest of this ratio for the monitoring of the quality of exercise recovery. Our hypothesis was that the assessment of overnight urinary cortisol and cortisone output may provide a potentially incisive approach to investigate the delicate balance between cumulative fatigue resulting from exercise training and its recovery period. Indeed, the nocturnal sleeping period represents the phase where the hormonal profile is the most anabolic (increased ratio of GH to cortisol and of testosterone to cortisol) (16) and therefore the most favorable to exercise recovery.

Changes in resting plasma and/or urinary catecholamines (epinephrine (EPI) and norepinephrine (NOR)) have been suggested as possible tools for monitoring the impact of training load and/or overload (22, 24). However, despite abundant literature, there is no consensus concerning overnight catecholamines excretion in response to the training stress.

Therefore, the aim of the present study was to examine over a 10-month season in triathletes the evolution of overnight urinary output of cortisol, cortisone, and catecholamines with the training load and fatigue. Saliva cortisol responses to awakening, a noninvasive marker of the activity and reactivity of the hypothalamo-pituitary-adrenal (HPA) axis (26), was also monitored throughout the season (26). To dissociate the effect of the last bout of exercise to the training effect, all measures were realized after a 24-h abstention from exercise as 24-h cortisol secretion is unchanged between sedentary and trained subjects 2–4 h after the end of exercise (9). Finally, to dissociate the effects of training to those of seasonal hormonal variations, endurance-trained men were compared to sedentary men.

MATERIALS AND METHODS

Subjects. The study was approved by the hospital ethics committee, and informed written consent was obtained from all the subjects. Two groups of healthy male adults volunteered for this study: 9 untrained men (UT) (performing less than 1 h wk⁻¹ of physical activity for more than 3 yr) and 10 endurance-trained men (triathletes). The triathletes were competing regionally and nationally, with a weekly training average of 12 km of swimming, 160 km of cycling, and 40 km of running. All subjects were free of any medication, none had a personal or family history of psychiatric disorders or diabetes mellitus or any endocrine disorders. Subjects with sleep–wake schedule disorders were excluded. The subjects did not smoke or consume alcohol abusively. The physical characteristics of the individuals are shown in Table 1.

Experimental design. The subjects were recruited at the beginning of their training season and were studied in a prospective way during the season 2002–2003, from November to September. They reported to the laboratory on three occasions: in the first 15 d of November 2002, in the second 15 d of March 2003, and in the third 15 d of June 2003. The triathletes did not complete the test for fatigue reasons.

### TABLE 1. Anthropometric data, maximal oxygen uptake ($\overline{V}_\text{O}_2\text{max}$), and peak lactate concentration during $\overline{V}_\text{O}_2\text{max}$ in untrained (UT) men, trained men (triathletes), and overtrained (OT) triathletes.

<table>
<thead>
<tr>
<th></th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>Body Fat (%)</th>
<th>$\overline{V}_\text{O}_2\text{max}$ (mL·min⁻¹·kg⁻¹)</th>
<th>Peak Lactate Concentration (mmol·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>November 2002</td>
<td>25.1 ± 1.4</td>
<td>67.9 ± 2.3</td>
<td>178.2 ± 2.0</td>
<td>15.5 ± 1.1</td>
<td>39.6 ± 1.0#</td>
</tr>
<tr>
<td>N = 9</td>
<td>November 2003</td>
<td>26.5 ± 2.7</td>
<td>71.1 ± 2.4</td>
<td>175.7 ± 1.7</td>
<td>14.3 ± 1.7</td>
<td>59.4 ± 2.8</td>
</tr>
<tr>
<td>March 2003</td>
<td>November 2002</td>
<td>70.1 ± 2.3</td>
<td>186.2 ± 1.1</td>
<td>12.4 ± 1.1</td>
<td>64.1 ± 1.6</td>
<td>10.7 ± 0.9</td>
</tr>
<tr>
<td>Triathletes</td>
<td>N = 8</td>
<td>OT</td>
<td>March 2003</td>
<td>November 2002</td>
<td>67 ± 1.0#</td>
<td>TNC</td>
</tr>
<tr>
<td>TNC</td>
<td>March 2003</td>
<td>67 ± 1.0#</td>
<td>TNC</td>
<td>TNC</td>
<td>TNC</td>
<td>TNC</td>
</tr>
<tr>
<td>TNC</td>
<td>March 2003</td>
<td>67 ± 1.0#</td>
<td>TNC</td>
<td>TNC</td>
<td>TNC</td>
<td>TNC</td>
</tr>
</tbody>
</table>

# Significant difference between UT and triathlete group.

TNC, triathletes did not complete the test for fatigue reasons.
first 15 d of March, and in the last 15 d of June 2003. In November, triathletes had resumed their training for 1 month (the preceding season ended in October followed by 4 wk of resting). From November to January, training was devoted to fundamental endurance and technical training. Between February to May, high-intensity and volume training were performed. In June (precompetition period), volume training decreased and specific training was privileged. In July, the first competitions began until mid-September.

The protocol of each test visit is described in Figure 1. The subjects were instructed to maintain a regular sleep–wake schedule for 3 d before each visit and to maintain total fluid intake between 1.5 and 3 L·d⁻¹. For each test visit, the subjects woke up at 0700 h (after a 9-h sleep) and immediately collected 3 mL of saliva (natural unstimulated flow) in a plastic test tube. Then, they voided their bladder to collect overnight urine. Between 0700 and 0730 h, subjects rested in a lying position, and at 0730 h they collected a second saliva sample (3 mL). At 0800 h they had a standardized breakfast (15% protein, 30% fat, 50% carbohydrate, 500 kcal = 2100 kJ, without chocolate, caffeine, tea, banana, vanilla, or orange). They arrived at the laboratory at 0900 h without having performed any training session on the previous day. A screening visit with interrogation (psychological evaluation), physical examination, electrocardiography, and measure of percentage of body fat mass by the skinfold method (10) was performed, and triathletes and UT men completed a questionnaire to determine the total score of fatigue (TSF). The questionnaire was validated by Atlaoui et al. (1). The eight questions focused on perception of training, sleep, leg pain, infection, mental concentration, efficacy, anxiety, irritability, and general stress on the previous week. The responses to the questions were collected to obtain the total score of fatigue (TSF). The TSF was weighted and calculated according to the relative importance of each question in the score as previously reported (1). Intrasubject variability was 2.4%.

Total score of fatigue. Each visit in the laboratory was completed with an eight-item questionnaire on fatigue. Concentrations of the urinary unconjugated cortisol and cortisone were determined by high-performance liquid chromatography (HPLC) followed by UV spectrometry (13). Urine was centrifuged for 15 min at 4000 × g at 4°C and filtered through a single-use filter unit (0.22 μm). The urine volume was adjusted according to its dilution (i.e., according to creatinine concentration). Creatinine levels were determined using spectrophotometry absorption according to the manufacturer’s instructions (Sigma Diagnostics, St Louis, MO). This method is based on the diminution of a color emission derived from the reaction between creatinine and alkaline picrate. Thus, the difference in color intensity measured at 500 nm before and after acidification of the mixture is proportional to creatinine concentration. Cortisol and cortisone were eluted with absolute ethanol. Eluates were then evaporated during 4 h at 50°C. Dried residues were dissolved in a mobile phase and were injected with HR resting corresponding to resting heart rate, HR ex to the mean heart rate measured during exercise, HR max to the maximal heart rate, and duration measured in minutes. Cardiac frequency was monitored (Polar S-210 heart rate monitor) during each training sessions and total training load corresponded to the addition of the average training load measured in the three modes of exercise (swimming, running, and cycling).

Measure of performances. At the end of the period of racing (in September), triathletes listed each race performed during the season (with an average of 11 races performed between July to September per triathlete), with their placing, their performances (in minutes), their feelings, and, if they had taken part in this triathlon the last year, their previous placing.

Urinary analysis. Urine samples were collected overnight (from 2200 to 0700 h, corresponding to a 9-h period), the diuresis was noted, and 50 mL was frozen at −80°C until analysis. Concentrations of the urinary unconjugated cortisol and cortisone were determined by high-performance liquid chromatography (HPLC) followed by UV spectrometry (13). Urine was centrifuged for 15 min at 4000 × g at 4°C and filtered through a single-use filter unit (0.22 μm). The urine volume was adjusted according to its dilution (i.e., according to creatinine concentration). Creatinine levels were determined using spectrophotometry absorption according to the manufacturer’s instructions (Sigma Diagnostics, St Louis, MO). This method is based on the diminution of a color emission derived from the reaction between creatinine and alkaline picrate. Thus, the difference in color intensity measured at 500 nm before and after acidification of the mixture is proportional to creatinine concentration. Cortisol and cortisone were eluted with absolute ethanol. Eluates were then evaporated during 4 h at 50°C. Dried residues were dissolved in a mobile phase and were injected.
in the HPLC system. Intraassay and interassay coefficients of variation were < 9%.

Concentration of the urinary free epinephrine (EPI) and norepinephrine (NOR) was determined by HPLC with electrochemical absorbance detection (14). After centrifugation (15 min at 4°C, 4000 × g), the urine concentration was adjusted according to its creatinine concentration. Urine was laid on disposable cation-exchange resin columns (Bio-Rad, France). After three washings with water, catecholamines were eluted with 8-mL boric acid. Intraassay and interassay coefficients of variation were < 3%. Both glucocorticoids and catecholamines were expressed in micrograms per nocturnal period (9 h).

**Saliva cortisol analysis.** Saliva concentrations of cortisol were measured by radioimmunoassay (RIA) (Cortisol TKCO, Dade Behring, Paris, France). The procedure of the manufacturer was used with the following adaptations: buffer (0.05M Na2HPO4, 2 H2O, pH 7.3; 0.1% human albumin) was used to dilute the supplied human serum-based calibrators. The final concentration of cortisol ranged from 0 to 25 ng·mL−1. Saliva samples were performed in duplicate and calibrators in quadruplicate. Radio competition between saliva cortisol (400 µL) and 125I cortisol (1 mL) was performed. After incubation overnight on a shaking platform at room temperature, supernatants were aspirated. Then, radioactivity was measured for 1 min using a gamma counter (Wizard 1470, Wallac Oy, Finland). Results were expressed in nanomoles per liter, and intraassay and interassay coefficients of variation were < 3%. All samples (saliva and urine) were run in the same assay at the end of the study.

**Statistical analysis.** Results are expressed as mean ± SEM. Statistical comparisons were performed with Statistics software. Statistical significance was accepted at P < 0.05. As anthropometric data, maximal oxygen uptake and total score of fatigue values were nonnormally distributed, Wilcoxon’s test was used for the calculation between the paired items, and Mann–Whitney’s test was used to compare triathletes (N = 8) and UT group (N = 9). Training level (UT vs triathletes) and period of sampling (November, March, June) differences on urinary hormonal values were tested by two-way analysis of variance followed by post hoc Newman–Keuls test. Saliva cortisol response to awakening differences were tested by three-way analysis of variance (training level period of sampling time of sampling) followed by post hoc Newman–Keuls test.

During the follow-up period, two triathletes (OT1 and OT2) developed an overtraining syndrome and were removed from the triathlete group. Therefore, for each value of overtrained triathletes, normalized deviation (ND) was calculated by subtracting the value of OT man from the mean values of the group of well-trained triathletes and dividing by the standard deviation (SD). Results of the overtrained triathletes were considered as significant, only when ND values were twice less or more than SD values (± 2SD).

**RESULTS**

Table 1 shows that the anthropometric characteristics were not different between UT and triathlete subjects. By contrast, compared with UT men, the triathletes had a significantly higher VO2max. In the triathlete group, two individuals developed an overtraining syndrome during the season (OT1 and OT2). They presented the following criteria of overtraining (30): 1) high score of fatigue during the season with mood disturbances (loss of positive feelings: energetic, vigorous, helpful, calm, relaxed; and increase of negative feelings: irritable, depressed, moody, fatigue, anxious, confused, excited, unable to concentrate), sleeping disorders, and poor appetite; 2) high training load performed during the season; 3) inability to complete test of VO2max; and 4) decrease in performances with unwillingness to train and feelings of inability to successfully participate in races. OT triathletes were thus removed from the triathlete group and evaluated individually.

**Clinical results.** The results of both the auto-questionnaire (eight-item questionnaire on fatigue) and of the interrogation with the physician do not suggest that any of the triathletes was subjected to greater amounts of lifestyle stress than others.

Total training load was identical between the triathlete group and the two OT men in November (Table 2). Triathletes training load increased between November and March by 61%, whereas in the two OT men total training load increased by 220%. Thus, total training load level of OT men was higher than the triathlete group level in March. In June the total training load of the 2 OT men decreased by 44% and did not differ from the triathletes’ level.

Table 2 shows the evolution of the TSF during the season. UT men and triathletes TSF did not significantly vary throughout the follow-up period, and the scores were not different between the two groups. TSF of overtrained athlete 1 (OT1) was similar to the mean values of the triathlete group only in November, because it increased by 91% from November to March and by 45% from November to June. TSF of overtrained athlete 2 (OT2) was higher than the mean value of the triathlete group during the whole follow-up period, and compared with November, it increased by 36% in March and 42% in June.

Table 3 shows the evaluation of the performances of the triathletes and the OT men during the competition period. Whereas the triathletes improved their performance, there were a dramatic number of poor results in the two OT men.

**Hormonal results.** Figures 2 and 3 show the effects of training level and of the season on overnight urinary glucocorticoids and catecholamines excretion. Two-way ANOVA (training level season) indicated significant seasonal effects on cortisol (F2,45 = 4.24, P = 0.04), cortisone (F2,45 = 9.50, P = 0.0003), and EPI (F2,45 = 6.77, P = 0.002) excretions. A training effect was depicted on cortisone and EPI overnight levels. No interaction between training level and season was observed.

**Clinical results.** The results of both the auto-questionnaire (eight-item questionnaire on fatigue) and of the interrogation with the physician do not suggest that any of the triathletes was subjected to greater amounts of lifestyle stress than others.
the UT men and in June compared with March in the triathlete group. The same profile was observed for overnight urinary cortisone with significant decreased output between November and June, and between March and June in both groups (Fig. 2A, 2B). Moreover, in conditions where urinary cortisol excretion was similar between both groups (ANOVA: training effect: F_{1,45} = 0.77, P = 0.38), overnight urinary cortisone excretion was significantly higher in triathletes compared with UT men (ANOVA: training effect: F_{2,45} = 9.50, P = 0.003), suggesting that during a resting day there is a higher inactivation of cortisol into cortisone in highly trained men compared with their sedentary peers. Urinary cortisol and cortisone concentrations of the two OT men were comparable to the mean values (± 2 ND) (normalized deviation) of triathletes and UT groups, except for June, where cortisone excretion of the two OT men was higher than triathletes values (OT1: ND = 4.6; OT2: ND = 2.1). Cortisol/cortisone ratio remained stable from November to June in the UT men and in the triathlete group (Fig. 2C). By contrast, the ratios of the two over-trained subjects were markedly increased during the period of high intensity training (March): +131% and +46% between November and March for OT1 and OT2 triathletes, respectively. In March cortisol/cortisone ratios of OT men were twice higher than the highest triathlete ratio (cortisol/cortisone ratio OT1: 1.04; OT2: 1.35; higher triathletes ratio: 0.56), corresponding to 7.2 and 11.1 ND compared with the triathlete group. A detailed analysis of the results showed that the rise of OT men ratios was due to a sharp decrease in cortisone in conditions of high level of cortisol output. It should be noticed that in OT2 this pattern was present since November (ND = 4.1). In June, OT men ratio decreased and returned to the values measured in the triathlete group.

Training load was determined by a professional coach and was adjusted for an optimal progression of the triathletes during the season. Therefore, as the training load differed between triathletes, the cortisol/cortisone ratio was also expressed per unit of training across the season with the following results: November: OT1: 0.144, OT2: 0.269, triathletes: 0.099 ± 0.026 [range: 0.04–0.269]; March: OT1: 0.102, OT2: 0.124, triathletes: 0.069 ± 0.007 [0.03–0.08]; June OT1: 0.032, OT2: 0.046, triathletes: 0.106 ± 0.02 [0.03–0.19]. Even expressed per unit of training, the differences in cortisol/cortisone ratio remained as OT2 presented in March a cortisol/cortisone ratio expressed per unit of training significantly increased (ND = 2.0) compared with the triathlete group, whereas the cortisol/cortisone ratio of OT1 approached 2ND (ND = 1.8). However, the value of OT1 remained superior to the highest value measured in the triathlete group (0.093 vs 0.124 highest value of the triathlete group vs OT1 value). For these reasons, we do not believe that the results obtained in OT1 and OT2 are explained by differences in their training load.

In UT men, overnight excretion of EPI was significantly increased between November and March (P < 0.05) and significantly decreased between March and June (P < 0.05). The same pattern was observed in the triathlete group,
except that the decrease in EPI output between March and June did not reach statistical significance. Consequently, EPI excretion was significantly increased in June in the triathlete group compared with UT men, explaining the training effects exhibited in EPI excretion (ANOVA: training effect: $F_{1,45} = 8.42, P = 0.005$). In both groups, over-

### TABLE 3. Performances of the triathletes and the two overtrained men.

<table>
<thead>
<tr>
<th>No. of Races (June to September)</th>
<th>Percentage of Poor Results</th>
<th>Progress</th>
<th>Break during the Competition Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triathlete 1</td>
<td>18</td>
<td>33%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 2</td>
<td>13</td>
<td>15%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 3</td>
<td>13</td>
<td>38%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 4</td>
<td>11</td>
<td>45%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 5</td>
<td>3</td>
<td>33%</td>
<td>No</td>
</tr>
<tr>
<td>Triathlete 6</td>
<td>2</td>
<td>0%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 7</td>
<td>5</td>
<td>20%</td>
<td>Yes</td>
</tr>
<tr>
<td>Triathlete 8</td>
<td>6</td>
<td>0%</td>
<td>Yes</td>
</tr>
<tr>
<td>Overtrained triathlete 1</td>
<td>11</td>
<td>72%</td>
<td>No</td>
</tr>
<tr>
<td>Overtrained triathlete 2</td>
<td>6</td>
<td>67%</td>
<td>No</td>
</tr>
</tbody>
</table>

These data summarized the major points of the responses of the triathletes to the questionnaire completed in September 2003. Percentage of poor results was determined from the triathletes placing during each race and in comparison with their previous results achieved the last year (competition period 2002). Progress was determined from the triathletes performances during the season races and in comparison with their previous performances achieved the last year. $\Delta$, normalized deviation (ND) > 2.0.

FIGURE 2—Overnight urinary excretion of cortisol (A) cortisone (B) and cortisol/cortisone ratio (C) in untrained men (UT), trained men (Triathletes), and overtrained men (OT). *$P < 0.05$ compared with June within the same group; normalized deviation > 2.0; normalized deviation > 7.0.
night excretion of NOR remained stable during the seasons, and no difference was measured between the two groups. The two overtrained triathletes showed concentrations similar to the mean value of the triathlete group (Fig. 3A, 3B), except in June where OT2 man showed a higher level of EPI output than the triathlete group (ND/H11005/6.2). Figure 3C shows that the EPI/NOR ratio tended to rise in March in the UT and triathlete group and to decrease thereafter in UT but not in triathlete group. In the case of OT1, the same trend was observed. On the contrary, EPI/NOR ratio of OT2 was decreased between November and March, with an inverse pattern in June.

Table 4 shows saliva cortisol concentrations after awakening throughout the 10-month follow-up period. Three-way ANOVA indicated only significant time of sampling effects ($F_{1,89} = 27.12$, $P < 10^{-6}$) on saliva cortisol concentrations (0730 h > 0700 h). No training ($F_{1,89} = 0.23$, $P = 0.62$) nor seasonal effects ($F_{2,89} = 2.61$, $P = 0.07$) were depicted. Consistent with earlier findings (17), post hoc analysis indicated that saliva free cortisol levels increased significantly between 0700 and 0730 h in the UT men. The same profile was observed in the triathletes men excepted in June. To obtain indices for the cortisol response to awakening, the mean increase (increment) of cortisol was calculated ($\left(\text{cortisol 0730 h} - \text{cortisol 0700 h}\right)/\text{cortisol 0700 h}$). Two-way ANOVA indicated significant training effects on saliva cortisol increment in response to awakening ($F_{1,44} = 4.37$, $P = 0.04$). Post hoc analysis indicated that when compared with November, this increment rose significantly in UT men in March and June, whereas this increment remained stable in triathlete men during the follow-up period. In November, cortisol increment of OT1 was higher...
than the triathletes mean value. It decreased dramatically between November and March and then did not differ from the triathlete values in March and June. The cortisol increment of OT2 decreased progressively during the season and was zero in June. His values did not differ from those of the triathlete group during the follow-up period (ND < 2.0), and it should be noticed that this null increment was also observed in some UT and triathlete men.

**DISCUSSION**

This study is the first to report in a population of untrained and highly trained subjects’ seasonal variations of overnight urinary GC and catecholamines outputs and of saliva cortisol responses to awakening, indicating that neglecting the hormonal seasonal variations may introduce errors in conclusion concerning the hormonal responses to training. Moreover, we report a parallel variation of cortisol and cortisone overnight urinary concentrations during seasonal variations, both in UT men and in well-trained men, suggesting that any significant increase in overnight cortisol secretion is balanced by its parallel inactivation into cortisone. The physiological importance of this mechanism may be protection against the deleterious effects of prolonged increased cortisol secretion. This is evidenced in triathletes, that is, in subjects subjected to repeated transitory hypercortisolism due to exercise-induced cortisol secretion (7,8,9), who present, in resting conditions, a higher inactivation of cortisol into cortisone than their untrained peers. The importance of this mechanism is also highlighted in the two OT men who presented in March a sharp decrease of inactivation of cortisol into cortisone.

In the triathlete group, two individuals developed an overtraining syndrome during the training season 2002–2003 (OT1 and OT2). As OTS is rare, in most of studies overtraining is voluntary induced by increasing the volume and the intensity of training. Therefore, it could be speculated that the hormonal modifications reported with this “voluntary” OT are not specific to the OT state but are instead caused by heavy training (29). In the present study, longitudinal follow-up depicted two OT athletes. Although this number is too weak to allow definite conclusion, we believe that longitudinal follow-up of athletes represents the only available tool to improve the (hormonal) diagnosis of OT. For this reason, and because the hormonal mechanisms underpinning the pathogenesis of OT remain unclear, we have reported and discussed herein the results of the two OT triathletes.

Contrary to venous forearm blood, where NOR is disproportionately influenced by forearm sympathetic activity (15), urinary excretion of catecholamines provides information of the whole sympathoadrenal system secretion integrated over the period of the urine collection. The same is true for urinary free cortisol (UFC) excretion because 24-h UFC represents an integrated measure of the 24-h cortisol secretion, and therefore an increased 24-h UFC corresponds to increased cortisol production rates (4). In the present study, we decided to measure nocturnal urinary catecholamines and GC excretion, instead of 24-h excretion, because the nocturnal sleeping time represents the period where the hormonal profile is the most anabolic (increased ratio of GH to cortisol and of testosterone to cortisol) (16). Therefore, overnight hormone output assessment represents a potentially incisive approach to investigate the delicate balance between cumulative fatigue resulting from exercise and the recovery period. It should be emphasized that urinary samplings were made after a 24-h abstention from exercise, therefore reflecting the effect of training and excluding the acute effect of the last exercise as 24-h UFC secretion is unchanged between sedentary and trained subjects 2–4 h after the end of exercise (9).

There is no report on overnight urinary free cortisol (UFC) values in endurance-trained men. On the other hand, despite abundant literature, there is no consensus concerning overnight catecholamines excretion for monitoring the impact of training load and/or overload. Declines in nocturnal urinary NOR have been shown after 4 wk of intensified training in runners becoming stale (20) and overtrained swimmers (24) displaying performance drops, whereas declines in urinary NOR have been reported in successful athletes (22). In contrast, increases in nocturnal urinary NOR have been shown in cross country skiers (18) with best competition results. Our results indicate that seasonal variations of catecholamines secretion, a too often forgotten physiological parameter, may explain some of these discrepancies. Indeed, a circannual rhythmicity of 24-h catecholamines excretion has been reported by Hansen et al. (12) with an increase in 24-h EPI excretion in June and July.

The table below shows the saliva cortisol concentrations at 0700 and 0730 h (nmol·L⁻¹) and its increment (%) between 0700 h and 0730 h (mean ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>UT</th>
<th>Triathletes</th>
<th>Overtrained</th>
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<tbody>
<tr>
<td><strong>Cortisol 0700 h (nmol·L⁻¹)</strong></td>
<td>13.0 ± 0.9</td>
<td>15.0 ± 0.0</td>
<td>12.0 ± 0.0</td>
</tr>
<tr>
<td><strong>Cortisol 0700 h (nmol·L⁻¹)</strong></td>
<td>15.8 ± 1.4</td>
<td>22.8 ± 2.5</td>
<td>15.7 ± 1.9</td>
</tr>
<tr>
<td><strong>Cortisol increment 0700–0730 h (%) (UT &gt; Triathletes)</strong></td>
<td>42.4 ± 13.9</td>
<td>8.3 ± 13.1</td>
<td>46.0 ± 23.0</td>
</tr>
</tbody>
</table>

The mean increase (increment) of cortisol was calculated as follows (results are expressed in %): [(cortisol 0730 h – cortisol 0700 h)/cortisol 0700 h] × 100. In some cases, a moderate decrease was found between cortisol 0700 h and cortisol 0730 h. In these cases, the increment was considered as zero (instead of negative) for two reasons: 1) these negative percentages were too weak to significantly affect the mean values of increment (data not shown), and 2) the lack of increment is more important than the magnitude of the negative change.

<table>
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</thead>
<tbody>
<tr>
<td><strong>Cortisol 0700 h (nmol·L⁻¹)</strong></td>
<td>13.0 ± 0.9</td>
<td>15.0 ± 0.0</td>
<td>12.0 ± 0.0</td>
<td>8.2 ± 0.0</td>
<td>5.5 ± 0.5</td>
<td>7.1 ± 0.7</td>
</tr>
<tr>
<td><strong>Cortisol 0700 h (nmol·L⁻¹)</strong></td>
<td>15.8 ± 1.4</td>
<td>22.8 ± 2.5</td>
<td>15.7 ± 1.9</td>
<td>12.6 ± 1.3</td>
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<td>9.9 ± 1.3</td>
</tr>
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</tr>
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The table above shows the saliva cortisol concentrations at 0700 and 0730 h (nmol·L⁻¹) and its increment (%) between 0700 h and 0730 h (mean ± SEM).
compared with the rest of the year, whereas no variation of NOR occurred (12). We report in this study seasonal variations of overnight EPI output in UT men with an increase in March compared with November and June, whereas NOR output exhibited no seasonal variation. Therefore, this study provides for the first time evidence that seasonal variations of overnight catecholamines differ between the 24-h collection (diurnal plus nocturnal phases) and the nocturnal phase. By contrast the overnight seasonal variations of cortisol and cortisone excretion are similar to those reported for 24-h urinary cortisol excretion (31) with a sharp decrease of the two glucocorticoids between November and June. In triathletes, seasonal variations of GC and EPI excretion are also found. Similarly to the UT men, the EPI output of triathletes increased between November and March, excluding a training effect. In contrast with UT men, triathletes showed no decrease of EPI excretion between March and June and consequently had a significant higher level of EPI output in June. This increase of overnight catecholamines output in the triathlete group probably resulted from exercise training (high total training load period followed by competition period), as it has been shown that excretion of catecholamines is correlated with training load (5) and emotional stress (21). In the present study, on the contrary, compared with UT, cortisone excretion was significantly increased during all the follow-up due to a training effect. This is associated with seasonal variations but without any interaction between training and seasonal effects. Therefore, the results of the present study indicate that neglecting the hormonal seasonal variations may introduce errors in conclusions about the hormone responses to training and/or overtraining. Although variations of overnight concentrations of EPI, cortisol and cortisone occurred in endurance trained men during the 10-month follow-up, a training effect was only depicted in June EPI and in overall cortisone excretion.

The HPA axis plays a pivotal role in both the response to acute exercise and the adaptation to endurance training. Repeated exercises induce adaptation of the HPA axis with reduced glucocorticoids receptor levels (11) and decreased tissular sensitivity to glucocorticoids (9). These adaptations could be beneficial for the athletes limiting prolonged exposure of their tissues to glucocorticoids. Studies of plasma cortisol levels are limited because in conditions of normal or subnormal plasma cortisol levels variations in extracellular and intracellular cortisol availability may occur. Indeed, although plasma cortisol concentrations can be measured accurately, the biological effect of cortisol on the target tissues is uncertain. Extracellular bioavailability depends on the free fraction of the hormone, that is, free cortisol. Cortisol largely binds to plasma proteins and especially to the cortisol-binding globulin (CBG) (4). Thus, plasma cortisol levels are modulated by variations of CBG and poorly correlate with cortisol production rates, unless differences in CBG are corrected for (4). Conversely, saliva and urinary cortisol concentrations are independent of CBG concentrations and thus closely reflect the free-active-plasma cortisol (19). However, there are also conflicting reports on the
tissues (27). The activity of 11β-HSD2, which inactivates cortisol to cortisone is constitutive and is mainly expressed in the kidney, in which it presents an effective barrier to cortisol access to mineralocorticoid receptors under all conditions (27). By contrast, the activity of 11β-HSD1 is regulated by hormones (mainly upregulated by glucocorticoids, and to a less extent downregulated by estrogens and IGF-I) and cytokines (upregulated by TNFα and IL-1β but only in adipose tissue) and is expressed in numerous tissues where it converts the inactive cortisone to active cortisol (27). The crucial physiological principle illuminated by the action of 11β-HSD is that cortisol action on target cells is determined by enzyme activity within the cells, rather than circulating cortisol levels alone. It has been shown that the peripheral metabolism of cortisol can be assessed accurately from the urinary free cortisol/cortisone ratio, which is a good index of whole body 11β-HSD activity (27). It should be noted that for determination of urinary free cortisol and urinary free cortisone, the HPLC method showed a better efficiency than the method of competitive binding and must be preferred in the measure of cortisol/cortisone ratio in urine (23). As the nocturnal period is essential for exercise recovery (16), we have decided to focus in the present study on overnight glucocorticoids output for monitoring the delicate balance between cumulative fatigue resulting from exercise training and its recovery period. The effect of the last exercise can be ruled out as all assessments have been realized after a 24-h abstention from exercise. In accordance with our previous findings reporting that during a day without exercise 24-h urinary cortisol excretion is similar between trained and untrained men (7), overnight urinary cortisol excretion was also unchanged between the UT and triathlete group. Intriguingly, we report for the first time a training effect on overnight urinary cortisone excretion with higher levels in trained subjects than in their sedentary peers, suggesting a higher tissular inactivation of cortisol into cortisone in conditions where cortisol production is similar. This interesting hypothesis of increased intracellular inactivation of cortisol during the night in well-trained men are broadly in keeping with our previous reports of the existence of a plasticity of tissular sensitivity to glucocorticoids in endurance-trained men, superimposed to systemic cortisol concentrations (9). The present results highlight the diversity of potential mechanisms developed by trained men to protect their tissues against the effects of exercise-induced increased cortisol secretion.

This tissular protection is also effective in UT men. Whereas overnight excretion of glucocorticoids showed significant seasonal variations, conversely, overnight urinary cortisol/cortisone ratio remained stable in the UT and triathlete group during the follow-up period, suggesting that in physiological conditions, any significant increase in cortisol secretion (seasonal-induced increased cortisol secretion) is balanced by the parallel increase in its tissular inactivation in cortisone. In a previous study, we have shown that, in swimmers, when measured during a competitive day, 24-h urinary cortisol/cortisone ratio was positively related to the total training load (1). Conversely, in this study overnight urinary cortisol/cortisone ratio, measured during a resting day, 24 h after the last exercise, was unchanged even if the training load increased. This apparent contradiction can be resolved if we assume that during an exercise day cortisol effects are necessary for the protein turnover and for its anti-inflammatory effects, especially if the training load is high (25). On the opposite, during the night, the effects of cortisol may be deleterious in the face of predominant protein anabolism. Therefore, the cortisol/cortisone ratio should be low to favor muscular recovery triggered by anabolic hormones like testosterone and growth hormone (16). Taken as a whole, the data of these two studies suggest that peripheral metabolism of cortisol may play a role in both acute (competitive day (1)) and chronic effects of exercise (24 h after the last exercise, present study). More intriguing, and perhaps the stronger evidence of the importance of this cellular inactivation of cortisol, is the association of high cortisol-to-cortisone ratio and overtraining. Low inactivation of cortisol into cortisone (cortisol/cortisone > 1) in overtrained triathletes suggests either an increased activity of 11β-HSD1 and/or an inhibition of 11β-HSD2. Whatever the mechanisms involved (hormones, cytokines), they translate into an increase in cortisol action on the target tissues even if circulating cortisol levels are stable. Finally, our results raise for the first time the possibility that the increase of overnight urinary cortisol/cortisone ratio above 1 might be a predicitive marker of overtraining or might translate an important risk for the athlete to develop an overtraining syndrome. However, future study with a greater number of overtrained subjects will be necessary to rigorously examine this hypothesis.

In conclusion, this study points to significant seasonal variations in overnight glucocorticoids and catecholamines concentrations and in saliva cortisol response to awakening, which when not taken into account may induce errors in the interpretation of hormonal variations with training. This is also the first report of an increase in intracellular inactivation of cortisol during the night in endurance trained men after a 24-h abstention from exercise. This is in agreement with our previous studies reporting the existence of a plasticity of tissular sensitivity to glucocorticoids, highlighting the diversity of the mechanisms set up by the organism to adapt to HPA axis activation associated with intensive training. Lastly, as biological research on the neuroendocrine system depends on the availability of noninvasive and clinically relevant markers, we propose that overnight urinary cortisol/cortisone ratio (assessed by HPLC) might be a valuable tool for uncovering even subtle changes in HPA function during training and/or OT.

We thank Nicoline Geverink for revising a previous version of this paper and Guillaume Nicolas for his help in recruiting the triathlete population. We also thank the subjects for their participation in this study.
REFERENCES


