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Profiling of urinary testosterone and luteinizing hormone in exercise-stressed male athletes, using gas chromatography–mass spectrometry and enzyme immunoassay techniques

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Abstract

Knowledge of the effects of episodic or short-term exercise-stress on endogenous testosterone and luteinizing hormone levels still remains fragmentary and inconclusive. In this study, an approach based on the absolute concentrations of urinary total testosterone (T), luteinizing hormone (LH) and the T/LH concentration ratios, was used to profile short-term exercise-stress responses in healthy drug-free male athletes. Testosterone and luteinizing hormone concentrations were measured using gas chromatography–mass spectrometry (GC–MS) and microparticle enzyme immunoassay (MEIA) techniques, respectively. Stress profiles derived from exercise-stress at VO_2max, 68.1% VO_2max and 51.6% VO_2max were plotted using the concentrations of T, LH and the ratios of T/LH found under non-stressed and stressed conditions. Significant changes in LH concentrations ($p<0.005$) and T/LH ratios ($p<0.005$) levels were observed between the pre-stress and post-exercise conditions during acute exercise-stress at VO_2max but the T concentration did not show any marked change relative to the non-stressed condition. Whilst exercise-stress appeared to reduce the change in T concentrations between the pre- and post-exercise states compared to that in the non-stressed control condition, the change in LH concentrations showed a moderate increase at submaximal oxygen uptake values. The stress profiles derived from this study facilitated an assessment of the relationship between the endogenous T, LH and T/LH ratio stress-responses over a short period of applied exercise-stress.

Keywords: Testosterone; Luteinizing hormone

1. Introduction

At present, an untimed urine sample is collected from an athlete for testing of prohibited substances. In the case of endogenous hormones such as testosterone (T), detection depends upon the resultant alteration in the pattern of hormonal excretion in epitestosterone (Epi-T) and luteinizing hormone (LH). For example, urine samples obtained from men dosed with supraphysiological doses of T have shown elevated ratios of T/LH and T/Epi-T [1]. Under the regulations of the International Olympic Committee (IOC) [2], a T/Epi-T ratio greater than the value of 6.0 may result in disqualification of, and disciplinary action on, the athlete. To date, the T/LH ratio is also employed as an additional tool to facilitate evaluation of abnormal T/Epi-T values.

Changes in the excretion of T and LH may also

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result from acute exercise-stress response in the human. The hypothalamus–pituitary–testicular system is affected by stress [3] and this may also lead to changes in the concentrations of plasma T [4] and LH [5], which may subsequently alter the T/LH ratio. At present, evaluation of the data presented in the literature [6–8] indicates that current knowledge of the effects of exercise-stress on the concentrations of T and LH appears to be fragmentary and inconclusive. Recent attempts by De Boer [9] to profile T/LH responses to exercise-stress in urine samples, that were collected without fixed time-points, showed no significant changes in the T/LH ratios in exercise.

As emotional, neural and hormonal input effect the androgenic steroids, LH and other target tissues under the direct control of the hypothalamic–pituitary–adrenal axis in the human (Fig. 1), it appears that the intensity of exercise-stress may alter both the T and LH responses. It may also be possible that these responses depend on the condition and training status of the athlete. For instance, untrained or lightly trained subjects who are subjected to similar exercise-stress as conditioned athletes may not produce similar T and LH responses. Therefore, assessment of the relationship between T, LH responses and training status may help expand the current knowledge of T and LH profiles in exercise-stress response.

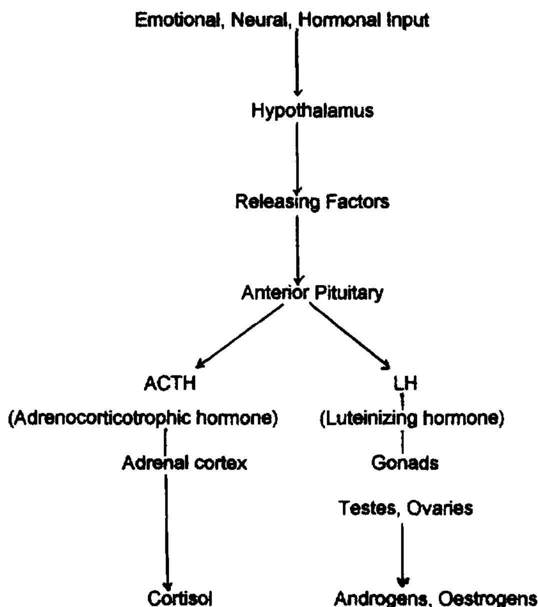

Fig. 1. Cortisol, androgenic steroids, luteinizing hormone and target tissues under the direct control of the hypothalamic–pituitary–adrenal axis.

To examine this relationship the urinary concentrations of T and LH were determined using gas chromatography–mass spectrometry (GC–MS) and multiple enzyme immunoassay (MEIA), respectively, following the application of a series of exercise-stress tests on a group of male athletes.

2. Experimental

2.1. Chemicals and reagents

N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), ammonium iodide (Sigma, St. Louis, MO, USA), β-glucuronidase *Escherichia coli* enzyme (Boehringer Mannheim, Mannheim, Germany). XAD-2 resin (0.1–0.2 mm mesh size, Serva, Heidelberg, Germany), testosterone and methyltestosterone (Sigma), potassium hydrogen carbonate (BDH Poole, UK), potassium carbonate and di-sodium hydrogen orthophosphate (BDH, Kilsyth, Victoria, Australia), potassium dihydrogen phosphate (May and Baker, W. Footscray, Victoria, Australia), methanol (Mallinckrodt, Paris, KY, USA), dithioerythritol (Sigma), diethyl ether (Mallinckrodt), IMx LH mode 1 calibrator (2239-40), IMx LH control (2239-10) (Abbott Diagnostics, Sydney, Australia), ultrafiltration centrifugal microconcentrators (10 000 molelular mass cut-off) (Amicon, Beverley, MA, USA) and Mulstitix 8 SG Reagent Strips (Bayer, Mulgrave, Victoria, Australia) were used.

2.2. GC–MS

Selected-ion monitoring (SIM) measurements were performed with a Hewlett-Packard 5890 gas chromatograph and a 5970 mass-selective detector coupled to the Unix data processing system. The mass-selective detector was set at 70 eV and focused on the following characteristic ion produced by electron impact: T, m/z 432 (M^+), methyltestosterone internal standard, m/z 446 (M^+).

2.3. GC–MS conditions

A HP Ultra-1 capillary cross-linked methyl silicone (17 m×0.22 mm I.D., 0.11 μm) column was used in the gas chromatograph. Ultra-high purity helium at a flow-rate of 1 ml/min was used as the carrier gas. A split-injection (10:1) mode was used, with the injector set at 300°C. The initial oven temperature was set at 162°C and programmed to increase by 3°C/min to 232°C, after which the temperature was increased by 50°C/min to 300°C and held for 2 min. The detector temperature was set at 300°C.

2.4. Sample preparation

The urine samples (5 ml) for the determination of testosterone were prepared and derivatized according to the established procedures [10] that are used in the IOC accredited laboratories. Briefly, the urine was eluted through a column of XAD-2 resin in a Pasteur pipette. The column was washed with demineralised water and the steroids were subsequently eluted with methanol. The eluent was vacuum-evaporated to dryness and enzymatically hydrolysed in a pH 7 buffer solution at 50°C for 1 h. The steroids were extracted at pH 9.5–10 with diethyl ether and the extract was vacuum-evaporated to dryness. The dried residue was derivatized with a MSTFA–TMS-iodide–dithioerythritol mixture at 60°C for 15 min and subjected to GC–MS analysis.

The urine samples (4 ml) for the determination of luteinizing hormone were prepared according to the procedure described by Cowan et al. [11]. Details of the procedures need not be repeated here as they have been described elsewhere [11].

2.5. Preparation of calibration graphs

Standard methanolic solutions (5 ml) containing 6, 12, 18, 30, 60 and 120 μg/l of testosterone and 100 μg/l of the methyltestosterone internal standard were used to prepare the testosterone calibration graphs. Peak-area response ratios were plotted against concentrations of testosterone. The graph was linear in the 0–120 μg/l concentration range with a correlation coefficient of 0.978. The intra-assay and inter-assay coefficients of variation at the 12 and 30 μg/l level ranged from 1.6 to 1.7% and 2.3 to 4.1%, respectively.

Standard solutions containing IMx LH calibrators of 0, 2, 10, 25, 100 and 250 mIU/ml of LH prepared in calf serum (supplied by Abbot Diagnostics) were used for calibrating the LH calibration graphs. The graph was linear in the 0 to 250 mIU/ml range with a correlation coefficient of 0.991. The intra-assay coefficients of variation were 2.02, 1.84 and 3.29% for 5.0, 40.0 and 80.0 mIU/ml of LH, respectively. The inter-assay coefficient of variation at 4.5–5.5 mIU/ml was 13.4%.

2.6. Test subjects

Groups of healthy, drug-free male athletes participated in the study. They were as follows: Group 1 (twelve subjects) was exercise-stressed at VO_2max; Group 2, containing nine subjects (seven were common with Group 1), was tested at 60–70% VO_2max; Group 3, with seven subjects (of whom three were part of Groups 1 and 2), was tested at 51% VO_2max; Group 4, with eight subjects (one subject had been part of Groups 1, 2 and 3, one participated in Groups 1 and 2, one was in Group 1 only and one was in Group 3 only) acted as the controls and did not exercise but sat quietly in the same room that was used by the pre- and post-test groups of athletes. All the athletes were selected from a pool of volunteers at the Human Movement Science Department of the Southern Cross University, Lismore, New South Wales. The reason for treating subjects in separate groups was because of the unavailability of many athletes to participate in the complete trial, due to sporting competition commitments during this study. All the submaximal exercise-stress tests were conducted in a random order following VO_2max. Ethical permission for the study was obtained from the Ethics Committee of the Southern Cross University and written informed consent was given by the athletes. The experimental procedures were fully explained to the subjects who also completed a Medical Questionnaire, which included medical history and training status. The subjects were given a full medical examination by a qualified Medical Practitioner and the tests also included an electrocardiogram check prior to the exercise-bout.

All the subjects were non-smokers and did not consume coffee or tea, cola or chocolate on a regular basis. The subjects abstained from exercise for 24 h and no food was consumed in the 2-h period before the test. Table 1 shows the mean physical characteristics of both test subjects and controls. Application of the two-tailed F-Test [12] to the data in Table 1 did not show any statistically significant difference ($p<0.05$) between all four groups of subjects for age, height and weight.

The test subjects were exercise-stressed at three levels of oxygen uptake, these being maximal oxygen uptake (VO_2max), 68.1±3.0% VO_2max and 51.6±3.0% VO_2max. The control group was not exercised, but sat quietly and relaxed in an air-conditioned room (22°C, 55% humidity) for the same duration as the exercise-bout. All the subjects were tested in the same time period of the day, to minimise the diurnal effects of the body. A summary list showing subject participation in the different groups tested at random is compiled in Table 2.

2.7. Sampling

The subjects were required to void their bladder approximately 1 h before the test. A pre-exercise urine sample was obtained at the end of a rest-period of 15 min in the air-conditioned room. Subsequently, the subject proceeded to the test-laboratory for stress-testing. After the exercise-bout, the subject returned to the room and rested for another 15 min after which a post-exercise urine sample was obtained. Immediately after sampling, the urines were stored at −4°C in the freezer. At the end of the test session, all the samples were transferred to a freezer kept at −20°C for storage until they were analysed.

A similar sampling protocol was applied to the control group but no stress-testing was performed. The controls sat and read quietly in a separate air-conditioned room for the same duration of time as the exercise-bout.

Renal function of the subjects throughout the study was monitored to detect any abnormal changes between the pre- and post-exercise conditions by using the Diagnostic Mulstitix (8 SG) reagent strips. The parameters on each reagent strip were glucose, ketone, blood, pH, specific gravity, protein, nitrite and leucocytes.

2.8. Maximal oxygen uptake (VO_2max)

All the testing was performed on a computerised treadmill in an air-conditioned laboratory maintained at 22°C and 55% relative humidity. The exercise protocol consisted of a 5-min warm-up period when the subject jogged at a speed of 5 km/h at a 1% gradient. The speed was subsequently increased by 2 km/h every 2 min up to 10 km/h. The treadmill gradient was subsequently increased by 1% each min until maximum oxygen uptake was obtained. Testing was terminated if the subject demonstrated an inability to maintain the desired workload, volitional termination due to exhaustion, or when a decline in peak VO_2 with increased workload was evident. The subject's heart rate, oxygen consumption and carbon dioxide levels were monitored electronically at 15 s intervals.

2.9. Submaximal oxygen uptake

The submaximal oxygen uptake values were calculated based on the previously determined VO_2max levels. A linear regression equation based on the subject's oxygen consumption rate and workload during the treadmill exercise-bout was used to predict the treadmill running speed that would elicit

Table 1
Physical characteristics of male Caucasian athletes who participated in the study

Subject group	Age (years)	Height (m)	Weight (kg)
Control (n=8)	20.4±1.8	1.79±0.10	74.0±9.6
VO_2max (n=12)	19.8±1.5	1.79±0.06	68.2±9.4
68% VO_2max (n=9)	19.5±1.3	1.77±0.05	65.0±7.0
51% VO_2max (n=7)	19.4±1.3	1.74±0.05	66.7±9.1

Values are mean±S.D.

Table 2
Summary list showing subject participation in the different groups of athletes tested at random

Subject	Group 1 VO_2max	Group 2 68% VO_2max	Group 3 51% VO_2max	Group 4 Control/non-exercised
LS	x	x		x
SC				x
CU				x
GH				x
AG	x	x	x	x
DC				x
SH	x			x
JW			x	x
BH	x	x	x	
DG	x			
BR	x	x	x	
KE	x			
1.3	x			
3.3	x			
4.3	x			
5.3	x			
9.3	x			
JW		x		
SB		x		
JM		x		
JH		x		
CC		x		
MF			x	
GA			x	
AB			x	

an oxygen uptake value in the range of 68–70% and 51–52% of the subject's VO_2max. Each subject was exercised on the treadmill at the calculated speed for approximately 25–30 min at the 1% gradient for each VO_2 value on separate days.

3. Results

Evaluation of the Diagnostic Mulstitix reagent strips used to test the urine samples did not reveal any marked changes in the renal function of the subjects during the pre- and post-exercise conditions.

The mean testosterone concentrations in the urine were not significantly different between the basal and post-exercise conditions for exercise-stress at VO_2max and submaximal oxygen uptake values (Fig. 2b–d). The post-exercise mean testosterone concentration for the control group appeared markedly suppressed compared to that in the basal state. This was due to the low testosterone concentration excreted by one subject under the control conditions (Fig. 2a). Fig. 5a illustrates the overall pattern of the mean T stress-response changes for the range of exercise-stress tests applied. Exercise-stress appeared to reduce the overall T responses, when compared to the resting profiles of the controls (Fig. 5a).

A survey of the change in the mean LH level in the subjects showed a significant increase in the mean LH concentration ($p<0.005$, paired t-tests) [12] at post-exercise relative to pre-exercise values, during exercise-stress at VO_2max (Fig. 3b). No significant changes between the pre-stress and post-exercise conditions were observed for the controls (Fig. 3a) and exercise-stress responses at submaximal VO_2 values (Fig. 3c–d). However, there appears to be a trend showing a moderate increase in the change in LH concentrations for the range of exercise-stress tests used relative to the non-exercised controls (Fig. 5b).

When the LH concentrations were ratioed to the testosterone concentrations, a significant decrease in

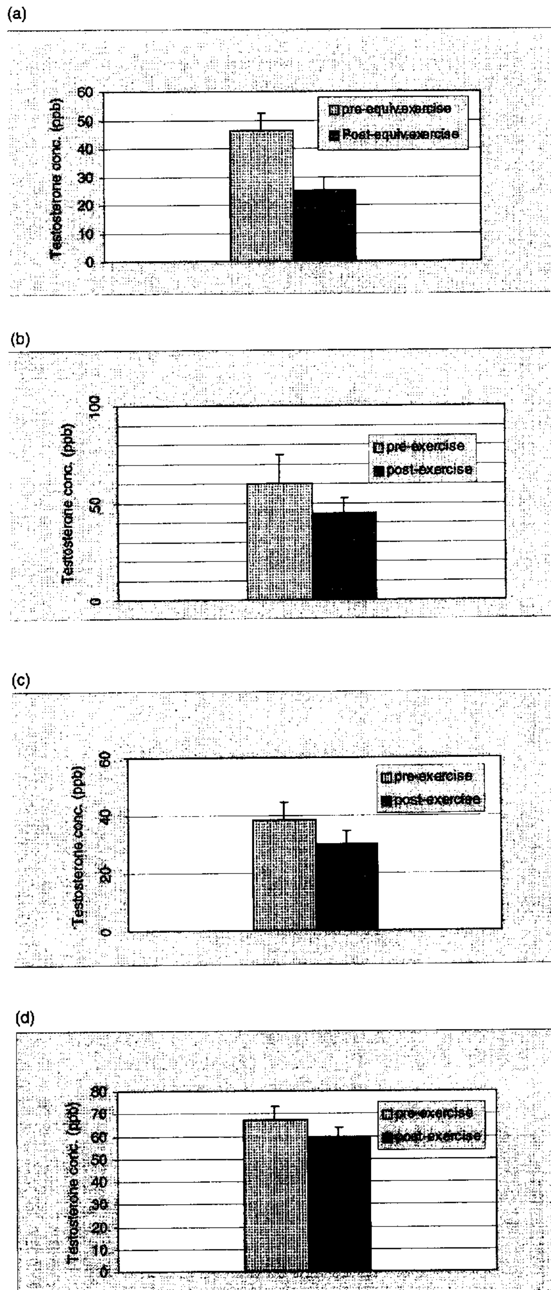

Fig. 2. (a) Mean testosterone resting-profiles of nine athletes (S.E.M.) in the control group. (b) Mean testosterone stress-profiles of nine athletes (S.E.M.) exercise-stressed at VO_2max. (c) Mean testosterone stress profiles of nine athletes (S.E.M.) exercise-stressed at 68.1±1.8% VO_2max. (d) Mean testosterone stress profiles of seven athletes (S.E.M.) exercise-stressed at 51.6±3.0% VO_2max.

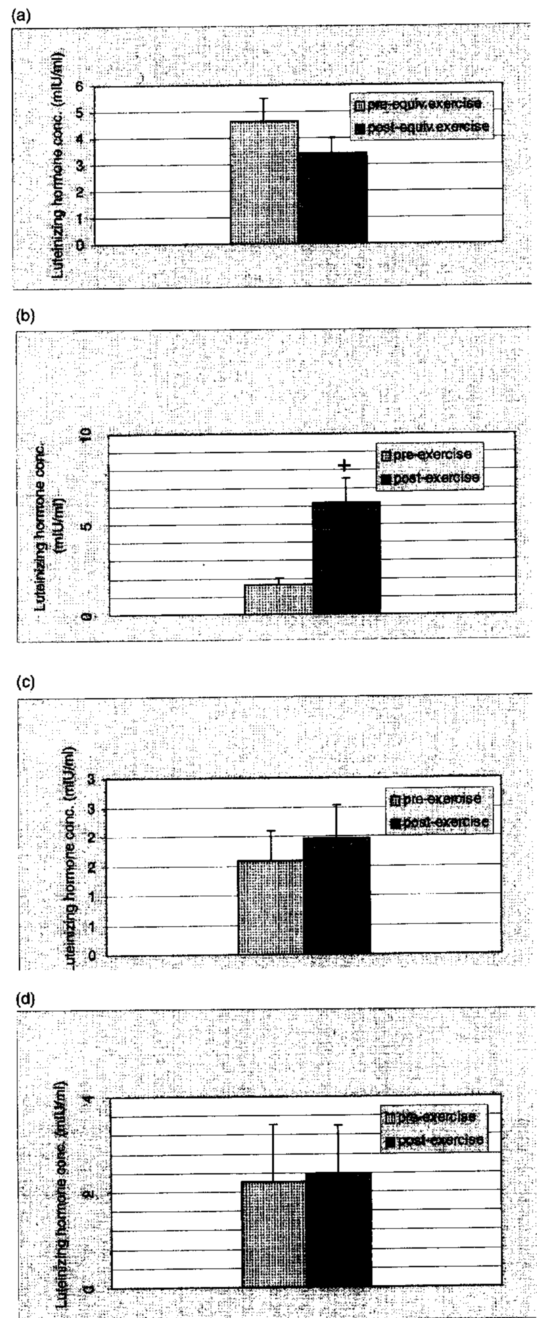

Fig. 3. (a) Mean luteinizing hormone resting profiles of nine athletes (S.E.M.) in the control group. (b) Mean luteinizing hormone stress profiles of twelve athletes (S.E.M.) exercise-stressed at VO_2max. (+) statistically significant at $p<0.005$. (c) Mean luteinizing hormone stress profiles of nine athletes (S.E.M.) exercise-stressed at 68.1±8.1% VO_2max. (d) Mean luteinizing hormone stress profiles of seven athletes (S.E.M.) exercise-stressed at 51.6±3.0% VO_2max.

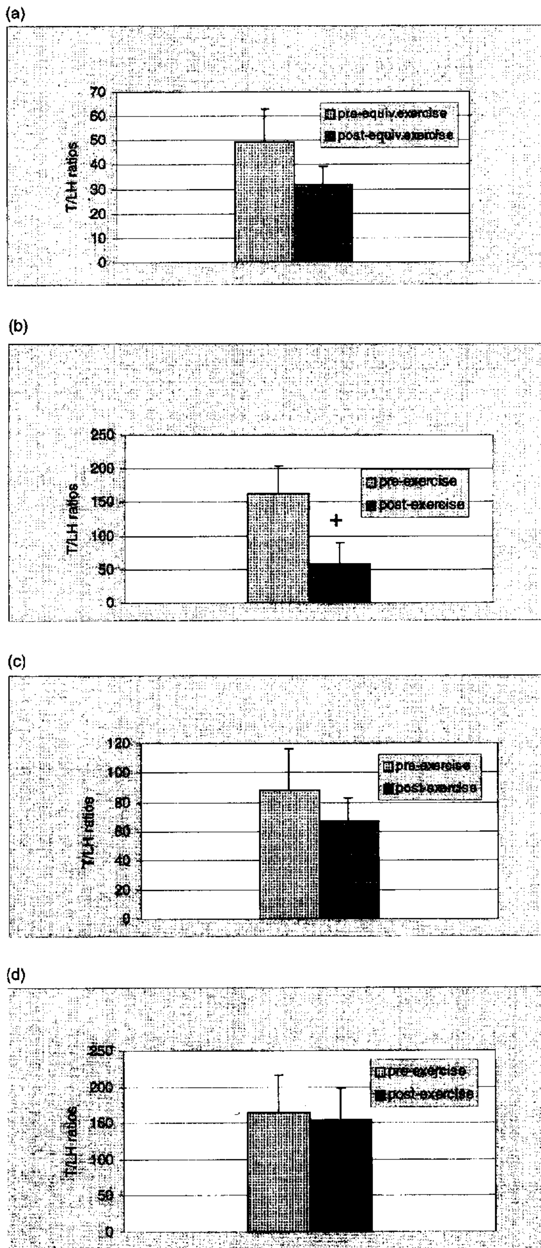

Fig. 4. (a) Mean T/LH ratio resting profiles of nine athletes (S.E.M.). (b) Mean T/LH ratio stress-profiles of twelve athletes (S.E.M.) exercise-stressed at VO_2max. (+) statistically significant at $p<0.005$. (c) Mean T/LH ratio stress profiles of nine athletes (S.E.M.) exercise-stressed at 68.1±8.1% VO_2max. (d) Mean T/LH ratio stress profiles of seven athletes (S.E.M.) exercise-stressed at 51.6±3.0% VO_2max.

the mean T/LH ratio ($p<0.005$, paired t-test) was evident in the stressed condition compared to the resting state for the group of subjects stressed at maximal oxygen uptake (Fig. 4b). The T/LH ratios were calculated as nmol/IU.

Exercise-stress response elicited at the submaximal oxygen uptake levels of 68.1±8.9 and 51.6±3.0%VO_2max did not produce any marked changes in any of the ratios (Fig. 4c–d). Similarly, the mean resting profiles of the control group did not demonstrate any significant change in the T/LH ratios (Fig. 4a).

4. Discussion

During short-term acute exercise-stress, there was no marked change in the total testosterone concentrations (Fig. 2b), although LH concentrations increased significantly (Fig. 3b) at post-exercise. The lack of any marked change in the T and LH concentrations was apparent in the exercise-stress responses at 68.1% VO_2max, 51.6% VO_2max and in the resting profiles of the controls. This observation seems to contradict an earlier finding that T levels were raised significantly following a short bout of strenuous exercise [13]. The short-term stress effects might be accounted for by the decrease in circulating plasma volume [14], possible reduced metabolic clearance of T by the liver [8] or decreased hepatic blood flow as a consequence of acute exercise-stress.

The lack of significance in the post-exercise T concentration changes despite the significant increase in LH during strenuous exercise, seems to indicate that acute stress is induced by a direct inhibitory effect on the testicular endocrine function or it could have been caused by insufficient stimulation by LH from the pituitary [3]. This evaluation appears consistent with the pattern of the profiles depicted in Fig. 5a, which shows a smaller change in the T concentrations during exercise-stress compared to that in the mean resting profiles of the controls. It is also possible that, during acute exercise-stress, prolactin may be involved in modifying the T response. Prolactin deficiency had been related to male hypogonadism [15] and the nocturnal rise in T in normal man was associated with the preceding increase in prolactin [16]. A possible shift in the

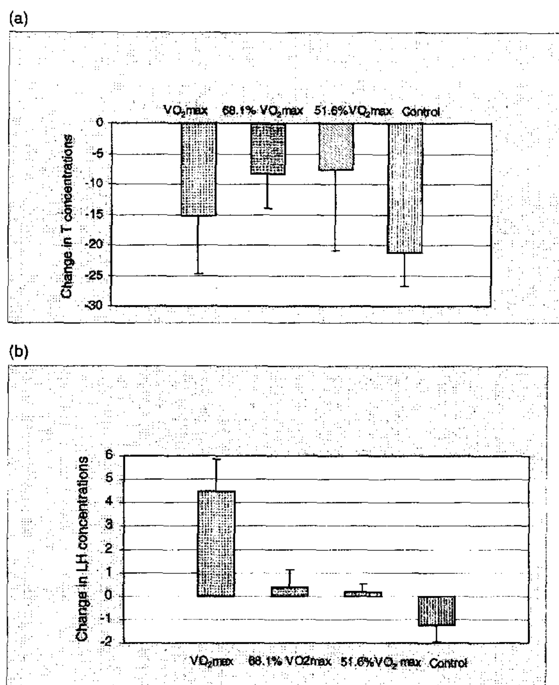

Fig. 5. (a) Comparison of the mean change in T concentrations between pre- and post-exercise states for the stress tests and controls. (b) Comparison of the mean change in LH concentrations between pre- and post-exercise states for the stress tests and controls.

biosynthetic pathway and a decrease in enzyme activities of the testis caused by stress [7] might also contribute to low T levels. This reasoning suggests that the variation in serum prolactin might be another factor that could have contributed to the decreased T response observed during intense exercise. This assessment also appears to be consistent with the evaluation by Tanaka et al. [17] who showed that strenuous exercise resulted in a persistent relative insensitivity of the T biosynthetic machinery to LH.

Evaluation of the mean LH profiles in Fig. 3b showed that acute exercise-stress resulted in a significant increase in the mean LH concentration ($p<0.005$) at post-exercise, while exercise-stress at submaximal VO_2 values appeared to promote moderate increases in the change in LH concentrations, relative to the non-stressed condition of the controls (Fig. 5b). The marked rise in LH concentrations during acute stress might be due to greater LH secretion [7]. It may also be possible that the decreased concentration of T might have stimulated a surge of LH release, due to the negative feedback effect of T on LH response. In particular, the post-exercise T/LH ratio (Fig. 4b) was found to decrease significantly ($p<0.001$) compared to the mean resting profile of the non-exercising control group. However, this finding appears to contrast with those of De Boer [9], who reported a reverse relationship in the urinary T/LH ratio for trained cyclists subjected to long-term exercise-stress in a marathon race. It would be difficult to compare De Boer's data with the results from this study, as the urine samples collected by De Boer were untimed. The reverse relationship could have been the consequence of the diurnal effects of the body during the marathon race, which lasted for more than 2 h.

5. Conclusions

The exercise-stress responses of a group of drug-free, healthy, male athletes were profiled using a combination of GC–MS and MEIA techniques. The urinary profiles showed that LH concentrations were significantly increased and that T/LH concentration ratios were markedly decreased by short-term acute exercise-stress, whilst T levels did not exhibit any dramatic change. Exercise-stress at submaximal oxygen uptake values of 68.1 and 51.6%VO_2max did not produce any marked change in the LH, T and T/LH values relative to the basal condition. The urinary profiles derived from these human stress studies facilitated a meaningful evaluation of the overall T and LH stress responses over a short period of applied stress.

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