Effects of a training program on resting plasma 2-hydroxycatecholestrogen levels in eumenorrheic women

CARL DE CRÉE,1 PETER BALL,2 BÄRBEL SEIDLITZ,2 GERRIT VAN KRANENBURG,3 PETER GEURTEN,3 AND HANS A. KEIZER3

Interuniversity Project on Reproductive Endocrinology in Women and Exercise,1 Department of Applied and Experimental Reproductive Endocrinology, B-3000 Lausen 3, Belgium; 2Department of Biochemical and Clinical Endocrinology, Medical University of Lübeck, D-23538 Lübeck, Germany; and 3Department of Movement Sciences, Faculty of Health Sciences, University of Maastricht, NL-6200 MD Maastricht, The Netherlands

De Créé, Carl, Peter Ball, Bärbel Seidlitz, Gerrit Van Kranenburg, Peter Geurten, and Hans A. Keizer. Effects of a training program on resting plasma 2-hydroxycatecholestrogen levels in eumenorrheic women. J. Appl. Physiol. 83(5): 1551–1556, 1997.—Catecholestrogens (CE) represent a major metabolic pathway in estrogen metabolism. Previous information on CE and training is limited to two cross-sectional studies that did not involve standardized training. Our purpose, by means of a prospective design, was to evaluate the effects of a brief, exhaustive training program on resting plasma concentrations of 2-hydroxy CE. The experimental design spanned two menstrual cycles: a control cycle and a training cycle. The subjects were nine previously untrained, eumenorrheic women [body fat: 24.8 ± 1.0 (SE) %]. Data were collected during the follicular (FPh) and the luteal phases (LPh). Posttraining FPh and LPh tests were held the day after the last day of a 5-day period of training on a cycle ergometer. Total 2-hydroxyestrogens (2-OHE) averaged 200 ± 29 pg/ml during the FPh and 420 ± 54 pg/ml during the LPh (P < 0.05). Levels of total 2-methoxyestrogens (2-MeOE) were 237 ± 32 pg/ml during the FPh and 339 ± 26 pg/ml during the LPh (P < 0.05). After training, although the plasma levels of 2-OHE significantly decreased (–21% (P < 0.05) during the LPh, the actual CE formation (as estimated from the 2-OHE-to-total estrogens ratio) increased (+29% (P < 0.05). CE activity, as expressed by the 2-MeOE-to-2-OHE ratio, showed significantly higher values in both phases (FPh, +14%; LPh, +13%) (P < 0.05). At the same time, resting levels of norepinephrine (NE) were increased by 42% (P < 0.05). CE strongly inhibit biological decomposition of NE by catechol-O-methyltransferase (COMT). Results of the present study suggest that, in response to training, CE are increasingly competing with the enzyme COMT, thus preventing premature NE deactivation.

amenorrhea; anovulation; catecholamines; catechol-O-methyltransferase; estrogens; 2-hydroxylase

It has been extensively documented that acute physical exercise and training in women provoke significant changes in the plasma concentrations of sex hormones (5). Strenuous exercise leads to an increased risk of irregular menses and anovulation. Premature osteoporosis has been identified as the most worrisome long-term effect (12). Despite the many published reports, the underlying mechanisms of the initial hypoestrogenemia are still unknown because of the many confounding variables (see Ref. 21 for a review). Some years ago, it was hypothesized that hypoestrogenemia in female athletes is not merely a secondary effect of hypoestrogenemia due to the hypothalamic disruption of the gonadotropin oscillator, as previously assumed, (30) but rather, perhaps, a primary consequence of increased catecholestrogen (CE) formation. As a consequence, normal gonadotropin release may be inhibited by negative CE feedback (23, 24).

CE are very unstable substances with both catecholamine and estrogen capabilities and represent the major metabolic pathway of estrogens. The name CE refers to the C2- and C4-hydroxylated metabolites of estrone (E1) and estradiol (E2) as well as to their O-methylated compounds. Most available research on CE has focused on either 2-hydroxyestrone (2-OHE1) or 2-hydroxyestradiol (2-OHE2). The reason for this choice is that the C4-hydroxylated estrogen metabolites circulate in smaller plasma concentrations and have a shorter half-life than the C2-hydroxylated estrogens, which makes their analysis even more critical than that of C2-hydroxylated estrogen metabolites. Because of the considerable difficulties involved in all CE-analysis procedures, it should not be surprising that only two published studies (26, 27), each with a cross-sectional design and partially the same subjects, are available that have examined the effects of physical training on 2-hydroxyestrogens. These authors found that, in the most strenuously training swimmers, plasma E2 concentrations were the lowest and 2-hydroxyestrogen levels were simultaneously the highest. These results suggested that physical training induces an increased turnover from primary estrogens to CE. However, these authors did measure only the nonmethylated 2-hydroxyestrogens.

After the formation of CE, the next biochemical step, the conversion of the 2-hydroxyestrogen to the 2-hydroxyestrogen-monomethylethers or 2-methoxyestrogens (2-MeOE), is catalyzed by catechol-O-methyltransferase (COMT). For a detailed overview of the metabolic pathways, see Ref. 9). Because CE competitively bind to COMT, the enzyme that decomposes catecholamines (1, 2), it has been speculated that these CE would prevent norepinephrine (NE) from premature decomposition (9). The information provided by measurement of 2-hydroxyestrogens alone is too limited to investigate the role CE may play. To have some indication concerning the actual physiological activity of CE, we also determined 2-MeOE and catecholamines.

Our purpose was to obtain preliminary data on the responses of 2-hydroxyestrogens and their monomethyl ethers in untrained, normally menstruating women.
after brief, standardized physical training. This represents a first step in thoroughly examining the CE sequence within the feedback system proposed earlier (9). In the present study, we have also computed the 2-hydroxyestrone-to-estradiol and the O-methylated-to-nonmethylated CE ratios, as introduced previously (10). In the absence of radiolabeling techniques, the formation and activity of CE may be estimated from these ratios. We hypothesized that exercise in women increases the turnover from primary estrogens into CE and the activity of CE (binding to COMT).

MATERIALS AND METHODS

Subjects. Nine young active but untrained women [age 20.4 ± 1.3 (SD) yr; height 172 ± 4.7 cm; body mass 61.4 ± 4.8 kg; percentage of body fat 24.8 ± 3.1%; age at menarche 13.2 ± 1.3 yr; menstrual cycle length 28.9 ± 2.9 days] volunteered for the study. Percentage of body fat was estimated by using the quadratic sum-of-three skinfolds equation of Thorland et al. (29), which has been cross-validated as a suitable alternative for underwater weighing (17). Therefore, skinfolds were measured at 11 sites (triceps, subscapular, axilla, supra ilioc, anterior supra iliilum, abdominal, thigh, medial calf) by a Holtain Skinfolds Calliper (Holtain, Crosswell, Crymch, UK). The same subjects participated in an earlier study (10). However, the trials used in the present and the previously published study are different.

Each subject filled out a questionnaire concerning her sports habits (discipline, intensity, years of training, and so on) and menstrual and gynecological history. Inclusion criteria were no gynecological, liver, renal, or thyroid disorders; no drugs or oral contraceptives; and normal ovulatory menstrual cycles for at least 1 yr before the study. Cycle normalcy was evidenced by plasma luteinizing hormone (LH) and progesterone measurements (data not shown) and was defined as a regular ovulatory cycle occurring each 26–32 days, with a cycle normalcy defined by the previously published study are different.

The experimental procedure was designed (Fig. 1). The training period started 5 days before the second FPh test and again 5 days before the second LPh test. Training was adjusted daily. Each subject had to report for a standardized lab training session on a cycle ergometer. Training started each day with 6 min of cycling at 100 W, and exercise intensity was increased 25 W at 2-min intervals until the individual’s maximum of that day was reached. This allowed us to accommodate the daily training program to the individual’s maximal physical working capacity (MPWC) on that day. MPWC was defined on the basis of the subject’s maximal power and heart rate. Immediately after the maximal exercise test, the subject was allowed to recover for 10 min at 50% MPWC. Afterward, the subject switched to interval training. This part of the training consisted of a 2-min series at 90% of the individual’s MPWC, followed each time by 2 min at 50% MPWC. Whenever the subject failed to continue (when she was not able to raise her pedaling rate again over 60 revolutions/min), the exercise intensity was lowered to 80% MPWC, and a 2-min interval series would continue at this intensity, followed again each time by a 2-min recuperation, still at 50% of the individual’s maximum, until the individual failed to continue at 80% MPWC. If necessary, each time the individual failed, intensity was lowered by 10% in a way similar to that described above. The training session...
stopped when the subject reached complete voluntary exhaustion (defined as the inability to keep the pedaling rate >60 revolutions/min at any intensity). This was usually after ~90 min of exercise.

Blood hormone and biochemical analysis. Blood for hormone analysis was drawn from the antecubital vein through a venous indwelling catheter by means of disposable syringes (20 ml) between days 7 and 10 in the FPh and between days 23 and 25 in the LPh. Analysis day was identical to the day of the exercise test. In the training phase, this day coincided with the first day after completion of the 5-day training period. Blood samples for CE analysis were thus obtained 5–7 days before ovulation and 10–14 days after ovulation. Blood samples were collected in prechilled lyophilized EDTA glass tubes stored in an ice bath at 2°C. Blood samples for estrogen and CE determination were immediately centrifuged at 2,000 x g and CE determination were extracted in duplicate in 3-ml Eppendorf cups, quickly frozen in liquid nitrogen, and stored at −80°C until assayed.

Free, i.e., the unconjugated, 2-hydroxyestrogen fraction in plasma is frequently near or below the detection limit of normal CE-assaying procedures (3). Therefore, to ensure validity and accuracy, we determined “total” 2-hydroxyestrogens (2-OHE), i.e., the sum of the unconjugated and conjugated fractions of 2-OHE1 and 2-OHE2. A similar approach was used for the total 2-OHE-monomethylethers (i.e., 2-MeOE). 2-OHE and 2-MeOE were determined altogether in one assay procedure, as detailed previously (10).

To have a comparable measurement, we also determined total estrogens (i.e., E), being the sum of the conjugated and unconjugated E1 + E2 fractions. Intra-assay coefficients of variation for 2-OHE and 2-MeOE are <10%. The interassay precision, as determined from pool plasma, resulted in a coefficient of variation of <17.2% for 2-OHE and 2-MeOE. The lower limit of detection for all CE assays was ~6 pg/ml.

The plasma for the determination of all other hormones was pipetted in 3-ml Eppendorf cups, quickly frozen in liquid nitrogen, and stored at −80°C until analysis. Plasma LH concentrations were determined by using an immunoradiometric assay purchased from Serono (Geneva, Switzerland). The sensitivity is 0.4 mIU/ml, and the interassay coefficient of variation is <10%. All samples were analyzed in duplicate in the same assay and were batched so that pre- and postexercise samples for both FPh and LPh were analyzed in the same assay.

Catecholamines were determined by electrochemical determination after separation by using high-performance liquid chromatography. These procedures, as well as the equipment and chemicals used, have all been described previously (10). The respective interassay coefficients of variation were 4.5% for NE, 11.3% for epinephrine (Epi), and 11.8% for dopamine (DA). The intra-assay coefficient of variation was <10% for each catecholamine.

Total protein was determined by the biuret method, and hemoglobin was determined spectrophotometrically with the hemoglobin cyanide method. Hematocrit was determined by the microcentrifuge method, and blood lactate was determined with an electrical-chemical-enzymatic method by using a semiautomatic lactate analyzer (model 640, Kontron, Zürich, Switzerland).

Statistical analysis. Results before and after training were compared by using paired t-tests. Comparisons between different menstrual phases were made by a two-way, mixed-model, repeated-measures analysis of variance. An α-level of 0.05 was set a priori. Phase, group, phase-group (interactive), and subject effects were evaluated independently by maximum likelihood procedures. This technique has proved to be more robust than conventional statistics in the presence of bias due to nonnormal distribution, missing values, radioimmunoassay imprecision, and so on (6, 8, 16). Values for comparisons of group means are given as means ± SE.

RESULTS

Physiological characteristics. In subjects before training, we found that in the LPh maximal power was significantly higher but the maximal VO2 (VO2max) was significantly lower (41.5 ± 1.4 vs. 45.7 ± 1.5 ml·kg−1·min−1 in LPh vs. FPh, respectively; P < 0.05). Power increased with training in both phases, but VO2max decreased in the FPh to 42.5 ± 1.1 ml·kg−1·min−1. Posttraining LPh values (43.5 ± 1.1 ml·kg−1·min−1) were higher than pretraining LPh values, although still lower than in the FPh (Table 1). There were no significant changes in body mass that could account for these differences.

Hormonal responses. An overview of plasma catecholamine and LH responses to training is given in Table 2. Posttraining plasma NE levels were significantly (P < 0.05) increased by 42% in both phases. A significant menstrual phase difference (P < 0.05) was observed for plasma DA and Epi levels but only so long as subjects were untrained. After training, NE, Epi, and DA levels did not significantly differ between menstrual phases. Resting plasma LH concentrations were not significantly altered by training.

Significantly lower (P < 0.05) posttraining plasma levels of primary estrogens (~53%) and 2-OHE (~21%) were only observed in the LPh (Table 3). To have a rough estimation of CE production and turnover, we calculated 2-OHE-to-E (2-OHE/E) and 2-MeOE-to-2-OHE (2-MeOE/2-OHE) ratios. 2-OHE/E (measurement of CE formation) increased after training but only in the LPh (+29%, P < 0.05). 2-MeOE/2-OHE (correlates with COMT activity) increased in both the posttraining FPh and LPh (+14 and +13%, respectively; P < 0.05).

DISCUSSION

Justification of experimental procedures. Research on CE is handicapped by their high instability. Even

Table 1. Comparison of anthropometric and physiological data before and after a 5-day exhaustive training program

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pretraining</th>
<th>Posttraining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FPh</td>
<td>LPh</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>24.8 ± 1.0</td>
<td>24.4 ± 1.2</td>
</tr>
<tr>
<td>LBM, kg</td>
<td>46.0 ± 0.7</td>
<td>45.8 ± 0.8</td>
</tr>
<tr>
<td>VO2max, ml/min</td>
<td>2.624 ± 7</td>
<td>2.365 ± 7</td>
</tr>
<tr>
<td>HRmax, beats/min</td>
<td>188 ± 3</td>
<td>184 ± 4</td>
</tr>
<tr>
<td>Wmax, W</td>
<td>241 ± 14</td>
<td>250 ± 10†</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>7.7 ± 1.2</td>
<td>8.6 ± 1.6†</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>39.1 ± 0.4</td>
<td>38.6 ± 0.5†</td>
</tr>
<tr>
<td>Hemoglobin, mmol/l</td>
<td>8.4 ± 0.2</td>
<td>8.2 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 women. FPh, follicular phase; LPh, luteal phase; LBM, lean body mass; VO2max, maximal oxygen consumption; HRmax, maximal heart rate; Wmax, maximal power. *Significantly different from pretraining value, P < 0.05. †Significantly different from FPh levels, P < 0.05.
subjects at rest before and after a 5-day exhaustive training program

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pretraining</th>
<th>Posttraining</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE, pg/ml</td>
<td>170 ± 17</td>
<td>167 ± 49</td>
</tr>
<tr>
<td>Epi, pg/ml</td>
<td>22.0 ± 3.3</td>
<td>43.5 ± 8.4†</td>
</tr>
<tr>
<td>DA, pg/ml</td>
<td>49.3 ± 15.6</td>
<td>&lt;10†</td>
</tr>
<tr>
<td>LH, mIU/ml</td>
<td>3.6 ± 0.4</td>
<td>4.8 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 9 women. LH, luteinizing hormone; NE, norepinephrine; Epi, epinephrine; DA, dopamine. *Significantly different from pretraining value, P < 0.05. †Significantly different from FPh levels; <10 below detection level for DA, P < 0.05.

extremely mild methods may often destroy CE to a considerable extent. Previously, CE were mostly measured in urine. However, new methods have made it possible to measure the much lower circulating amounts in plasma. By adding ascorbic acid to samples, by using refrigerated centrifuges and precooled tubes, and by immediate deep-freezing with liquid nitrogen and subsequent storage at −80°C, we have employed maximal guarantees to protect the samples from premature decomposition (13) and have avoided some of the flaws of previous studies. Furthermore, the present study offers specific data on the metabolic pathway of CE by including measurements of the monomethylethers. This is particularly important for our hypothesis because without these values it is hardly possible to speculate on the actual physiological activity of CE. Because CE are heavily conjugated, and the inactivity of the conjugated fraction has not been proved, we have used the sum of conjugated and unconjugated fractions in this study. Most literature on estrogens and exercise only considered before the actual trial started. Therefore, this method would be inappropriate here because it would be an extremely difficult method of obtaining any indication whatsoever on the transfer toward total CE.

Standard laboratory data on CE that have been published previously have all been based on very few subjects because of the laborious and expensive assaying procedures (13). Our values for basal total CE appear to be lower than ranges previously suggested for 2-hydroxy CE in some of the previous papers (3, 13). In fact, values were akin to assumed ranges for the mere unconjugated fraction in sedentary women. The data were also lower than our estimated reference laboratory values calculated from our earlier studies (3). They were, however, within the ranges of our pool values. Although, we do not have any conclusive explanation for these differences, it must be emphasized that sample collection and assaying procedures were conducted with extreme rigor. In addition, most data in the literature that mention CE concentrations date back 15 years or more. In these studies, plasma collection and treatment often did not involve immediate freezing by liquid nitrogen, and samples were mostly stored at −20°C only. The type of training was chosen because studies from Keizer et al. (20) had shown earlier that by using this aerobic and anaerobic training schedule it was possible to provoke a disturbance of normal gonadotropin pulsatility.

Catecholamines and training. Although posttraining samples were obtained under resting conditions (at least 24 h after the last training had taken place), plasma NE levels were significantly higher than before training. This finding is important because there is substantial evidence in support of noradrenergic mediation of LH release (18). Also, acute exercise-induced NE concentrations are known to be higher in hypoestrogenemic female athletes (7). However, no previous explanation has been offered as to why training produces higher resting levels of NE. It is speculated, therefore, that this is a direct effect of the increased competition for COMT, as suggested by the increased resting 2-MeOE/2-OHE values found in both menstrual phases. Recent experiments in rats have demonstrated that CE indeed increase NE concentrations via the above-mentioned mechanism (25).

Primary estrogens and training. Resting LPh plasma E levels, after a brief, exhaustive training program, were significantly lower. This finding is in association with many earlier findings on unconjugated E₂ behavior (19, 20, 27). Only a limited number of studies, for example by Bullen and colleagues (5), did not find any differences in resting plasma E₂ after a 2-mo period of endurance training. However, the training intensity of the subjects involved in that trial appeared to be lower than in most studies, including ours. Of considerable importance is a comparison with the studies by Russel et al. (26, 27) because they are the only other group of investigators who has reported responses of plasma CE to training. Like Bullen et al. (5), these authors did not find any differences in simple estrogens after strenuous training. However, this should not be surprising because their subjects were already highly trained, and many of them were oligomenorrheic and estrogen deficient before the actual trial started.

CE and training. In the present study, only the LPh levels of CE (both 2-OHE and 2-MeOE) were significantly lower than pretraining values. Conversely, Rus-
Physiology of CE. It was speculated before that CE would participate in energy metabolism in response to heavy exercise by preventing NE from premature decomposition by COMT. When more primary estrogens are converted into CE, this is likely to produce lower circulating plasma estrogen concentrations and eventually hypoestrogenemia, unless estrogen production would simultaneously increase. This is unlikely to happen. Therefore, exercise-induced menstrual problems might rather be the consequence of hypoestrogenemia than vice versa. However, a second simultaneously active mechanism causing hypoestrogenemia has also been suggested. This involves the documented stimulatory action of CE on prostaglandin F₂α (15, 22). This prostaglandin is known to dramatically increase in response to exercise (11) and to exert strong luteolytic effects on the developing corpus luteum. Furthermore, the low amounts of body fat previously considered as a key process in the development of exercise-induced amenorrhea may induce crucial changes in CE metabolism. Indeed, the 16α-hydroxylation that dominates estrogen metabolism in women with higher percentages of body fat shifts to 2-hydroxylation (i.e., formation of CE) in women with lower percentages of body fat. This was already observed in obese sedentary women (14) and was recently confirmed in female athletes (28).

To the best of our knowledge, no data on the actual formation or activity of 2-hydroxyestrogens in response to training are available. We observed that before training there seemed to be no differences in the rate of CE formation (expressed as 2-OHE/E) between menstrual phases. However, the rate of methylation of CE (and thus COMT activity, expressed as 2-MeOE/2-OHE) is apparently higher during the FPh. Both formation (but only in the LPh) and activity (in both phases) of CE seem to be boosted by training. The two studies by Russel et al. (26, 27) provided enough data to compute the (unconjugated) CE-to-estrogen ratio as a measurement of CE formation. This leads to results of actual CE formation that are in agreement with those of the present study.

In conclusion, the results of the present study show that brief periods of exhaustive training in previously untrained eumenorrheic women decreased the total primary estrogen levels but were unable to raise total CE levels. A longer period of intensive training is probably needed to provoke increments in resting CE levels. However, despite the absence of significant changes in plasma CE concentrations, there appears to be an increase in their formation and especially in their activity. We suggest that the training-induced increases in resting NE levels, previously shown to inhibit hypothalamic gonadotropin-releasing hormone release (4), may be a direct effect of increased CE activity (i.e., competition for COMT). However, as yet there is not sufficient evidence either to accept or to reject the hypothesis that assigns a crucial role to CE in the etiology of exercise training-induced hypoestrogenemia.

We are indebted to all of the women who kindly volunteered for this study. We gratefully acknowledge Drs. Alex Vermeulen (Dept. of Endocrinology and Metabolism, State Univ. of Ghent, Ghent, Belgium) and Mich Ostyn (Institute of Physical Education, Catholic Univ. of Leuven, Leuven, Belgium) for scientific advice. Yvonne Janssen, Monique Van Der Heyden, and Kerstin Mannheimer provided excellent technical assistance. Kristin Hibler (Dept. of Speech Communication, Univ. of Washington, Seattle, WA) and Dr. Gary Hibler (Portland, OR) are acknowledged for proofreading the manuscript.

Address for reprint requests: C. De Créé, Dept. of Applied and Experimental Reproductive Endocrinology, The Institute for Gynecological Endocrinology Research, PO Box 134, B-3000 Leuven 3, Belgium. Received 11 February 1997; accepted in final form 16 June 1997.

REFERENCES