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HUMAN STUDIES: These were designed to determine (a) if short-term estradiol supplementation (ES) improves heat dissipation and if HSP70 can serve as a biomarker to assess this improvement; and (b) if ES for 7 days of heat exposure enhances the process of heat acclimation (HA). We also determined if HSP70 measurements could predict the rate of HA. Results showed that ES in premenopausal women during the follicular phase of the menstrual cycle (a) did not enhance sweating, cutaneous blood flow, or the time required to achieve HA. Moreover, neither ES nor HA alone induced the synthesis of HSP70. ANIMAL STUDIES: These studies were designed to determine if ES would enhance exercise performance in the heat and what combination of ES and exercise training would enhance the acclimation of female ovariectomized rats to the heat. These studies showed that (a) ES had no effect on heat transfer to the skin or heat dissipation by evaporative cooling, but that ES increased thermotolerance in ovariectomized rats exercising at high ambient temperatures; and (b) the combination of exertional heat exposure and ES, when compared to ES alone, enhances thermotolerance in rats exercising at a high ambient temperature.

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I. HUMAN STUDIES

A. Effect of estrogen supplementation on exercise thermoregulation in premenopausal women

Abstract

This study examined the effects of 3 days of estrogen supplementation (ES) on thermoregulation during exercise in pre-menopausal (20-39 yr) adult females during the follicular phase of the menstrual cycle. Subjects (11 control, 10 experimental) performed upright cycle ergometer exercise at 60% $\dot{V}O_2$ max in a neutral environment (25°C, 30% RH) for 20 min. Subjects were given placebo (P) or ß-estradiol (2 mg•tablet^{-1}, 3 tablets•day^{-1} for 3 days). All experiments were conducted between 6:30 and 9:00 a.m. following ingestion of the last tablet. Heart rate, forearm blood flow (FBF), mean skin temperature, esophageal temperature (T_{es}) and forearm sweat rate (SR) were measured. Blood analysis for estrogen and progesterone reflected the follicular phase of the menstrual cycle. Maximal O_2 uptake (37.1 ± 6.2 ml•kg^{-1}•min^{-1} in P vs. 38.4 ± 6.3 ml•kg^{-1}•min^{-1} in ES) and body weight to surface area ratio (35.58 ± 2.85 in P vs. 37.3 ± 2.7 in ES) were similar between groups. Synthesis of heat shock protein 70 (HSP70) was not induced by 3 days ES. Neither the threshold for sweating (36.97 ± 0.15°C in P vs. 36.90 ± 0.22°C in ES), the threshold for an increase in forearm blood flow (37.09 ± 0.22°C in P vs. 37.17 ± 0.26°C in ES) , the slope of SR-T_{es} relationship (0.42 ± 0.16 in P vs. 0.41 ± 0.17 in ES) nor the FBF-T_{es} relationship (10.04 ± 4.4 in P vs. 9.61±3.46 in ES) were affected ($P > 0.05$) by 3 days of ES. We conclude that 3 days of ES by young adult women in the follicular phase of their menstrual cycle has no effect on heat transfer to the skin, heat dissipation by evaporative cooling, or leukocyte synthesis of HSP72.

B. Effect of estrogen supplementation on heat acclimation in pre-menopausal females

Introduction

Heat acclimation can usually be achieved by performing mild to moderate exercise (30-55% VO_2 max, 2-4 $h \cdot day^{-1}$) in a hot environment for 5-12 days. The state of acclimation is characterized by decreased core and skin temperature, decreased heat storage, increased sweating rate, and a lower sweating and vasodilation threshold (24, 25, 35, 37). The acclimation process is similar in women and men of comparable aerobic fitness (1), and is independent of the menstrual cycle (20).

Reducing the duration of time needed to achieve heat acclimation would be beneficial under situations where exercise in the heat was necessary after limited notice. Recent evidence suggests that estradiol supplementation (ES) should accelerate the heat acclimation process. In post-menopausal women ES improves thermoregulatory responses during exercise in the heat (33). Furthermore, recent animal studies demonstrate that estradiol therapy increases evaporative water loss at all levels of core body temperature, reduces the core body temperature threshold for onset of saliva spreading (2), and elevates heat shock protein 70 (HSP70) and heat shock protein 90 (HSP90) concentrations in the ventromedial hypothalamus in ovariectomized female rats (19). No study to date has examined the time required to develop heat acclimation in menstruating women receiving estrogen supplementation or in post-menopausal women undergoing estrogen replacement therapy.

The most temperature sensitive and highly conserved of the heat shock proteins is HSP70. At the cellular level, all organisms respond to stress by synthesizing HSP's which are considered the central component of acquired heat tolerance (16, 17, 18). Ryan et al. (27) demonstrated that HSP70 is produced in humans exercising in the heat, and thus HSP70 maybe a useful biomarker to investigate the adaptation to thermal stress.

Wright (36) reported that the maximal core temperature of mice can be increased by heat acclimation; however, the mechanisms for this observation were not examined. Highly trained human marathon runners can tolerate core temperatures between 40°C - 42°C without signs of heat illness (22, 37). Two species of antelope, Grant's gaelle and orynx, can survive the rigors of desert life, in part, by allowing their core temperature to rise above over 46°C (34). These observations suggest that tissue tolerance to high temperature may be developed in organisms repeatedly exposed to heat stress.

Thus, the purpose of this study was to determine: a) whether ES will reduce the time required to achieve heat acclimation in pre-menopausal females during the early follicular phase of the menstrual cycle, and b) whether levels of leukocyte HSP70 can serve as a biomarker of heat acclimation in these individuals. Because ES may increase HSP70 and improves thermoregulation in post-menopausal women, we hypothesized that ES will reduce the time required to achieve heat acclimation and that the level of leukocyte HSP70 will serve as a convenient and rapidly assayed biomarker of heat acclimation. This study therefore allows investigation of cellular mechanisms in the heat acclimation process.

Methods

Subjects. Fourteen females, aged 18-35 yrs, volunteered to participate in the study. Subjects were sedentary, did not smoke, and did not use oral contraceptives for at least 2 months prior to the experiment. All subjects exhibited a normal menstrual cycle. Each volunteer was informed of testing procedures, written consent was obtained, and each received a physical examination. All experimental procedures were approved in advance by the Human Subjects Committee at the University of Iowa.

Experiments were conducted between February and May 15 to ensure that subjects were not heat acclimatized prior to the study. Subjects recorded their basal rectal temperature for at least one cycle prior to the experiment. There was no significant difference between groups for maximal oxygen consumption (VO_2max), age, percent body fat, and body weight/surface area ratio (Table 1). Serum estradiol and progesterone concentrations were obtained on the first day of menstruation to confirm the follicular phase of the period (Table 2).

Estimation of maximal oxygen uptake and submaximal oxygen uptake. Before the experimental trials, each subject's VO_2max was determined using a graded incremental treadmill

test to volitional fatigue. Oxygen uptake (VO_2) and carbon dioxide production were analyzed continuously (Q-Plex I Metabolic System, Quinton Instruments Co., Seattle, WA), and heart rates were measured every min. VO_2max was defined as achieving of at least two of the following three criteria: a plateau or decrease in VO_2 despite an increase in workload, a respiratory exchange ratio greater than or equal to 1.1, and/or attainment of at least 90% of predicted maximum heart rate (5). The workload required to elicit 35-45% VO_2max was calculated from submaximal VO_2 values.

Study design. Subjects were studied during the follicular phase of the menstrual cycle to minimize possible effects of endogenous progesterone and to maximize differences in estradiol levels between ES and control subjects. There were two experimental time periods. One group (n=6) started experiments at 6:00 a.m., whereas another began at 3:30 p.m. Each subject exercised at the same time of the day throughout the study. Subjects who exercised in the same group were paired based on VO_2max, percent body fat, and body weight to surface area ratio. Body surface area was determined by the Dubois formula (6). Using a double-blind design, subjects were randomly assigned to either the ES (n=7) or placebo (P; n=7) groups. To minimize training effects, subjects walked on a treadmill (35-45% VO_2max for 2 h) in a cool environment (22-24°C, 20% RH) for 5 days beginning 3 days before the expected start of their menstrual cycle. On days 2-8 of the menstrual cycle either estradiol (Estrace®, 2 mg·tablet^{-1}, Bristol-Meyers Squibb Co., Princeton, NJ) or placebo tablets were ingested (3 tablets·day^{-1}). On day 4 of their menstrual cycle, subjects began heat acclimation in a heat chamber (45° C, 20% RH) by walking at 35-45% VO_2max for 2 h. Heat acclimation was identified when rectal temperature (T_r) stabilized (≤ 0.1°C) during the last 30 min of the 2 h walk in the heat. To ensure subject safety, exercise was terminated when: a) rectal temperature (T_r) exceeded 39.5°C; b) symptoms of impending syncope, nausea, dizziness, or headache occurred; c) heart rate (HR) exceeded 95% of the predicted maximum HR; or d) volitional fatigue.

Blood samples (20 ml) were obtained on the first day of the menstrual cycle, immediately before and after exercise on the last day in a cool environment and on the first, fifth, and final day in a hot environment. These were analyzed for leukocyte HSP70, serum estradiol and serum progesterone.

Experimental protocol. Upon arriving to the lab, subjects ingested 6 ml·kg^{-1} of water to ensure hydration. Subjects then stood for 20 min for plasma volume equilibration and a blood sample (20 ml) was taken. A nude body weight was obtained after voiding, a rectal thermocouple probe (ESO-1, Physitemp Instruments, Inc., Clifton, NJ) interfaced to a thermocouple digital recorder (Model HH201, Omega Engineering, Inc., Stamford, CT). was inserted 10 cm beyond the anal sphincter, and a heart rate monitor (Polar Vantage XL, Polar USA, Stamford, CT) was attached. Subjects walked 2 h in the cool environment ingesting 2.5 ml·kg^{-1} of water every 20 min. Immediately after exercise, a nude body weight, and blood and urine samples were obtained. Exercise in the heat began on day 4 of the menstrual cycle. In these experiments, skin temperature was monitored and water intake was increased to 5 ml·kg^{-1} every 20 min.

Measurements. T_r, mean skin temperature (MST), perceived exertion (PE), thermal sensation (TS), and HR were measured on day 1 and day 5 in the cool environment (22-25°C), and on each day of heat acclimation. T_r and HR were measured every 5 min, TS and PE every 10 min, and MST every 2.5 min. VO_2 was measured 5-10 min into experiments on the first and last day in the cool environment, and on the first, third, fifth and last day in the hot environment.

Percent body fat was estimated from the sum of triceps, suprailiac, and thigh skinfolds (21). Skin temperatures were collected each min by an on-line computer system (IBM AT Personal Computer, IBM Corp., Armonk, NY). Mean skin temperature was reported as the weighted sum of 4 sites (chest 0.3 + upper arm 0.3 + thigh 0.2 + lower leg 0.2) (23). Measurements of PE and TS were obtained as described by Borg (3) and Gagge (10). Sweat rate (SR) was determined from the change in nude body weight pre- to post-exercise corrected for water intake and urine output.

Blood analysis. Serum estradiol and progesterone were measured by ^{125}I radioimmunoassays. Leukocyte HSP70 was determined as described previously (27). Briefly, 5 ml of Histopaque (Sigma Chem. Co., St. Louis, MO) was added to ~10 ml blood and centrifuged at 3000 rpm for 25 min to isolate the leukocytes. The leukocytes were then washed (gentle aspiration and centrifugation at 1,500 rpm for 5-7 min) twice with phosphate buffer solution (PBS). The final leukocyte pellet was suspended in 0.25 ml PBS, and stored at -70°C until analysis. This isolation procedure was initiated immediately on attainment of a blood sample. HSP70 analysis was performed using western blot analysis.

Statistical analysis. One and two-factor analysis of variance (ANOVA) with repeated measures were utilized to determine if significant differences existed between groups and across time. The Scheffe post-hoc test was employed to determine the location of any significant differences ($P < 0.05$). Values presented are mean ± SE.

Results

Serum estrogen concentration was significantly elevated after 3 and 7 days of ES compared to P. Serum progesterone was not significantly different from day 1 to 7 and was similar between groups (Table 2).

The number of days required to achieve heat acclimation was not reduced by ES (6 ± 0.5, 6 ± 0.5 days in ES and P groups, respectively). T_r on the first and last days in the heat was also similar between groups (Fig. 1C). These results indicate that 7 days of ES had no effect on T_r during rest or exercise in the heat in pre-menopausal women during the follicular phase of the menstrual cycle.

Synthesis of HSP70 also did not significantly differ between ES and P on any day of blood measurement. HSP70 was elevated on the last day of ES before exercise, however, due to high variability among subjects, there were no significant differences between or within groups (Fig. 3).

SR on day 1 and the final day of heat acclimation was not significantly different between the ES and P groups and no significant difference existed between day 1 and the final day of heat acclimation (Fig. 4). HR, MST, T_r , TS, and PE (Fig. 1A, 1B,1C and Fig. 2A, 2B) were significantly lowered on the last day of heat acclimation. VO_2 was similar on days 1, 3, 5, and the final day in the heat for each group and no significant difference was found between groups on any day (Table 3).

Discussion

Results from this study show that the time required to achieve heat acclimation was not reduced by 7 days of ES in pre-menopausal women during the follicular phase of their menstrual cycle. T_r was not altered at rest or during 2 h exercise in the heat and the synthesis of HSP70 was not induced by ES. Heat acclimation significantly reduced HR, MST, TS and PE, but these physiological responses were not altered by ES. SR and VO_2 were not altered by heat acclimation or ES.

HR was significantly reduced on the final day of acclimation following 60 min of exercise in the heat (Fig. 1A). Several mechanisms have been proposed for lowering HR over the course of heat acclimation: 1) increased plasma volume (14, 31, 35); 2) redistribution of cardiac output away from cutaneous vascular beds (32); and 3) decreased thermal drive associated with the fall in core and skin temperatures (26). The time course of the decrease in HR is roughly similar to that of the increase in plasma volume, and the two changes are significantly correlated (31). Furthermore, Senay (29) has hypothesized that even after plasma volume at rest returns toward normal, plasma volume during exercise in the heat is still larger after acclimation, because of an enhanced hemodilution response during exercise in the heat. Since HR at a given core temperature is the same or higher after acclimation than before (8, 9), lower body temperatures produce lower HR. However, the evidence available is not sufficient to establish a specific mechanism(s) to explain the cardiovascular improvements resulting from heat acclimation (35).

Several studies have demonstrated that SR significantly increases with heat acclimation (7, 14, 15, 30, 37). An increase in SR, however, is not a prerequisite for heat acclimation. In this study SR was not significantly elevated ($p > 0.05$) after heat acclimation in either group, and no significant difference occurred between groups. Sawka et al. (28) also did not find an increased SR in pre-menopausal women following 10 days of heat acclimation to alternate hot-dry (49°C, 20% RH)/hot-wet (35°C, 79% RH) environments. Horstman and Christensen (11) did not find increases in SR after 6 days of heat acclimation (45°C dry-bulb, 23°C wet-bulb environments) in pre-menopausal women, however, they did observe a significant increase after 11 days. Unfortunately, menstrual cycle phase was not indicated in either study. Avellini et al. (1) demonstrated that after 10 days of heat acclimation in a 36°C dry-bulb/32°C wet-bulb environment, SR was similar at 30, 90, and 120 min of exercise-heat exposure. Furthermore, SR during pre- and post-ovulation was similar before and after heat acclimation. Maher (13), and Robinson (25) did not find increased SR in men after heat acclimation. These experiments were conducted in a dry heat environment. Increases in SR after heat acclimation may be related to high ambient water vapor pressures. Collins et al. (4) reported that in dry heat nearly all of the sweat produced is evaporated and acclimation increases SR only enough to reduce heat storage and to compensate for the alteration in sensible heat exchange associated with lowered skin temperature. Another possibility is that sweating is more evenly distributed after heat acclimation leading to enhanced evaporative cooling. These data may explain significantly lower MST after heat acclimation without increases in SR. Fortney and Senay (7) reported an increase in SR did not occur until after 7 days of acclimation for most subjects. In the present study, our subjects reached heat acclimation in less than 7 days.

MST has a strong relationship with the sensation of comfort, and the consequent use in models predicting comfort (12). Fortney and Senay (7) suggested that a reduction in peripheral blood flow may explain the significantly lowered MST after heat acclimation, since only part of which could be accounted for an increase in SR. Roberts et al. (24) have also reported lowered skin conductances following heat acclimation. Since our subjects' MST during exercise in the heat were higher before, than after acclimatization, it is obvious that heat exchange was more favorable following heat acclimation.

TS and PE were improved after heat acclimation (Fig. 2A, 2B). This improvement is attributed to a lower core temperature and HR (9). It is also possible that the lowered skin temperature achieved with acclimation (12) allowed a greater thermal gradient from core to surface, reducing demands for cutaneous blood flow.

Synthesis of HSP70 was not induced by 7 days of ES or by heat acclimation. Although, there was a trend for HSP70 synthesis to rise on the last day of ES, there were no significant differences between groups or between blood samples within a group. Ryan et al. (27) demonstrated that synthesis of HSP70 in humans was induced when the core temperature reached 40.2 to 40.7°C. In our study, the highest T_r was 39.3°C. These data suggest that 5 to 8 days of exercise in the heat is not sufficient to induce HSP70 when T_r's are below 39.3°C. Olazabal et al. (19) reported that estradiol significantly induced HSP70 synthesis in the ventromedial hypothalamus of females rats after 12 h of hormone treatment. In the present study, human leukocytes did not increase synthesis of HSP70 with increased plasma estrogen concentrations suggesting that tissue (16) and/or species differences may exist.

We conclude that 7 days of ES had no effect on the time required to reach heat acclimation in pre-menopausal women during the follicular phase of the menstrual cycle. T_r, HR, MST, SR, TS, and PE responses were similar throughout the heat acclimation protocol between the two groups. Furthermore, synthesis of HSP70 was not induced by ES or by the heat acclimation process.

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Table 1. Subject characteristics.

Group	Height (cm)	Weight (kg)	Age (y)	VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	% Body Fat	BW/SA
Estrogen	168.2± 3.3	62.5 ± 4.0	24.3±1.4	39.5 ± 1.3	24.1 ± 2.6	36.9 ± 1
Placebo	169.2±2.1	64.1 ± 3.3	25.1±1.9	40.8±1.9	24.8 ± 2.2	36.9 ± .9

Values are means ± SE.

Table 2. Plasma estradiol and progesterone concentrations on the day before, and on days 3 and 5 of estrogen supplementation.

Group	Estradiol ($pg \cdot ml^{-1}$)	Progesterone ($ng \cdot ml^{-1}$)
Estrogen		
Pre	30.9 ± 6.8	0.8 ± 0.1
Day 3	870.2 ± 148.3 *	0.6 ± 0.1
Day 7	1062.7 ± 168.1*	0.7 ± 0.1
Placebo		
Pre	31.7 ± 4.9	2.2 ± 1.6
Day 3	61.2 ± 21.6	0.6 ± 0.2
Day 7	59.0 ± 10.7	0.6 ± 0.1

* Significantly different from placebo groups ($P < 0.001$). Values are means ± SE.

Table 3. Oxygen uptake during treadmill walking on day 1, 3, 5, and the final day in the heat.

	VO_2 ($ml \cdot kg^{-1} \cdot min^{-1}$)			
Condition	Day 1	Day 3	Day 5	Final Day
Estrogen	41.2±2.48	39.3±2.28	41.8±2.55	39.48±2.31
Placebo	38.1±1.22	39.2±3.74	42.0±3.36	42±5.29

Values are means ± SE.

Figure Legends

Fig. 1. Heart rate, mean skin temperature, and rectal temperature on day 1 and the final day of heat acclimation. (ES: estrogen supplementation, group; P: placebo group). Values are ± SE.

Fig. 2. Perceived exertion, and thermal sensation on day 1 and the final day of heat acclimation.

Fig. 3. Leukocyte HSP70 concnentration. Data are expressed as a percentage of day 2 pre-exercise ES or P supplementation. Abbreviations: HSP70: heat shock protein 70; ES: estrogen supplementation group; P: placebo group; 1 pre Ex: day 1 in the heat before exercise; 1 post Ex: day 1 in the heat after exercise; 5 pre Ex: day 5 in the heat before exercise; 5 post Ex: day 5 in the heat after exercise; F pre Ex: final day in the heat before exercise; F post Ex: final day in the heat after exercise.

Fig. 4. Sweat rate on day 1 and the final day of heat acclimation in each group.

Fig. 1

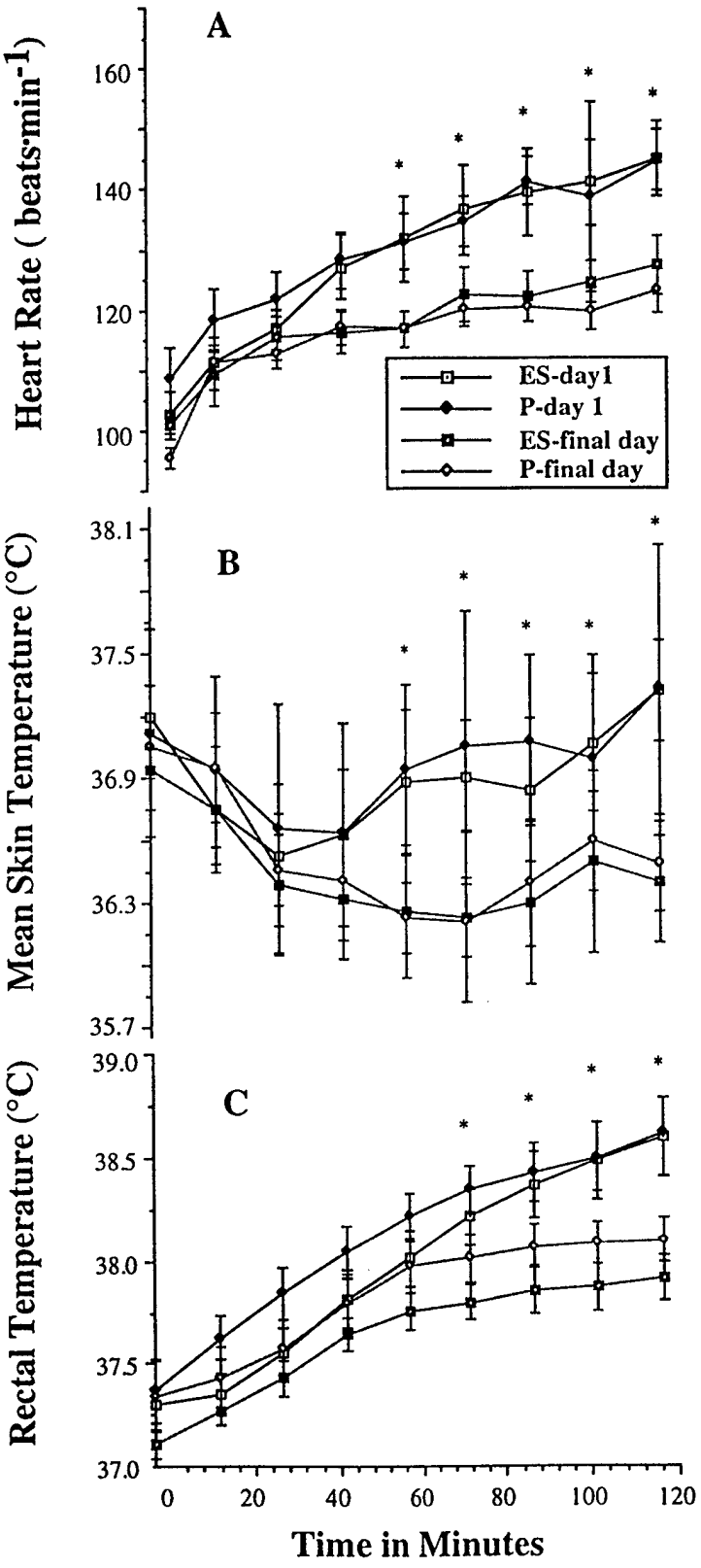

A
Heart Rate (beats·min-1)
160
140
120
100
ES-day1
P-day 1
ES-final day
P-final day
B
Mean Skin Temperature (°C)
38.1
37.5
36.9
36.3
35.7
C
Rectal Temperature (°C)
39.0
38.5
38.0
37.5
37.0
0
20
40
60
80
100
120
Time in Minutes

Fig. 2

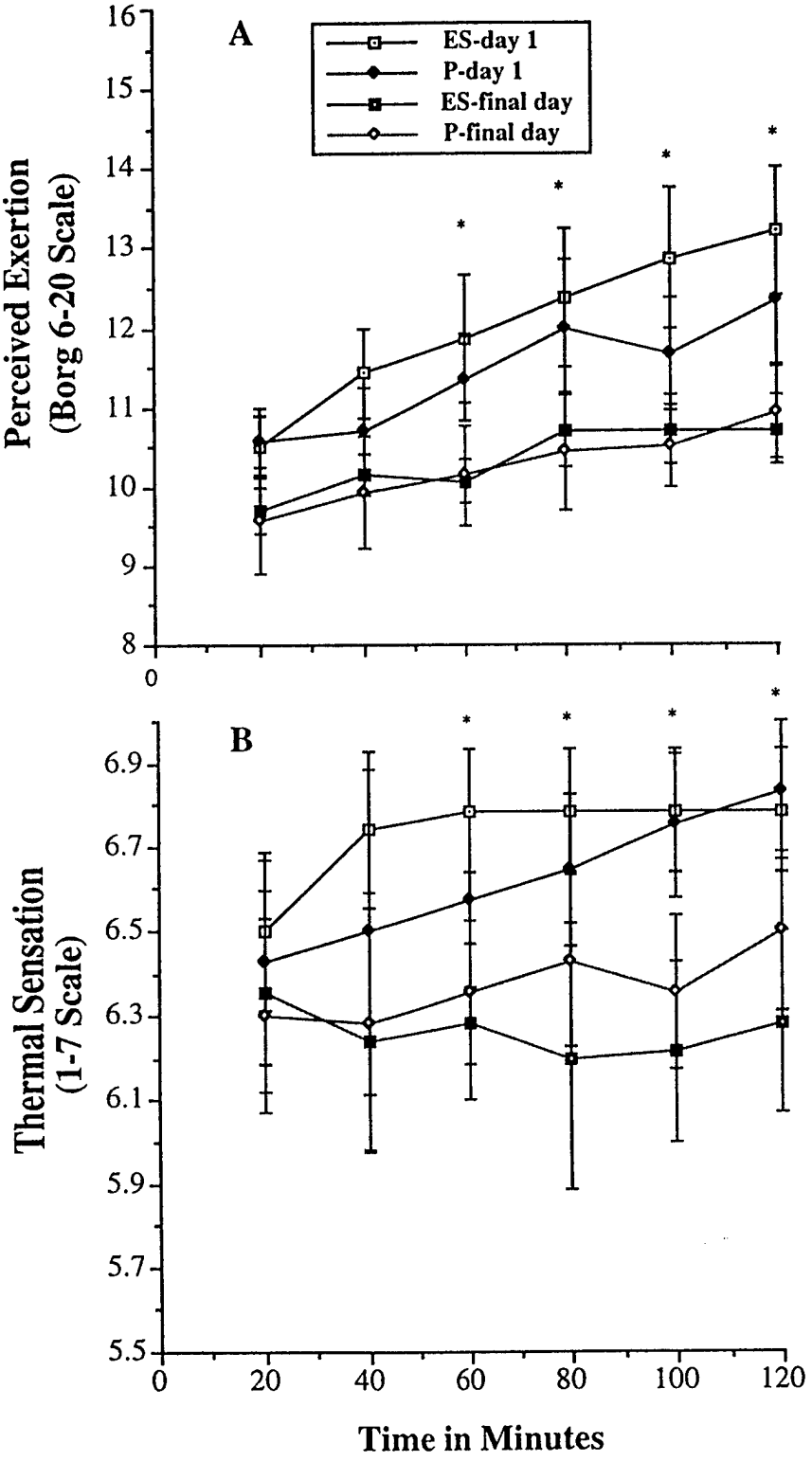

A
ES-day 1
P-day 1
ES-final day
P-final day
Perceived Exertion
(Borg 6-20 Scale)
16
15
14
13
12
11
10
9
8
B
Thermal Sensation
(1-7 Scale)
6.9
6.7
6.5
6.3
6.1
5.9
5.7
5.5
0
20
40
60
80
100
120
Time in Minutes

Fig. 3

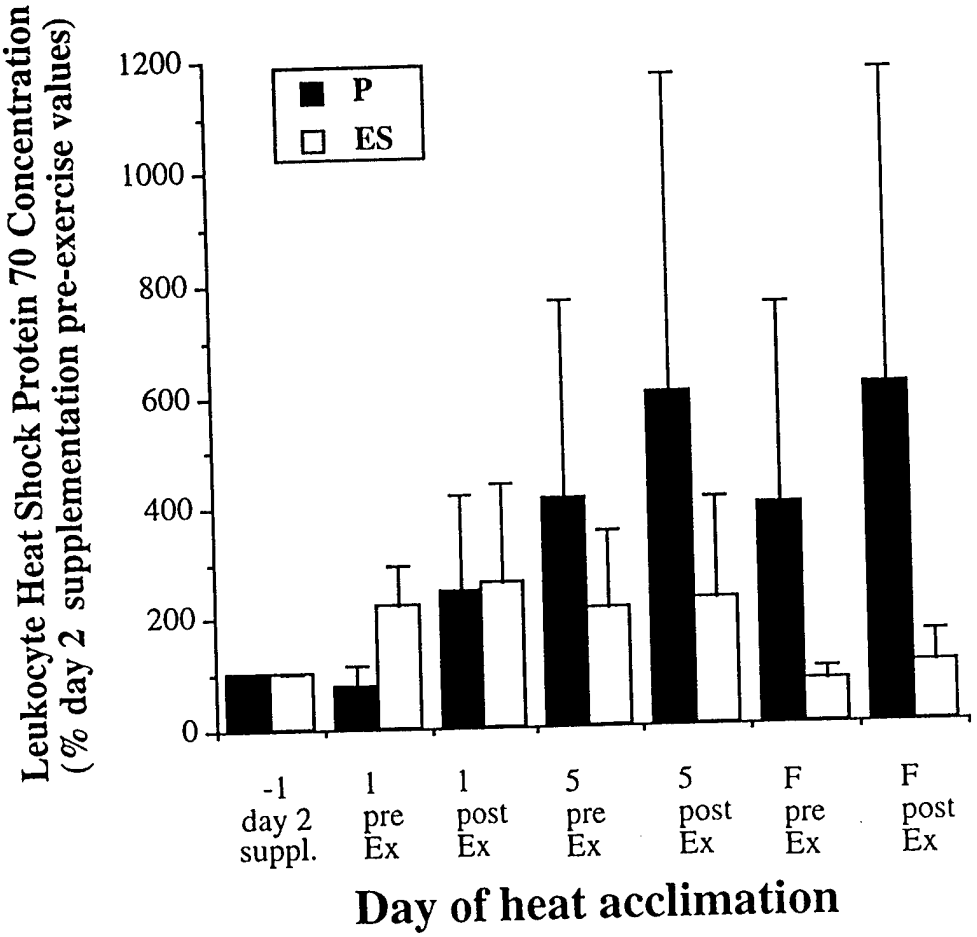
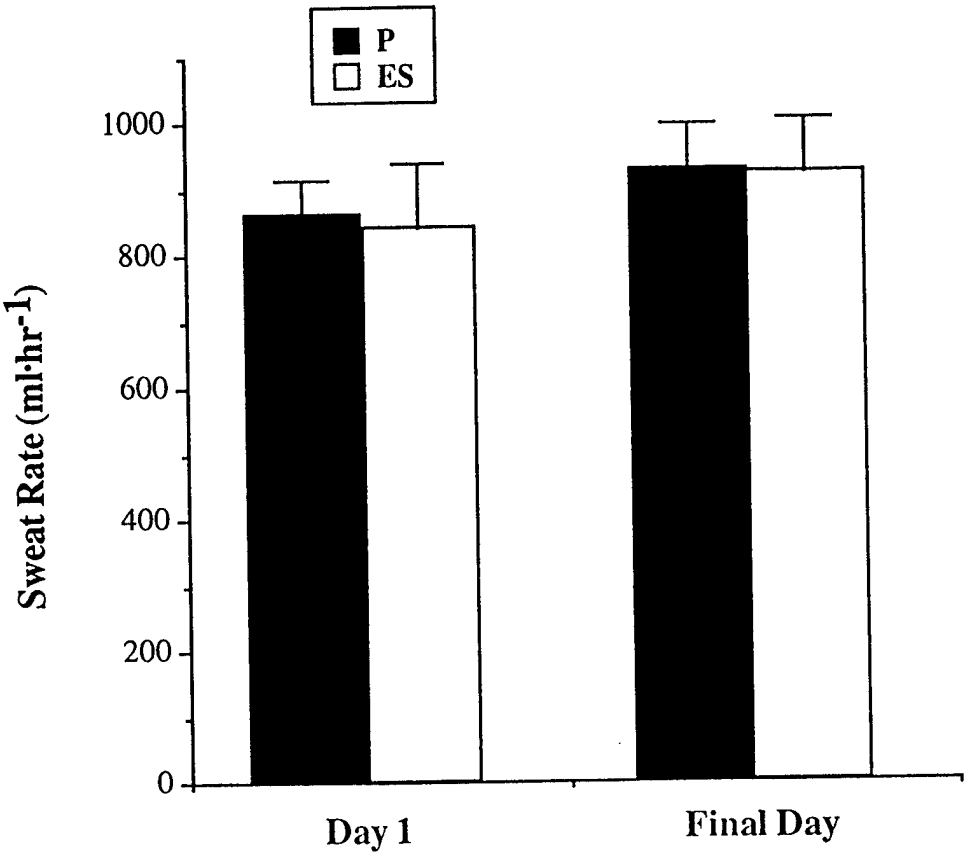

Leukocyte Heat Shock Protein 70 Concentration
(% day 2 supplementation pre-exercise values)
1200
1000
800
600
400
200
0
P
ES
-1
day 2
suppl.
1
pre
Ex
1
post
Ex
5
pre
Ex
5
post
Ex
F
pre
Ex
F
post
Ex
Day of heat acclimation

Fig. 4



II. ANIMAL STUDIES

A. Thermotolerance during exercise and heat stress: Impact of exercise and heat challenge

Introduction

Over the past decade, there has been a dramatic increase in the number of women participating in competitive, strenuous sports activities. This has lead to a parallel rise in research devoted to the exercise responses and adaptations observed in women. In particular, it has become apparent that sex differences in temperature regulation and heat tolerance exist in humans (22). Furthermore, there are conflicting reports regarding the thermal adaptive response of women, especially related to the menstrual cycle. Therefore, it is of considerable interest to investigate the mechanisms associated with heat adaptation responses observed in women .

Very little work has focused on the influence of the hormonal cycle on exercise performance. Given that estrogens and progestins can have individual or interactive effects on a variety of metabolic processes, the potential exists for an influence on athletic performance. The role of estrogen in body temperature regulation has only recently been addressed in post-menopausal women. It has been demonstrated that estrogen is significant in maintaining proper body temperature regulation in post-menopausal women, reducing hot-flashes in addition to increasing heat dissipatory mechanisms during exercise (34). These data suggest a beneficial role of estrogen thermoregulatory responses in post-menopausal women.

Due to the link that has been established between an improvement in exercise performance in the heat as a result of hormone replacement therapy in the form of estradiol, and estradiol supplementation increasing plasma volume and thermoregulatory mechanisms, we postulate that estradiol will enhance exercise performance in a warm environment. Our specific purpose is to test the hypothesis that estrogen therapy in ovariectomized female rats is associated with enhanced thermotolerance to exercise in the heat. The unique contribution of these experiments lies in our methodology and approach to the problem. Hyperthermia is a physiological stress regularly experienced by a majority of our population. It is noninvasive and does not make use of exogenous pharmacological manipulation. Moreover, a variety of questions including hormonal contributions to thermoregulation and exercise performance in the heat and cellular stress responses may be addressed. The rat is a useful animal for studying the mechanisms of action of estrogen on thermoregulation in heat stress, and can be utilized to define the upper limits of temperature exposure and the tissue specificity of the response. The information derived from these studies may in turn be an indication of therapeutic interventions for athletes competing in the heat.

Methods

Animals. Two groups of 8 week old female Sprague-Dawley rats (Harlan, Madison, WI) were utilized to conduct these experiments: a vehicle control group (251-300 g, n=18) and an estradiol treated group (220-263 g, n=18). Ovariectomized rats were used to precisely control for the effects of female reproductive hormones on the HSP response. OVX rats have no estrous cycle and no circulating plasma estrogen and progesterone, thereby enabling experimental manipulation of hormone concentrations. Ovariectomies were performed by the supplier prior to shipment. Rats were housed in group cages in a temperature-controlled animal facility with a 12:12-h light-dark cycle and were provided standard rat chow and water ad libitum. Experiments and animal care procedures were performed in accordance with institutional guidelines. All rats were familiarized with the testing environment and a colonic thermistor probe several times over the week preceding an experiment. Animal weights were recorded immediately before and after each experiment.

Experimental Protocol. Within each of the two groups, animals were randomly divided into three sub-groups each with a separate protocol dependent upon the duration of injections: 1) Four day treatment protocol, 2) Eight day treatment protocol, and 3) Twelve day treatment protocol. Each injection was administered by one investigator at the same time of day to ensure consistency of hormone treatment throughout the protocol. The vehicle control group was administered a 0.1 ml sesame oil/100 g body weight dose and the estradiol treated group was given

a pharmacological dose of 17 13-estradiol 3-benzoate (Sigma Chemical, St. Louis, MO): 10 μg •0.1 ml of sesame oil^{-1} · 100 g body weight^{-1} injected into the subcutaneous dorsal neck skinfold. Four hours following the final daily injection for each protocol, the rats were prepared for the experimental intervention. All experiments were conducted on conscious unrestrained rats. Internal body temperature was monitored continuously during each experimental protocol by a thermistor probe inserted 6-7 cm beyond the anal sphincter into the colon (T_c). Following attachment of the probe, each rat was placed inside an enclosed small animal treadmill unit maintained at an ambient temperature (T_a) 35°C. The rats were exposed to an exertional heat tolerance test (HTT) consisting of continuous treadmill exercise at a velocity of 21.5 m/min (0% grade). The experimental protocol was terminated when the animals attained a T_c =40.4°C. This Tc was chosen because it was demonstrated in the rat that T_c values of 41.0 and 40.4°C are the minimal lethal temperatures for death due to nonexertionally and exertionally induced heat exhaustion, respectively (15). Upon completion of the exercise challenge, the rats were removed from the treadmill unit and placed into their home cage for four hours prior to collection of tissue samples. The rationale for choosing this time point was that previous data indicate that maximal HSP70 induction to estradiol occurs in this 4-12 hour post-injection period (23, 27, 31). In general, the heat tolerance test was administered four hours following the last injection of either estradiol or vehicle.

Assays. Following the four hour waiting period to allow adequate time for protein synthesis, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg). It has been shown that this procedure will have no effect on HSP70 synthesis (5). A midline incision was made on the ventral surface of the rat. A 2 ml sample of heparinized blood was obtained via exsanguination through the inferior vena cave and placed into a bullet tube for centrifugation. Blood samples obtained during exsanguination were used for measurement of hematocrit, and plasma osmolality. Hematocrit was measured in duplicate immediately after bleeding using microhematocrit tubes and a microcentrifuge. The blood samples were centrifuged for six minutes and the ratio of red blood cells to total blood volume was determined. For the measurement of plasma osmolality, the heparinized, whole blood sample was centrifuged immediately after collection and aliquots of plasma were stored at -70°C. The aliquots were then thawed and analyzed for plasma osmolality using a vapor pressure osmometer.

Analysis. There were 36 ovariectomized female rats separated into two treatment groups consisting of a vehicle control group and an estradiol treated group. These groups were in turn divided into three sub-groups depending upon the duration of the treatment protocol. All results are presented as means + SE. Appropriate statistical analysis was performed to determine significant differences in animal weight (pre- vs. post-Heat Tolerance Test), initial and final T_c values, heating rate, and heating rate as a function of body mass. In addition, hematocrit and plasma osmolality were also analyzed. Significant differences were determined with an analysis of variance for two factors design (Comparison between protocols within each treatment group and comparison between treatment groups within a given protocol) followed by Duncan's post hoc analysis. Significance was established at the $P < 0.05$ level.

Results

As shown in Table 1, the administration of sesame oil vehicle had no effect on either the initial T_c or the change in T_c during the heat tolerance test within any of the three protocols. In addition, there were no significant differences among the three vehicle-treated groups with respect to heating rates and the length of time to reach 40.4°C.

The administration of estradiol for eight and twelve days had significant effects on baseline body temperature (Table 1). The eight day and twelve day estradiol-administered animals had significantly lower initial T_c values when compared to the four day treated group ($P<0.05$). A reduction in resting core temperature was also demonstrated in middle-aged women undergoing estrogen replacement therapy, suggesting that estrogen acts centrally to lower the set point about which T_c is regulated (34). Therefore, the change in T_c to reach 40.4°C was significantly elevated in both the eight and twelve day estradiol-treated animals. In addition, the length of time to reach 40.4°C was significantly increased in the eight and twelve day estradiol-treated protocol. The heating rate and heating rate as a function of body mass was also significantly lower in the eight and twelve day treated animals when compared to the four day treated estradiol protocol. Furthermore, the eight and twelve day estradiol-treated groups displayed a

significantly lower initial T_c when compared to the vehicle-treated animals undergoing the similar time-matched treatment protocol (i.e., 8 day estradiol vs. 8 day vehicle and 12 day estradiol vs. 12 day vehicle). The change in T_c and the time to 40.4°C in the eight and twelve day estradiol-administered animals were significantly higher than the time-matched vehicle-treated animals within the comparable protocol. In addition, estradiol administration significantly reduced the heating rate and heating rate as a function of mass in the eight and twelve day protocols when compared to animals treated with sesame oil vehicle for the same duration of time. The four day estradiol-treated animals had a significantly elevated heating rate and heating rate/mass when compared to the four day vehicle-treated animals.

Vehicle-administered animals within the eight and twelve day protocols had significantly elevated body weights both before and after the heat tolerance test when compared to the four day vehicle-treated group (Table 2). Body weight was lower in estradiol-treated animals, an effect of estrogen that is commonly observed in ovariectomized rats (2, 20). Both the pre- and post-weight measurements within each of the three protocols in the estradiol-treated animals were significantly reduced when compared to animals undergoing sesame oil vehicle treatment for the same duration of time. Furthermore, the body weight measurements obtained prior to the exercise intervention were significantly lower in the eight and twelve day estradiol-treated animals when compared to the four day estradiol-treated group. Estradiol treatment significantly decreased body weight following the heat tolerance test in the eight day protocol compared to the four day protocol. Animals administered estradiol for twelve days displayed a trend for a reduction in post-weight measurements when compared to the four day estradiol-treated animals ($P<0.10$). However, the administration of estradiol for any of the three protocols had no effect on the change in body weights of the animals during the heat tolerance test. Estradiol administration had minimal effects on hematocrit and plasma osmolality (Table 2). There was a tendency for hematocrit to be lower in estradiol-treated animals when compared to each protocol's time-matched vehicle-treated animals. However, this difference was only significantly in the four day estradiol-treated group. Furthermore, a trend for an elevated plasma osmolality was observed in the estradiol-treated animals with respect to the time-matched vehicle-treated animals. Again, this was only significant during the four day estradiol treatment protocol.

Discussion

The effects of hormonal therapy in females exercising in the heat has only recently been addressed. To study the effects of estrogen in regulating core temperature, several investigators have examined the consequences of exogenously administered estrogen in middle-aged women exhibiting menopausal symptoms. This approach is utilized because estrogen withdrawal during menopause has been associated with signs and symptoms that are analogous to heat loss responses (19). Specifically, the sensation of hot flashes coincides with increased skin blood flow and sweat rate (19). The onset of heat loss responses has been demonstrated to be lower in middle-aged women administered estrogen replacement therapy during exercise exposure in a warm environment, thereby reducing the thermoregulatory strain (34). This was determined by a decrease in the threshold body temperature for the onset of both sweating and cutaneous vasodilation, measured via forearm blood flow (34). It is suggested that the elevated plasma estrogen concentration acts centrally to lower the set point about which T_c is regulated at rest and during a heat and exercise challenge (34). The mechanisms of these effects of estrogen during heat stress are not known and could involve direct or indirect actions on peripheral effectors or on central nervous thermoregulatory control regions. In addition, these effects could be secondary either to systemic actions involving alterations in body fluid balance, or to the cellular contributions to thermotolerance via heat shock proteins. It was the purpose of this study to measure the effects of chronic estrogen replacement throughout three different time courses of therapy on thermotolerance in ovariectomized rats exercising in a hot environment. Not only did we evaluate estrogen's effects on thermotolerance, but also we delineated a time course of action of estrogen therapy on heat loss mechanisms.

The present experiments demonstrated that plasma estrogen levels obtained through both the eight and twelve day treatment protocols had a marked effect on thermotolerance in ovariectomized rats exercising at a high ambient temperature. Estrogen-treated animals within the eight and twelve day groups had a lower heating rate, and the length of time that was

necessary to elevate T_c to 40.4°C was significantly increased when compared to the four day estradiol treated animals, in addition to the time-matched vehicle-treated animals for each protocol. Furthermore, estrogen administration for the eight and twelve day groups resulted in significantly reduced resting colonic temperatures compared to the four day estradiol-treated animals and each group's time-matched vehicle-treated animals, indicating that estrogen acts centrally to lower the set point about which body temperature is regulated in ovariectomized rats. This is indicative of a time course of estrogen administration that is required to enhance thermotolerance in ovariectomized rats, as measured by the animals ability to maintain exercise in the heat for a significantly longer duration of time. further, because there were no statistical differences observed between the eight and twelve day estradiol-treated groups, it is suggested that estrogen's effects on thermotolerance reach a plateau within eight days of hormone therapy.

The enhanced thermotolerance in the eight and twelve day estradiol-treated animals could be a function of the significantly reduced body weight observed when compared to vehicle-treated animals. Because we utilized the same absolute workload in all the animals without assessing VO_2 max, the decrease in body weight would allow the animals to run longer to perform the same amount of work as heavier vehicle-administered animals (18). The reduced body weight would enable the animals to be exercising at a lower intensity, or lower percent of VO_2 max, when compared to the heavier oil-treated animals, which would contribute to the significant increase in the exercise duration throughout the heat tolerance test. Kendrick et al. (17) suggested that the differences in body weight may possible affect the recruitment of muscle fibers and alter the muscles' pattern of fuel use while the same amount of work has been performed. However, this may not be a critical factor to the enhancement of thermotolerance in this study because when normalized for body weight, the animals within the eight and twelve day estradiol groups still displayed a significant reduction in heating rate as a function of mass.

Body Temperature Regulation. Enhanced thermotolerance in ovariectomized rats could be related to the influence of estrogen on thermoregulation in heat stress. The effects of estrogen replacement on thermoregulatory evaporation and body core temperature in ovariectomized rats resting in a hot environment have been previously evaluated (2). It was demonstrated that the rate of evaporation at a given core temperature for estrogen-treated animals was higher during the passive heat exposure period, and the threshold core temperature at which the estrogen-treated animals initiated spreading saliva was lower than untreated animals (3). Evaporative water loss was utilized to measure thermoregulatory responses because the rat uses saliva spreading for evaporative cooling (33). Therefore, administration of estradiol to ovariectomized rats in the present study should enable the animals to maintain a lower colonic temperature during an exercise challenge in the heat. Moreover, evaporative water loss should be higher at any given core temperature after estrogen replacement therapy due to a lower core temperature threshold for the onset of heat loss responses. This would allow the animals to sustain exercise in a warm environment for a longer duration of time, as was observed during the heat tolerance test in the eight and twelve day estradiol-treated groups. In addition, similar effects of estrogen on body temperature and on evaporative cooling have been observed in human females during exercise in a warm environment (34). Baker et al. (2) suggest that central neural regions controlling body temperature could be mediating the influence of estrogen on thermoregulatory evaporation, indicated through the similarity of the hormonal therapy on heat loss mechanisms in two difference species, each possessing distinct methods of evaporative heat loss. The hypothalamus is involved in regulating appetite, thirst and water conservation, temperature, sleep, autonomic balances, and endocrine functions (12). Specifically, due to the presence of thermosensitive neurons in the preoptic anterior hypothalamus, this region of the brain plays a significant role in thermoregulation (4). When brain slices of the preoptic area of the hypothalamus in the rat were bathed in an estradiol (E2) perfusate, 26% of the warm-sensitive neurons responded with an increased firing rate (32). This result suggest that E2 can stimulate a greater heat loss response in the rat, and its site of stimulation may involve the preoptic hypothalamic neurons that are known to regulate body temperature (32). However, preoptic estrogen implants had no effect on T_c of rats resting in a euthermic environment at an ambient temperature of 24°C. In addition, normal fluctuations of the hormonal environment during the menstrual cycle may influence thermoreuglatory responses during exercise and heat stress. Both estrogen and progesterone levels are elevated during the post-ovulatory luteal phase

when compared to the follicular phase. There appears to be a thermogenic effect of the increased progesterone levels during the menstrual cycle, causing an increase in basal metabolic rate (19). This is believed to be a result of the increased concentration of circulating progesterone during the phase. Therefore, changes in estrogen and progesterone concentrations during the menstrual cycle may influence thermoregulatory function in females during an exercise challenge. Although we did not directly measure thermoregulatory responses during the exercise intervention in the heat, estrogen's effects following eight and twelve days of therapy in the present experiment indicate that the elevated thermotolerance could be indicative of enhanced heat loss responses. Indeed, the reduction observed in resting, baseline core temperature suggests that estrogen could play a role in centrally lowering the set point about which body temperature is regulated, which in turn could exert its effects during exercise, blunting the rise in core temperature throughout the duration of the heat tolerance test. This would allow for an increase in heat loss responses at any given core temperature during the exercise intervention.

Body Fluid Composition. Plasma volume expansion is closely associated with estrogen therapy in both humans and rats (3, 21, 34). An increase in plasma volume corresponds with a reduction in hematocrit in estradiol-treated animals, with no alterations in erythrocyte volume (3). Previous reports in the literature indicate that estrogen is effective at lowering Hct measurements in resting animals (2,3). It is suggested that a lower Hct following estradiol administration is a strong indicator of plasma volume expansion (2). Plasma volume in both resting and exercise conditions is a major factor influencing thermoregulation. During exercise, interstitial fluid levels are reduced due to sweat formation and fluid shifts which tend to induce hypovolemia, compromising circulatory and thermal regulation (6). Therefore, hypervolemia, commonly associated with trained or heat acclimatized subjects, allows considerable loss of body water before plasma volume returns to pre-heat exposure or pretraining values (14). Consequently, it is reasonable to postulate that hypervolemia can improve performance by inducing better muscular perfusion, by limiting the reduction in cardiac output due to the decrease in plasma volume during exercise, and by facilitating thermoregulation by increasing skin blood flow (26). Plasma volume expansion observed as an adaptive response to acclimation to heat is associated with increased sweat rate, increased evaporative heat loss, and decreased skin temperature during heat (6, 26). These thermoregulatory adaptations correlate highly with plasma volume and appear to play significant roles in enhancing heat tolerance by producing a decreased core temperature and reduced strain as acclimation occurs (6). By increasing the cutaneous perfusion, hypervolemia enhances the body's ability to regulate body temperature during exercise. Conversely, it has been demonstrated that the core temperature in dehydration is always found to be higher than in euhydration (7). The role of hypervolemia as a function of estradiol-induced plasma volume expansion as it relates to thermoregulatory responses has not been fully established. Marked reductions in Hct after estrogen therapy in ovariectomized rats is associated with an elevation in thermoregulatory responses, determined by evaporative water loss, while resting in the heat (2). Therefore, plasma volume expansion could play a beneficial role in heat loss mechanisms in the rat. Unfortunately, we did not directly measure baseline plasma volume or hematocrit values. However, we may speculate that estrogen therapy elevates plasma volume, reducing hematocrit, which in turn would increase evaporative cooling and enhance the animal's ability to perform exercise in the heat.

Metabolic Responses. The administration of estrogen may improve exercise performance in the heat by promoting liver and muscle glycogen sparing. It is well established that during prolonged exercise glycogen stored in liver and muscle tissue is utilized as a metabolic fuel source (8, 11, 13, 28). Depletion of muscle glycogen may play a significant role in the development of exhaustion during prolonged strenuous exercise (1, 10). Furthermore, when previously sedentary individuals engage in aerobic exercise training, the duration of time they can maintain a given submaximal work load is typically elevated (11). One mechanism whereby this may occur could involve a reduction in the rate of carbohydrate utilization by elevated oxidation of fatty acids. Early studies in the literature indicate that fatty acid oxidation inhibits carbohydrate utilization in isolated perfused rat heart tissue, limiting both glucose uptake and glycolysis (24, 25, 29). In addition, high concentrations of fatty acids has been demonstrated to slow carbohydrate utilization in the fast-twitch skeletal muscle in exercising rats (30). Further, the rate of muscle tissue uptake of plasma fatty acids is proportional to the concentration of fatty acids to which it

is exposed (9, 16). When plasma free fatty acid levels were artificially elevated in rats prior to an exercise exposure, the animals ran for a significantly longer duration of time when compared to the controls, suggesting a marked influence on endurance perhaps due to enhanced muscle fatty acid oxidation (11). Therefore, an increase in the availability of fatty acids could be a significant factor in reducing glycogen depletion and enhancing endurance in trained compared to untrained individuals.

Summary

In conclusion, the evidence presented in this study suggests that administration of estradiol for eight and twelve days increases thermotolerance in ovariectomized rats. In addition, the data are indicative of a time course of hormonal therapy necessary to enhance performance during an exercise and heat challenge in ovariectomized rats, and that estrogen's effects on thermotolerance reach a plateau within eight days of treatment. These results could be due to enhanced thermoregulatory responses during exercise in a warm environment. Furthermore, estradiol-induced plasma volume expansion could also contribute to improving thermoregulation. Both of these factors would play a role in elevating evaporative cooling and heat loss mechanisms. In addition, estradiol treatment may result in a decreased rate of tissue glycogen utilization secondary to an estradiol-mediated increase in the availability of lipid substrate during the exercise intervention in the heat.

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Table 1. Effects of estradiol treatment on T_c, heating duration, heating rate, and heating rate as a function of mass in ovariectomized rats.

Group	Initial T_c, °C	ΔT_c, °C	Time to 40.4°C	Heating Rate, °C/min	Heating Rate/Mass, $°C \cdot kg^{-1} \cdot min^{-1}$
Vehicle					
4 Day	37.3±0.1	3.1±0.1	20.99±1.05	0.151±0.005	0.541±0.020
8 Day	37.3±0.1	3.1±0.1	20.30±1.19	0.155±0.007	0.550±0.020
12 Day	37.4±0.0	3.0±0.0	20.41±0.87	0.148±0.006	0.526±0.024
Estradiol					
4 day	37.4±0.1	3.0±0.1	17.66±1.53	0.175±0.008†	0.7078±0.034†
8 Day	36.8±0.2*†	3.6±0.2*†	44.77±8.93*†	0.091±0.011*†	0.383±0.042*†
12 Day	37.0±0.1*†	3.4±0.1*†	31.00±2.01*†	0.112±0.004*†	0.454±0.018*†

Values are means ± SE; n = 6 rats per group. T_c, colonic temperature. *Significantly different from 4 Day within a treatment group. †Significantly different from time-matched Vehicle. Differences were considered significant at the $P<0.05$ level.

Table 2. Effects of estradiol on body weight, hematocrit, and plasma osmolality of ovariectomized rats.

Group	Pre-Weight, g	Post-Weight, g	ΔWeight, g	Hct, %	Posm, mosmol/kgH_2O
Vehicle					
4 Day	273±2.2	269±3.5	3.7±0.3	38.1±0.01	336.7±1.36
8 Day	281±3.9*	276±3.9*	4.8±0.8	37.3±0.01	341.2±4.45
12 Day	283±5.6*	278±5.5*	4.3±0.8	36.5±0.01	334.2±1.67
Estradiol					
4 Day	248±3.0†	243±1.8†	5.3±1.7	35.8±0.01†	355.2±6.34†
8 Day	237±4.0*†	230±5.1*†	6.7±1.5	35.6±0.01†	346.0±3.15
12 Day	237±1.5*†	232±1.5†	4.0±0.4	35.5±0.01†	343.8±4.14

Values are means ± SE; n = 6 rats per group. Hct, hematocrit; P_{osm}, plasma osmolality. Note that P_{osm} was lower in estradiol- compared with vehicle-treated rats. *$P<0.05$ vs. 4-day treatment within a group. †$P<0.05$ vs. time-matched vehicle group.

B. The effects of estradiol and training on thermotolerance during exercise and heat stress

Abstract

Ninety female ovariectomized rats, approximately 10 weeks old were randomly divided into 5 groups. In each group, at least five rats received treatment for 4, 8 and 12 days.

	Conditioned Heat Treatment	Hormone During Pre-HTT	Hormone During HTT
Group I	No	Estradiol	Vehicle
Group II	Yes	Estradiol	Vehicle
Group III	Yes	Vehicle	Vehicle
Group IV	Yes	Estradiol	Estradiol
Group V	No	No	No

Treatment consisted of daily injections of either estradiol (10 micrograms in 100 microliters of sesame oil per 100 grams body weight) or vehicle (sesame oil). At the end of the treatment period, a heat tolerance test was performed. Rats were instrumented with colonic thermistor probes for core body temperature measurements, then exercised on a treadmill at 21.5 meters per minute with an ambient temperature of 35°C until core temperature rose to 41.5°C. The initial weights and initial temperatures were recorded and heating rates were calculated. The results indicate that the training alone, estradiol alone, and a combination of estradiol and training enhanced heat tolerance, as evidenced by decreased resting core body temperatures and slowed heating rates compared to the vehicle treated and naive groups. These data suggest that a combination of training and estradiol treatment for at least 8 days best enhances heat tolerance in ovariectomized female rats.

Introduction

Women having to exert force in warm climates is growing increasingly more common. In particular, female athletes and women in the military are forced to adapt very rapidly to work in the heat. Previous studies have examined the effects of estrogen on the thermoregulatory and metabolic responses to exercise, but these studies utilized only one pre-established duration of hormone administration (1, 2). It is difficult to resolve the time course of estradiol-induced changes in body fluid composition, thermoregulation and metabolism. Therefore, it would be advantageous to know the best way to acclimate a female to physical activity in the heat.

Gender differences in temperature regulation and heat tolerance exist in humans. Women have a lower sweating rate than men and are at a disadvantage for work in hot climates (1, 3). It has been noted that heat tolerance (assessed by core body temperature and time to onset of sweating) is improved in post-menopausal women by estradiol supplementation; however, the effects of estrogen supplementation on heat tolerance have not been studied in normally menstruating women. Furthermore, no study to date has examined the time required to develop heat acclimation in females (4).

Estrogen plays a significant role in maintaining proper body temperature regulation in post-menopausal women by reducing hot-flashes and by increasing heat dissipatory mechanisms during exercise (1). Studies indicate that a relationship exists between elevated circulating estrogen levels in middle-aged women given estrogen replacement therapy and the maintenance of a lower temperature at rest and during exercise in the heat (1). Furthermore, estrogen withdrawal during menopause has been associated with signs and symptoms that are analogous to heat loss responses. For example, the sensation of hot flashes coincides with increased skin blood flow and sweat rate (5). It has been suggested that the elevated plasma estrogen concentration acts centrally to lower the set point at rest and during a heat and exercise challenge (1).

Although these studies have evaluated the effects of estrogen in post-menopausal women, few have addressed the thermoregulatory adjustments associated with thermotolerance that accompany the hormonal changes in normally menstruating females. Because estradiol improves exercise performance in the heat in post-menopausal women receiving estradiol supplementation, it is likely that estradiol will be an important factor in accelerating heat

tolerance and will reduce the duration of time required to achieve heat acclimation (1). A recent study evaluated estrogen's effects on thermotolerance. The results of this study showed that plasma estrogen levels have a marked effect on thermotolerance in ovariectomized rats exercising at high ambient temperatures (4). The study also showed that estrogen-treated animals had a lower heating rate, and the length of time that was necessary to elevate T_c to 40.4°C was significantly increased (4).

It has also been hypothesized that aerobic training enhances endurance by increasing the capacity of muscles to oxidize fatty acids (6), with utilization of more fat and less carbohydrate during exercise. It has been observed that male animals administered estradiol for five days have significantly greater plamsa fatty acid contents at rest and following two hours of sub-maximal exercise (2). Therefore, with increased utilization of free fatty acids as an energy source during prolonged exercise, estrogen exerts a glycogen sparing effect which enhances exercise performance and duration (2).

Laboratories (7, 8) have documented that the rat is an excellent model for the study of heat stress and exercise acclimation, and testing protocols for the rat have been developed (7, 8). The rat is a useful animal for studying the mechanisms of action of estrogen on thermoregulation in heat stress, and can be utilized to define the upper limits of temperature (4). In addition, the rat can be subjected to a variety of severe stressors that cannot be safely undertaken in humans, although humans in the field are frequently exposed to these stressors and achieve the high core body temperature that will be utilized in this experiment.

The overall purpose of this study is to determine the best possible method to acclimate female ovariectomized rats to physical stress in warm environments, specifically to test the hypothesis that: 1) exercise training will enhance thermotolerance to exercise in the heat, 2) the length of training will affect thermotolerance, 3) the duration of estradiol therapy will affect thermotolerance, and 4) a combination of exercise training and estradiol supplementation will work best to acclimate a female ovariectomized rat to exercise in a warm environment.

Methods

Ovariectimized female Sprague-Dawley rats from a commercial vendor (Harlan, Indianapolis, IN) were used for this experiment. Ovariectomies were to be performed by the supplier prior to shipment and verified by luteinizing hormone, follicle stimulating hormone, estradiol and progesterone levels. The rats were approximately ten weeks old and weighted between 220 and 250 grams. The rats were stored in cages in the Medical Laboratory Animal Care Unit, with free access to water and food (standard rat chow) and a twelve hour light-dark cycle.

After the rats arrived at the Animal Care Unit, they spent three days being familiarized to the testing environment. They were handled and taught to run on the treadmill with a colonic thermistor probe. After the initial conditioning, the study began.

The rats were randomly divided into five groups and groups I-IV were further divided into three sub-groups. 1) four day treatment, 2) eight day treatment and 3) twelve day treatment. At least five rats were in each of the sub-groups. On the final day of the treatment (either the fourth, eighth, or twelfth day) a heat tolerance test (HTT) was administered.

At 8 a.m., the rats were weighed and the amount of estradiol or vehicle to be injected was calculated. The vehicle control group was administered 10 micrograms of sesame oil per 100 grams body weight and the estradiol group was given a pharmacological dose of 17 Beta-estradiol 3-benzoate (Sigma Chemical, St. Louis, MO; 10 micrograms in 100 microliters of sesame oil per 100 grams body weight). The vehicle and estradiol serums were injected into the subcutaneous dorsal neck skinfolds. Each injection was administered by one investigator at the same time of day to ensure consistency of hormone treatment throughout the protocol.

Three hours after the injections a colonic thermistor probe was inserted 6 cm beyond the anal sphincter and into the colon (T_c) in the conditioned heat treatment group. The pre-exercise temperatures were recorded (T_1). The rats were then placed on a treadmill and walked at a pace of 21.5 meters per minute at a 0% grade and timed until their temperature reached 40.4°C. At this time the rats were removed from the treadmill, the time taken to raise their temperatures to 40.4°C was recorded, and their masses were again recorded.

There was a shock grid at the back of the track of the treadmill to keep the rats walking continuously. A reverse air conditioner was used to pump warm air into the treadmill and a probe monitored the temperature in the treadmill. The treadmill temperature (T_a) was kept at a constant 32°C.

On the final day of the study (either the fourth, eighth, or twelfth day) a heat tolerance test (HTT) was administered. The appropriate injection was given at 8 a.m. and four hours later the rats were weighted, the colonic thermistor probe was inserted and T_1 were recorded. The rats were placed on the treadmill with T_a = 35°C. The rats walked at 21.5 meters per minute at a 0% grade and the time it took to raise their temperatures to 41.5°C was recorded. The rats were then removed from the treadmill and weighed again.

Results

The three variables examined in this study were exercise training, the treatment during the study and the treatment on the day of the heat tolerance test (Table 1). The notation for group designation will be composed of three parts: 1) trained or untrained, 2) treatment prior to heat tolerance test and 3) treatment on the day of the heat tolerance test. For example, Group I will be referred to as untrained-E_2-vehicle.

Comparing the untrained-E_2-vehicle with the trained-E_2-vehicle will demonstrate the effects of training on heat tolerance, while comparing the trained-Vehicle-Vehicle group with the trained-E_2-E_2 will demonstrate the effects of estradiol on thermotolerance (Table 2).

Student's-tests were used to compare the variables. Statistical significant was observed when the p-value was less that .05.

Weight. Between Groups: The trained-E_2-E_2 versus the trained-vehicle-vehicle group had significantly lower weights regardless of length of treatment. The untrained-E_2-vehicle group versus the trained-E_2-vehicle group had similar weights, with only the 8-day trained group having significantly lower weight than the comparable untrained group. Within groups: The general trend was for the weights to decrease as the length of treatment increased. Interestingly, the trained-vehicle-vehicle group had weights increase with length of training duration increased.

Pre-Heat Tolerance Test Temperature. Between Groups: The trained-E_2-vehicle group versus the untrained-E_2-vehicle group had lower temperatures when comparing the 4-day and 8-day treatment groups. The trained-E_2-E_2 versus the trained-vehicle-vehicle group had lower temperatures when comparing the 8-day treatment groups. The trained-E_2-vehicle group had significantly lower initial temperatures than the trained-E_2-E_2 group. Within Groups: The 8-day treatment groups in the trained-E_2-vehicle group and the trained-E_2-E_2 were significantly lower than the comparable 4-day treatment groups. No other statistical differences were noted.

Run-time During Heat Tolerance Test. Between Groups: The trained-E_2-vehicle rats ran significantly longer until their temperatures reached 41.5°C compared to the untrained-E_2-vehicle rats for the 4- and 12-day treatment groups. The trained-E_2-E_2 rats also ran significantly longer than the trained-vehicle-vehicle for the 4- and 12-day treatment groups. All of the treatment groups ran significantly longer than the naive group. Interestingly, the trained-E2-vehicle group always ran longer than the trained-E2-E2 group. *Within Groups:* In all but the trained-E_2-E_2 groups, the general trend was to increase the run-time with the length of treatment. The untrained-E_2-vehicle 12-day rats ran significantly longer than the 4-day rats. In the trained-E_2-vehicle group, the 12-day rats ran longer than the 4- or 8-day rats.

Heating Rate. Heating rate is defined as the change in temperature (°C) divided by the time necessary to raise the rats' temperature to 41.5°C (minutes). *Between Groups:* The trained-E_2-vehicle rats' heating rate was significantly lower compared to the untrained-E_2-vehicle rats for the 4- and 12-day treatment groups. The trained-E_2-E_2 rats also had significantly lower heating rates than the trained-vehicle-vehicle rats for the 4- and 12-day treatment groups. For the 4- and 12-day treatment groups, the trained-E_2-vehicle rats and the trained-E_2-E_2 rats had heating rates that were less than the untrained-E_2-vehicle rats and the

trained-vehicle-vehicle rats. All groups had significantly lower heating rates than the naive group. Within Groups: Again, the general trend was for the heating rate for the 12-day treatment groups to be lower than the 4- or 8-day groups or both. The untrained-E_2-vehicle 12-day rats' heating rate was significantly lower than the 8-day. In the trained-vehicle-vehicle group, the 12-day rats had significantly lower heating rates than the 4-day rats. In the trained-E_2-vehicle group, the 12-day rats had significantly lower heating rates than the 4- or 8-day rats. The trained-E_2-E_2 12-day rats also had significantly lower heating rates than the 4- or 8-day rats.

Heating Rate/Kg. The heating rate/kg is calculated as the change in temperature (°C) divided by the product of the mass (kg) and the time it took to raise the rats' temperature to 41.5 °C (minutes). Between Groups: The trained-E_2-vehicle rats' heating rate/kg was significantly lower compared to the untrained-E_2-vehicle rats for the 4- and 12-day treatment groups. The trained-E_2-E_2 rats also had significantly lower heating rates/kg than the trained-vehicle-vehicle rats for the 4-day treatment group only. All treatment groups were significantly lower than the naive control group. The heating rate/kg for the trained-E_2-vehicle rats was lower than the trained-E_2-E_2 rats. The trained-E_2-vehicle rats and the trained-vehicle-vehicle rats had heating rates/kg that were less than the untrained-E_2-vehicle rats and the trained-vehicle-vehicle rats. Within Groups: Again, the general trend was for the heating rate/kg to decrease when the length of treatment increased. The untrained-E_2-vehicle 8-day rats' heating rate/kg was significantly lower than the 4-day. In the trained-vehicle-vehicle group, the 8- and 12-day rats had significantly lower heating rates/kg than the 4-day rats. In the trained-E_2-vehicle group, the 12-day rats had significantly lower heating rates than the 4- or 8-day trained-E_2-E_2 group, no statistical differences were observed.

Discussion

The purpose of this study was to determine the best possible method to acclimate females to physical stress in warm environments, specifically to test the hypotheses that: 1) exercise training will enhance thermotolerance to exercise in the heat, 2) the length of training will affect thermotolerance, 3) the duration of estradiol therapy will affect thermotolerance and 4) a combination of exercise training and estradiol supplementation will work best to acclimate a female ovaiectimized rat to exercise in a warm environment.

The present experiments demonstrated that exercise alone enhanced thermotolerance, as measured by the slower heating rate during a heat tolerance test and lower pre-heat tolerance test temperature. We also observed that the estradiol therapy had a marked effect on thermotolerance, by decreasing the core temperature of the rats and slowing the heating rate in these animals. Furthermore, it was found that a combination of estradiol therapy and exercise training had the greatest effect on enhancing thermotolerance.

Temperature Regulation. The trained-E_2-vehicle group versus the untrained-E_2-vehicle group had lower temperatures when comparing the 4- and 8-day treatment groups. The body's thermoregulatory mechanisms are primarily geared to protect against overheating. During sustained vigorous exercise, the metabolic rate can increase 20 to 25 times above the resting level; this theoretically can increase core body temperature by about 1°C every 5 to 7 minutes (9). In hot weather, a competition exists between mechanisms that maintain a large muscle blood flow and those that provide skin blood flow for adequate thermoregulation. In humans exercising at 50% of VO_2 max, increases in core temperature to a steady level of approximately 37.3°C are observed, whereas working at 75% VO_2 max elevates core temperature to 38.5°C, regardless of the absolute level of oxygen uptake (10). Therefore, at the same percentage of VO_2 max, a fit person generates more total energy during exercise but still has about the same core temperature as a less fit person (11). This extra metabolic heat is dissipated via a larger sweat output by the person working at the higher absolute level of exercise. Therefore, at any given workload, a trained person exercises at a lower core body temperature. The data obtained from out studies support these statements.

Body weight is another factor entering into the equation of heat tolerance. The trained-E_2-vehicle rats had significantly reduced weight compared to the untrained-E_2-vehicle rats and had

lower heating rates. Because the same absolute workload was utilized in all animals without assessing VO_2 max, the decreased body weights would allow the animals to run longer to perform the same amount of work as heavier animals (2). The reduced body weight would enable the animals to be exercising at a lower intensity, or lower percent of VO_2 max, which would generate less heat.

The trained-E_2-E_2 versus the trained-vehicle-vehicle group had lower temperatures when comparing the 8-day treatment groups. The trained-E_2-E_2 animals also had longer run times during the heat tolerance test than the trained-vehicle-vehicle and, therefore, lower heating rates. The effects of estrogen replacement on thermoregulatory evaporation and core body temperature in ovariectomized rats resting in a hot environment have been previously evaluated (12). Evaporate water loss was utilized to measure thermoregulatory responses because the rat uses saliva spreading for evaporative cooling (13). It was demonstrated that the rate of evaporation at a given core temperature was higher during passive heat exposure, and the threshold core temperature at which estrogen-treated animals initiated saliva spreading was lower than in untreated animals (12). Therefore, the estradiol-treated rats in this study should maintain lower colonic temperature during exercise in the heat.

Furthermore, in humans, evaporate water loss should be higher at any given core temperature after estrogen replacement therapy due to a lower core body temperature threshold for the onset of heat loss responses. This would allow the humans to sustain exercise in a warm environment for a longer duration of time. In prior studies, similar effects of estrogen on body temperature and on evaporative cooling have been observed in human females during exercise in warm environments.

Estrogen's effects in this experiment indicate that the elevated thermotolerance could be indicative of enhanced heat loss responses. The estradiol-treated groups had reduced baseline core temperatures. Because the animals had a lower initial core body temperature in the estradiol treated groups, during the heat tolerance test their rise in core temperature could be blunted. This would allow for an increased heat loss response at any given temperature during the exercise bout compared to the vehicle groups.

One potential explanation for the lower pre-heat tolerance tests temperature in the estradiol treated rats is that estradiol is lowering the set point. The hypothalamus contains the central coordinating center for the carious processes of temperature regulation (14). Thermosensitive neurons in the preoptic anterior hypothalamus play a significant role in thermoregulation (14). When brain slices of the preoptic area of the hypothalamus in the rat were bathed in estradiol (E_2) perfusate, 26% of the warm-sensitive neurons responded with increased firing rate (15). This result suggests that E_2 can stimulate a greater heat loss response in the rat and its site of stimulation may involve the preoptic hypothalamic neurons that are known to regulate body temperature (15).

Body Fluid Composition. In general, our 12-day trained rats had a significantly slower heating rate than untrained rats that trained for 4- or 8-days. One cause of this phenomenon might involve differences in plasma volume between the two groups of rats. One of the advantages of training is a significant increase in plasma volume. The rate of thermoregulatory evaporation in warm environments, as determined my forearm blood flow and sweating rate, is reduced when plasma osmolality is elevated by dehydration (5, 13). Also, plasma osmolality at the same absolute workload in a trained versus an untrained group is lower post-exercise in the trained group. This suggests that trained rats would have greater heat dissipatory mechanisms without compromising their plasma volume.

In humans, this increase in plasma volume might have greater benefits. During exercise, interstitial fluid levels are reduced due to sweat formation and fluid shifts, which tend to induce hypovolemia. Consequently, it is reasonable to think the hypervolemia can improve performance by inducing better muscular perfusion, by limiting the reduction in cardiac output due to the increase in plasma volume during exercise, and by facilitating thermoregulation by increased sweat rate, increased evaporative heat loss, and decreased skin temperature during heat exposure (16,17). At the same absolute workloads, a trained and an untrained individual would lose the same amount of plasma volume. However, the trained individual would have a higher pre-exercise plasma volume and , therefore, a higher post-exercise plasma volume compared to the untrained individual. At the same relative percent of VO_2 max, a trained individual loses

more plasma volume, but this individual's post-exercise 4 plasma volume is still higher than the untrained post-exercise plasma volume level(18).

Our estradiol treated groups also had significantly slower heating rates than the naive or the vehicle groups. It has been observed in previous studies that estrogen therapy lowers plasma osmolality in rats (17). As stated previously, the lower the osmolality, the higher the plasma volume expansion, which would reduce plasma osmolality and enhance heat loss mechanisms. Thermoregulatory adaptations correlate highly with plasma volume and appear to play significant roles in enhancing heat tolerance by producing a decreased core temperature and a reduced strain as acclimation occurs (16). Plasma volume in both resting and exercise conditions is a major factor influencing thermoregulation (18).

Metabolic Responses. Another possible mechanism to explain why the 12-day trained rats had significantly lower heating rates than untrained and 4- or 8-day trained rats involves metabolism . It is well established that during prolonged exercise glycogen stored in liver and muscle tissue is utilized as a metabolic fuel source (2, 19, 20). Depletion of muscle glycogen may play a significant role in precipitating exhaustion during prolonged strenuous exercise (21). Furthermore, when previously sedentary individuals engage in aerobic exercise training, the duration of time they can maintain a given submaximal work load is typically elevated (20). This could occur by a reduction in the rate of carbohydrate utilization from an increase in oxidation of fatty acids.

High concentrations of fatty acids have been demonstrated to slow carbohydrate utilization in the slow oxidative skeletal muscle in exercising rats (22). Further, the rate of muscle tissue uptake of plasma fatty acids is proportional to the concentration of fatty acids to which it is exposed (22). When plasma free fatty acid levels were artificially elevated in rats prior to an exercise exposure, the animals ran for a significantly longer time period when compared to the controls. This enhancement could be due to increased muscle fatty acid oxidation (20). Therefore, an increase in the availability of fatty acids could be a significant factor in reducing glycogen depletion and enhancing endurance in trained compared to untrained individuals.

The mechanism of free fatty acid levels decreasing the rate of depletion of glycogen stores in the body has been attributed ton inhibition of glycolysis and glucose uptake in muscle and also to an accumulation of citrate (23, 24). Biochemically, citrate is an inhibitor of the enzyme phosphofurctokinase-1 (PFK-1) (23), which catalyzes the conversion of fructose 6-phosphate to fructose 1,6 *bis*phosphate. Inhibition of PFK-1 results in an accumulation of glucose 6-phosphate (25), a potent inhibitor of hexokinase. An increase in the levels of glucose 6-phosphate reduces glucose uptake by inhibiting glucose phosphorylation (26). This proposal is supported my the commonly observed accumulation of intracellular free glucose (27).

Exercise training results in a host of positive effects on the body. First, there is an increase in glycogen synthetase, which increases the body's ability to store glycogen (18). If more glycogen is stored, more glycogen will be available for use during exercise. Lactate dehydrogenase decreases with exercise training (18). This hormone converts pyruvate to lactate, which is associated with fatigue. Furthermore, hormone sensitive lipase is elevated with training and it elevates the concentration of free fatty acids. Training also increases carnitine and the size and number of mitochondria, where oxidation of fatty acids occur. The number of capillaries also increases, which results in an increased blood flow to working skeletal muscles which will then allow more fat to be burned as a fuel (18). These affects seen with training may help explain the decreased heating rate in the exercise trained groups.

These mechanisms also help explain why estradiol therapy enhances thermotolerance, and why a combination of training and estrogen treatment had the greatest effects on heat acclimatization. Estrogen treatment has been reported to enhance exercise performance and reduce the rate of utilization of glycogen during exercise (2, 19). This may occur through the ability of estrogen treatment to influence both carbohydrate and lipid metabolism of myocardial, skeletal muscle, and adipose tissue (27, 28, 29). It has been demonstrated that plasma free fatty acid levels at rest and following tow hours of submaximal exercise are significantly elevated in male rats undergoing five day s of estrogen therapy (2). Furthermore, tissue glycogen content of estradiol treated animals following tow hours of exercise was significantly increased in slow-twitch and fast twitch skeletal and myocardial muscle fibers and liver tissue when compared to sesame oil-administered animals (2). These results indicate that

estradiol administration enhances the availability and oxidation of free fatty acids, thereby reducing carbohydrate utilization (2).

Considerations and Limitations

It was curious finding that the trained-estradiol-vehicle group had significantly lower heating rates than the trained-estradiol-estradiol group. One possible explanation for this observation would be that there is a shift in vascularization due to the estrogen towards the visceral and away from the skeletal muscles. Also, because prolonged exposure to estradiol results in an increase in body weight, on might see a plateau in the heating rate with estradiol administration. A previous study saw no difference in the heating rates after 8 day and after 12 days of estradiol administration period would better enhance thermotolerance.

Conclusions

Training alone and estradiol alone both enhance heat tolerance in ovariectomized female rats. A combination of the two enhances thermotolerance to the greatest degree, as evidenced by a decreased initial temperature and a reduced heating rate. The 8- and 12-day treatment groups consistently demonstrated better acclimation than the 4-day treatment groups. Hence, based on the results of this study, to best acclimate a female ovariectomized rat to exercise in the heat, one should administer estradiol (10 micrograms in 100 microliters of sesame oil per 100 grams body weight) and exercise the rat daily in a warm environment for a minimum of 8 days.

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Table 1

	Conditioned Heat Treatment	Hormone During Pre-HTT	Hormone During HTT
Group I	No	Estradiol	Vehicle
Group II	Yes	Estradiol	Vehicle
Group III	Yes	Vehicle	Vehicle
Group IV	Yes	Estradiol	Estradiol
Group V	No	No	No

Table 2

	WEIGHT (gm)	Initial Temp (°C)	Time to 41.5 (min)	Heating Rate (Δ°C/min)	Heat Rate/kg (Δ°C/kgmin)
GROUP I					
4 day (n=7)	226 ± 1.543	37.725 ± 0.101	40.109 ± 8.259	0.111 ± 0.0142	0.494 ± 0.0619
8 day (n=7)	223.42 ± 2.618	37.428 ± 0.206	61.716 ± 9.042	0.0771 ± 0.0154	0.333 ± 0.0601*
12 day (n=8)	217.17 ± 2.015*¤	38.298 ± 0.159	31.518 ± 5.721¤	0.124 ± 0.0194	0.562 ± 0.0851
GROUP II					
4 day (n=8)	223 ± 4.082	37.583 ± 0.141	84.328 ± 14.287†	0.054 ± 0.0092†	0.245 ± 0.0407†
8 day (n=8)	215.2 ± 2.825*†	36.922 ± 0.0709*†	85.508 ± 13.61*	0.0617 ± 0.0108	0.293 ± 0.0518
12 day (n=7)	211.6 ± 2.364*	37.754 ± 0.0911†	115.193 ± 4.803*¤†	0.0325 ± 0.0017*¤†	0.155 ± 0.00877*¤†
GROUP III					
4 day (n=6)	238.3 ± 6.499	37.957 ± 0.106	28.268 ± 3.138	0.136 ± 0.176	0.567 ± 0.0689
8 day (n=6)	256.3 ± 2.403	38.23 ± 0.142	27.673 ± 4.483	0.128 ± 0.0149	0.5 ± 0.0484
12 day (n=7)	268 ± 3.968	37.99 ± 0.166	38.134 ± 4.45*	0.0988 ± 0.0095*	0.371 ± 0.0357*¤
GROUP IV					
4 day (n=7)	220.6 ± 3.329‡	38.068 ± 0.186	49.107 ± 4.666‡	0.0737 ± 0.0092‡	0.331 ± 0.0316‡
8 day (n=4)	218 ± 2.738‡	37.47 ± 0.0981*‡	49.75 ± 10.973	0.094 ± 0.0197	0.433 ± 0.0822
12 day (n=5)	224.6± 3.28‡	37.776 ± 0.232	66.104 ± 13.827‡	0.0205 ± 0.00092*‡	0.282 ± 0.0399
NAIVE (n=5)	241.8 ± 3.139	37.732 ± 0.0929	21.324 ± 0.0115	0.176 ± 0.0115	0.729 ± 0.0516

* $p<0.05$ 4-day vs. 12-day
¤ $p<0.05$ 4 day vs. 8-day
† $p<0.05$ Group I vs. group II
‡ $p<0.05$ Group III vs. Group IV

APPENDIX

Galleys of accepted manuscript:

"Effect of estrogen supplementation on exercise thermoregulation in premenopausal women"

Abstract presented at 1996 Intersociety Conference: The Integrative Biology of Exercise:

"Effects of estradiol on thermotolerance during exercise and heat stress"

Effect of estrogen supplementation on exercise thermoregulation in premenopausal women

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[1]*Department of Physical Education, National Ping Tung Teacher's College, Ping Tung, Taiwan; Departments of* [2]*Exercise Science and* [4]*Obstetrics and Gynecology, University of Iowa, Iowa City, Iowa 52242; and* [3]*Department of Internal Medicine, University of New Mexico, Albuquerque, New Mexico 87131*

Chang, R.-T., G. P. Lambert, P. L. Moseley, F. K. Chapler, and C. V. Gisolfi. Effect of estrogen supplementation on exercise thermoregulation in premenopausal women. *J. Appl. Physiol.* 85(6): ■■■■–■■■■, 1998.—This study examined the effects of 3 days of estrogen supplementation (ES) on thermoregulation during exercise in premenopausal (20–39 yr) adult women during the follicular phase of the menstrual cycle. Subjects (11 control, 10 experimental) performed upright cycle ergometer exercise at 60% of maximal O_2 consumption in a neutral environment (25°C, 30% relative humidity) for 20 min. Subjects were given placebo (P) or β-estradiol (2 mg/tablet, 3 tablets/day for 3 days). All experiments were conducted between 6:30 and 9:00 AM after ingestion of the last tablet. Heart rate, forearm blood flow (FBF), mean skin temperature, esophageal temperature (T_{es}), and forearm sweat rate were measured. Blood analysis for estrogen and progesterone reflected the follicular phase of the menstrual cycle. Maximal O_2 consumption (37.1 ± 6.2 in P vs. 38.4 ± 6.3 ml·kg^{-1}·min^{-1} in ES) and body weight-to-surface area ratio (35.58 ± 2.85 in P vs. 37.3 ± 2.7 in ES) were similar between groups. Synthesis of 70-kDa heat shock protein was not induced by 3 days of ES. Neither the threshold for sweating (36.97 ± 0.15 in P vs. 36.90 ± 0.22°C in ES), the threshold for an increase in FBF (37.09 ± 0.22 in P vs. 37.17 ± 0.26°C in ES), the slope of sweat rate-T_{es} relationship (0.42 ± 0.16 in P vs. 0.41 ± 0.17 in ES), nor the FBF-T_{es} relationship (10.04 ± 4.4 in P vs. 9.61 ± 3.46 in ES) was affected ($P > 0.05$) by 3 days of ES. We conclude that 3 days of ES by young adult women in the follicular phase of their menstrual cycle have no effect on heat transfer to the skin, heat dissipation by evaporative cooling, or leukocyte synthesis of 70-kDa heat shock protein.

core body temperature; forearm blood flow; sweat rate; heat shock protein; skin temperature; exercise

SEVERAL STUDIES INDICATE that estrogen replacement therapy in postmenopausal women reduces not only the physiological symptoms of estrogen withdrawal, such as the occurrence of hot flashes (21), but also the incidence of cardiovascular disease (37). Interestingly, there is controversy over the thermal adaptive response of women, particularly in relation to the menstrual cycle. Whereas some studies show no effect of sex hormones on adaptation to heat (15, 17, 20), more recent data demonstrate that estrogen supplementation (ES) lowers the core temperature threshold for the increase in forearm blood flow (FBF) and the initiation of sweating in postmenopausal women exercising in a warm environment (39). Similarly, rats treated with estrogen regulate their core temperature at a lower level during heat exposure and show an increased evaporative water loss at all levels of core temperature and a reduced core temperature threshold for the onset of saliva spreading (1).

Previous studies have shown that ES decreases (9, 16, 39), has no effect on (10, 22), or increases core temperature (2, 3, 24). Silva and Boulant (36) found that estradiol excited 26% of warm-sensitive neurons and inhibited 4% of cold-sensitive neutrons in preoptic tissue slices. This suggests that the hypothermic effects of estradiol are due to the direct actions of estradiol on preoptic neurons. In contrast, Cagnacci et al. (3) demonstrated that ES significantly increased the hyperthermic role of endogenous opioid peptides in postmenopausal women. However, the data of Czaja and Butera (6) indicate that the effects of estradiol on rectal temperature (T_{re}) may be related to changes in peripheral blood flow rather than to a shift in core temperature.

At the cellular level, heat shock proteins (HSPs) are produced in response to heat stress and are the central component of acquired heat tolerance (26). HSPs are differentially regulated in men and women, and increases in 70-kDa HSP (HSP 70) and 90-kDa HSP (HSP 90) occur in ovariectomized female rats following ES (28). Our laboratory was the first to show that HSP 70 may be produced in humans exercising in the heat, and thus HSPs may be useful biomarkers of both thermal history and thermal tolerance (33).

Because of the important link between ES conferring improved thermoregulatory responses in postmenopausal women and rats and ES increasing HSP 70 in ovariectomized rats, we sought to determine whether ES would alter thermoregulatory responses in premenopausal women during exercise. Previous research indicates that an assay of HSP 70 in blood mononuclear cells may be a useful biomarker to evaluate the potential relationship between ES and thermoregulatory responses in premenopausal women during exercise (33).

The purposes of this study, therefore, were to determine whether short-term ES improves thermoregulatory responses in premenopausal women and whether HSP 70 can be used as a biomarker to assess these responses. Because ES has been shown to enhance HSP synthesis and improve thermoregulatory responses during exercise in the heat, we hypothesized that *1*) short-term ES could improve thermoregulatory responses in exercising women and *2*) HSP might serve as a convenient and rapidly assayed biomarker of thermal tolerance and therefore could be used to develop therapeutic strategies to optimize temperature regulation in women.

METHODS

Subjects. Twenty-one women, aged 21–39 yr, volunteered to participate in this study. Subjects were nonsmokers and reported having recurrent, normal menstrual cycles. They refrained from oral contraceptive use for at least 2 mo before the experiments. Each volunteer was informed of the testing procedures and the risk factors associated with the experiments, and written consent to participate was obtained. Subjects were given a physical examination, and experimental procedures were approved in advance by the Human Subjects Committee at the University of Iowa.

To minimize the possible interaction of progesterone and to maximize differences in estradiol levels between ES and control subjects, this study was conducted with women in the early follicular phase of their menstrual cycle. In the follicular phase, estradiol levels average 40–80 pg/ml. A short-term ES protocol (2 mg estradiol 3 times/day for 3 days), developed and tested for safety in in vivo fertilization programs, was used to elevate estradiol levels >1,000 pg/ml.

Serum estradiol and progesterone concentrations were measured to reflect levels associated with the follicular phase of the menstrual cycle, specifically, a low level of estradiol and an extremely low level of progesterone, if any at all. These values were demonstrated in the preexperiment levels in all subjects. All estradiol levels were between 18 and 40 pg/ml, which is appropriate for the early part of the follicular phase. All progesterone levels were <1.5 ng/ml, which is also normal for a follicular-phase level. In addition, the women were menstruating during the experiments and showed a normal luteal elevation in basal body temperature measured rectally.

With the use of a double-blind design, subjects were randomly assigned to ES (n = 10) or placebo (n = 11) groups. Experiments were conducted between November and December to preclude heat acclimatization. Age, maximal O_2 consumption ($\dot{V}O_{2max}$), and body weight-to-surface area ratio were not significantly different between groups (Table 1).

Estimation of $\dot{V}O_{2max}$ and submaximal O_2 consumption. Before the experiment, each subject's $\dot{V}O_{2max}$ was determined by an incremental cycle ergometer (The Bike, Cybex, Ronkonkoma, NY) test. Subjects completed three 3-min workloads of 75, 100, and 125 W. After the last 3-min period, the workload was increased by 25 W each minute until volitional fatigue. O_2 consumption ($\dot{V}O_2$) and CO_2 production ($\dot{V}CO_2$) were analyzed continuously (Q-Plex I Metabolic System, Quinton Instruments, Seattle, WA), and heart rates (HRs) were measured every minute. A maximal test was defined by the achievement of at least two of the following three criteria: *1*) a plateau or decrease in $\dot{V}O_2$ despite an increase in workload, *2*) a respiratory exchange ratio (RER) ≥1.1, or *3*) attainment of at least 90% of predicted maximum HR (5). The workload required to elicit 60% $\dot{V}O_{2max}$ was determined from submaximal $\dot{V}O_2$ values.

Study design. Each experiment consisted of a 5-min rest period followed by 20 min of cycle exercise at 60% $\dot{V}O_{2max}$. Cycling was performed in an upright posture in an environmental chamber maintained at 25°C and 30% relative humidity (RH). Subjects ingested two tablets of estradiol (Estrace, 2 mg/tablet; Bristol-Meyers Squibb, Princeton, NJ) on *day 1* and three tablets on *days 2* and *3* of their menstrual cycle. A final tablet was ingested 1–2 h before the experiment. All experiments were carried out between 6:30 and 9:00 AM. On the first day of the subject's menstrual cycle, a blood sample was obtained to determine estradiol and progesterone concentrations and for analysis of leukocyte HSP 70. Additional blood samples for HSP 70 analysis were obtained before the experiment and 6 and 24 h after ingestion of the last tablet. Serum estradiol and progesterone concentrations were also measured on the second blood sample.

Experimental protocol. Subjects were instructed to fast overnight and to refrain from heavy exercise for 24 h before the experiment. On arrival at the laboratory, subjects ingested 6 ml water/kg body weight to ensure hydration. A blood sample was obtained for HSP 70 determination and for estradiol and progesterone measurement. Body height and weight were measured, and a HR monitor (Polar Vantage XL, Polar USA, Stamford, CT) was placed around the chest at the level of the xyphoid process. Subjects then entered the environmental chamber and sat upright on the cycle ergometer for ≥45 min, allowing for instrument setup and postural equilibration. An esophageal thermocouple was orally positioned at a depth equal to 25% of each subject's height (40) to measure esophageal temperature (T_{es}). Copper-constantan thermocouples were placed on the upper arm, chest, thigh, and lower leg for skin temperature [mean skin temperature ($\bar{T}_{sk}$)] measurements (29). For FBF measurements, a double-latex cuff and two pressure cuffs were placed on the forearm, arm, and wrist, respectively, and a capsule was placed on the right forearm 2 cm below the antecubital fossa for determination of the local sweating response by resistance hygrometry.

Measurement. T_{es} was monitored continuously with a T_{es} sensor (ESO-1, Physitemp Instruments, Clifton, NJ). Changes in T_{es} were plotted by a direct writing oscillographic recorder (model 1508A Visicorder, Honeywell, Denver, CO). Because swallowing saliva may lower T_{es}, subjects expectorated all saliva into a cup.

$\bar{T}_{sk}$ was calculated by a weighted sum of four different skin temperature measurements (29). $\bar{T}_{sk}$ was calculated as upper arm (0.3) + chest (0.3) + thigh (0.2) + lower leg (0.2). Skin temperatures were collected each minute by an on-line computer system (IBM AT personal computer, IBM, Armonk, NY).

Venous occlusion plethysmography was used to measure FBF on the left arm by using an air-filled latex plethysmographic cuff fitted for each subject (Plethysmography Products, Korsor, Denmark). During the experiment, FBF measurements were obtained with the forearm extended laterally away from the torso and supported above the venostatic level. The left hand was excluded from the circulation with a pneumatic cuff at the wrist inflated to 180–220 mmHg. A second cuff placed around the upper arm was inflated to 50 mmHg twice per minute (15 s on/off interval). FBF was computed from the slopes of plethysmographic curves.

Local sweat rate (SR) of the right forearm was continuously determined from a ventilated lithium chloride sensor within a 6.605-cm² capsule (12). Flow rate was adjusted to optimize sensitivity and to ensure adequate data range for the chart

Table 1. *Subject characteristics*

Treatment	n	Weight, kg	Height, cm	Age, yr	$\dot{V}O_{2max}$, ml·kg^{-1}·min^{-1}	Body Wt/ Surface Area
Estrogen supplementation	10	63.6 ± 2.2	166.1 ± 2.0	25.8 ± 1.8	38.4 ± 2.0	37.30 ± 2.70
Placebo	11	59.0 ± 2.8	166.2 ± 1.8	26.4 ± 1.8	37.1 ± 1.9	35.58 ± 2.85

Values are means ± SE for n = no. of women. $\dot{V}O_{2max}$, maximal O_2 consumption; Body wt/surface area, ratio of body weight to surface area.

recorder. Calibration of the hygrometer system involves determining the trace deflection from baseline for a given change in air flow through the humidification system. A regression equation is determined for the relationship between trace deflection and saturated air flow. SR was calculated from the regression line by converting rates of air flow through the humidification system into equivalent SR values.

The FBF-T_{es} and SR-T_{es} thresholds were defined as the point after which a continuous increase in FBF or SR occurred. The slope of the linear regression relating the rise in FBF and SR to T_{es} was used to characterize the sensitivity of the skin blood flow and SR responses. Calibration procedures for FBF, T_{es}, $\overline{T}_{sk}$, and SR measurements were conducted immediately before each experiment.

HSP analysis. Human leukocytes were isolated at room temperature (22°C) by adding 5 ml of Histopaque (Sigma Chemical, St. Louis, MO) to the blood (10 ml) and were centrifuged (3,000 rpm, 25–30 min). Leukocytes were collected and washed (gentle aspiration and centrifugation at 1,500 rpm for 5–7 min) twice with PBS. The final leukocyte pellet was suspended in 0.25 ml PBS and stored at −70°C until analysis. This isolation procedure was initiated immediately on attainment of a blood sample. HSP 70 analysis was performed as previously described by using Western blot analysis (33). Serum progesterone and estradiol concentrations were measured by using a radioimmunoassay (14).

Statistical analysis. Although a power test revealed that six subjects would yield 90% power, we opted to study four additional women for a total of 10 subjects. One- and two-factor ANOVAs were utilized with repeated measures as necessary to determine whether significant differences existed between groups and across time. Values presented are means ± SE. Linear-regression analysis was employed for each experiment to obtain thresholds and slopes of the SR vs. T_{es} and FBF vs. T_{es} relationships. A post hoc power analysis was conducted by using actual values obtained from this study to verify acceptance of the null hypothesis.

RESULTS

Serum estradiol concentration was unaffected by placebo treatment but was significantly elevated ($P < 0.05$) after ES (Table 2). Serum estradiol concentrations were similar ($P > 0.05$) between the two groups before ES and after placebo treatment. Serum progesterone concentration was not significantly different between the two groups or between pre- and post-ES ($P > 0.05$).

HR, $\overline{T}_{sk}$, and T_{es} responses during exercise were not significantly different between ES and placebo groups (Fig. 1). Preexercise T_{es} values were 36.80 ± 0.06 and 36.81 ± 0.01°C for the ES and placebo groups, respectively. $\overline{T}_{sk}$ was lower in the ES group than in the placebo group, but the difference was not significant. Both groups showed a fall in $\overline{T}_{sk}$ at the onset of exercise and then an increase as T_{es} rose. The rate of rise in $\overline{T}_{sk}$, T_{es}, and HR during exercise was not affected by ES.

FBF and SR responses during exercise were not significantly different between groups (Fig. 2). T_{es} threshold for SR, increase in FBF, and slope of T_{es}-SR and T_{es}-FBF relationships were plotted, with each line representing the data on a representative subject (Fig. 3, *A* and *B*). There were no significant differences between groups (Table 3).

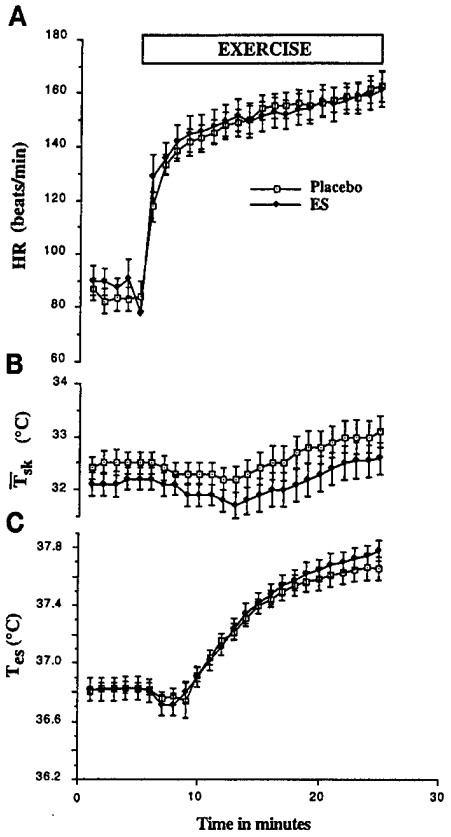

Fig. 1. Heart rate (HR; *A*), mean skin temperature ($\overline{T}_{sk}$; *B*), and esophageal temperature (T_{es}; *C*) responses during placebo and estrogen supplementation (ES) trials. Data are means ± SE; n = 10 women in ES and 11 women in placebo group.

Table 2. *Serum estradiol and progesterone concentration*

	Preplacebo	Postplacebo	Pre-ES	Post-ES	Early Follicular Phase (Normal Range)
Estradiol, pg/ml	34.8 ± 2.4	42.5 ± 4.2	32.55 ± 3.6	1,166.6 ± 114*	20–60
Progesterone, ng/ml	0.86 ± 0.07	1.02 ± 0.12	0.98 ± 0.11	0.99 ± 0.14	0.1–1.5

Values are means ± SE. Preplacebo and postplacebo, before and after placebo treatment; pre-ES and post-ES, before and after estrogen supplementation. *Significantly different from pre-ES and pre- and postplacebo trials.

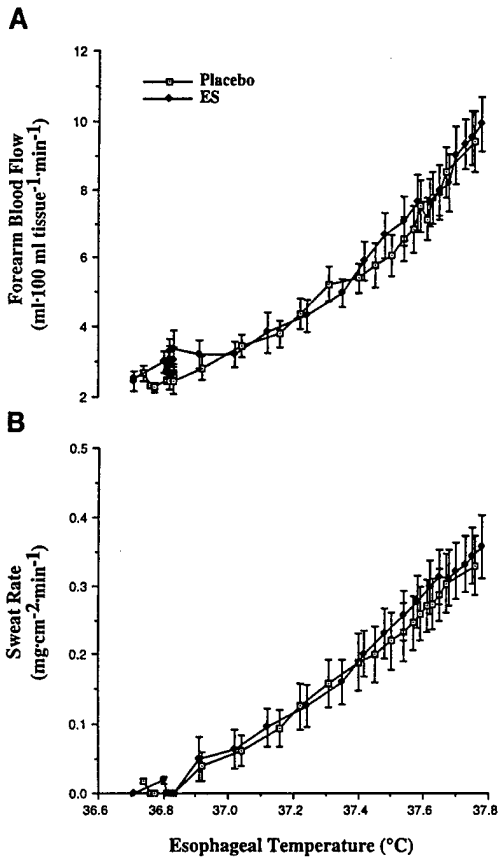

Fig. 2. Mean forearm blood flow (FBF; *A*) and mean sweat rate (SR; *B*) as a function of T_{es} (means ± SE). FBF and SR increase with the increment of T_{es}. There were no significant differences between groups. n = 10 Women in ES and 11 women in placebo group.

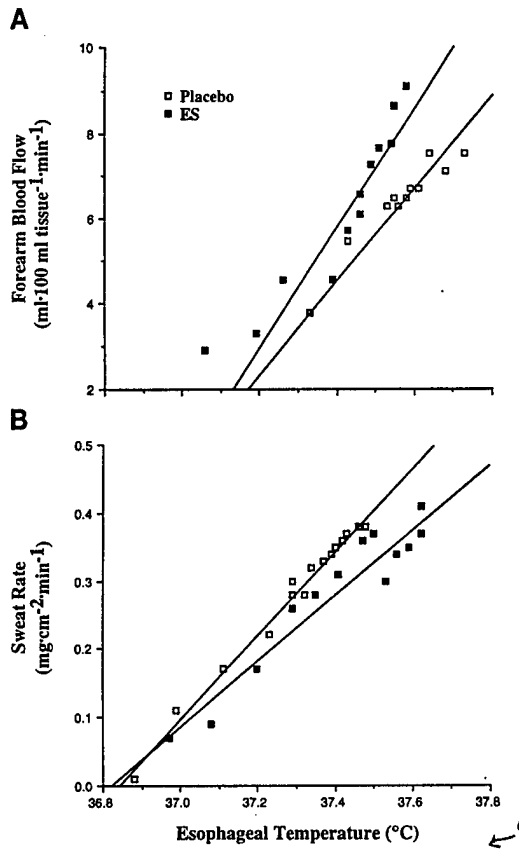

Fig. 3. T_{es} threshold for increase of FBF (*A*) and SR (*B*) and slope of T_{es}-FBF (*A*) T_{es}-SR (*B*) relationship are plotted. Each line represents 1 subject from each group.

Estradiol supplementation had no significant effect on blood mononuclear cell accumulation of HSP 70 after exercise. Figure 4 shows the HSP 70 accumulation in placebo (*A*) or ES (*B*) subjects before and 2 h after exercise. The post hoc power analysis using the HSP 70 values obtained from the present investigation revealed that 80 subjects would be necessary to achieve 80% power. Thus the null hypothesis was accepted.

DISCUSSION

The primary contribution of this study is that it provides evidence indicating that 3 days of ES during the early follicular phase of the menstrual cycle of premenopausal female subjects have no effect on thermoregulatory responses during exercise at 60% $\dot{V}O_{2max}$ in a neutral environment (25°C, 30% RH). T_{es} thresholds for the initiation of sweating and increase in FBF were not altered by ES. T_{es} also was not significantly different between groups during rest or after 20 min of cycle exercise. SR, $\bar{T}_{sk}$, HR, and FBF were similar between the two groups throughout exercise. Furthermore, HSP 70 synthesis was not induced by 3 days of ES.

Effect of ES on core body temperature. Previous studies have shown that ES decreases (9, 16, 39), has no effect on (10, 22), or increases core body temperature (2, 3, 24). These divergent findings are attributed, in part, to the following factors. *1*) Differences in subject

Table 3. *Slope and T_{es} threshold of FBF and SR responses*

Treatment	FBF		SR	
	Threshold, °C	Slope	Threshold, °C	Slope
Estrogen supplementation	37.17 ± 0.08	9.61 ± 1.10	36.97 ± 0.05	0.41 ± 0.05
Placebo	37.09 ± 0.08	10.04 ± 1.33	36.90 ± 0.07	0.42 ± 0.05

Values are means ± SE. T_{es}, esophageal temperature; FBF, forearm blood flow; SR, sweat rate. Slope is expressed in ml·100 ml tissue^{-1}·min^{-1}·°C^{-1} and in mg·cm^{-2}·min^{-1}·°C^{-1} for FBF and SR, respectively.

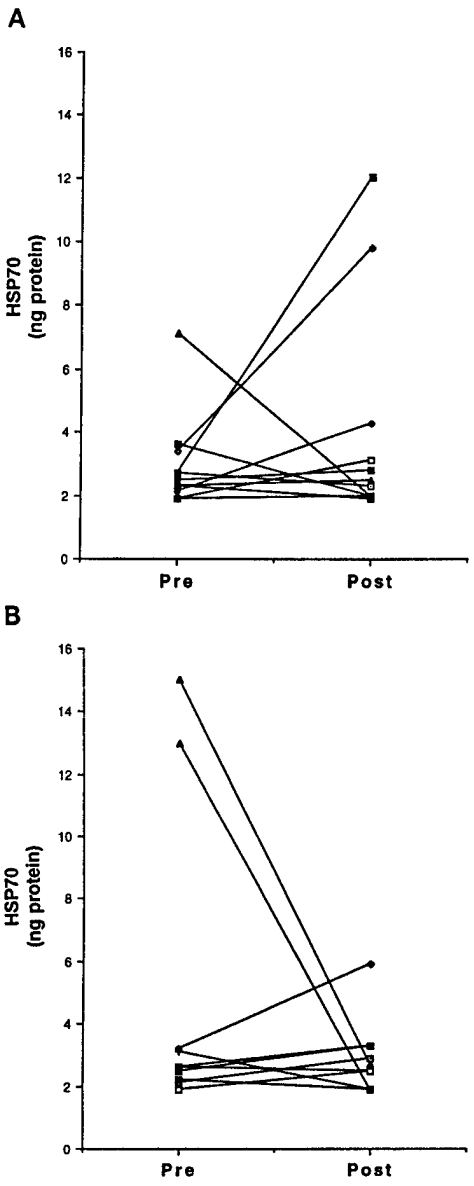

Fig. 4. Effect of placebo (*A*) or estrogen (*B*) on 70-kDa heat shock protein (HSP 70) accumulation in peripheral blood mononuclear cells of subjects before (Pre) and 2 h after (Post) exercise. Symbols, individual subjects; ■, mean for each group. Data are presented as nanograms of HSP 70 per lane based on densitometric comparison with recombinant human HSP 70 standards run on same immunoblots.

populations and/or species: Premenopausal, postmenopausal, and ovariectomized animals have served as experimental subjects in the above studies. *2*) Phase of the menstrual cycle: It has been demonstrated (8, 13) that body temperature is regulated at a higher level during the luteal than the follicular phase of the menstrual cycle in women with normal periods; therefore, it is critical to control for both the time of day experiments are performed and the phase of the menstrual cycle, along with factors such as aerobic power, age, and body composition, when studying thermoregulatory responses to exercise in women (38). *3*) Dosage of ES: Israel and Schneller (16) injected subjects with 1.5 mg estradiol compared with 18 mg given orally over 3 days in the present study or over 14–23 days in the study by Tankersley et al. (39). *4*) Type of ES: It is not known what effect differences in estrogen formulations have on blood vessels (35). *5*) Previous studies have not been conducted in similar environmental conditions. Based on the above, the effects of ES on temperature regulation in women warrant further investigation.

Effect of ES on FBF. In the present study, ES had no effect on FBF among premenopausal women (Fig. 2). However, previous studies suggest that acute (within 60 min) ES elevates blood flow in several vascular beds in both postmenopausal women and ovariectomized animals (11, 30, 41, 44, 45). The mechanism likely involves the production of nitric oxide from endothelial cells (4). In the present study, 3 days of ES is a longer term therapy relative to other acute ES studies (within 60 min). Long-term ES acts through a different cellular mechanism and includes regulation of gene expression, which opposes the acute potentiation of endothelium-dependent vasodilation (11). Thus it is possible that acute (within 60 min) administration could increase vasodilation in premenopausal women. However, evidence suggests that blood vessel integrity is well protected in premenopausal women, and estrogen may *1*) inhibit the release of a vasoconstrictor substance such as endothelin (25), *2*) decrease lipoprotein-induced smooth-muscle proliferation (7) in blood vessels, and *3*) inhibit intimal proliferation associated with mechanical injury to endothelium (31, 32, 42). Thus relatively longer term ES had no effect on FBF in our subjects. This is likely due to the effects of longer term ES acting through a different mechanism compared with acute ES. It is also possible that blood vessel integrity is already well protected in the premenopausal woman.

Effect of ES on SR and skin temperature. In the present study, the slope of the T_{es}-SR relationship was nearly identical in the ES and placebo groups. This agrees with the work of other investigators (8). In normal menstruating women without ES, however, Kawahata (18) demonstrated that there were rather large changes in the latent period of thermal sweating (time required to start sweating) measured at different times during the menstrual cycle. At the time of menstruation, the latent period was much shorter than the time of ovulation, and estrogen administration lengthened the latent period of sweating in men (18). Sargent and Weinman (34) repeated Kawahata's (18) experiments on women at different times of the menstrual cycle, but they reported that sweating onset did not vary as a function of the menstrual-cycle phase. Thus experimental results indicate that neither ES nor menstrual-cycle phase influences SR. However, it is

possible that our forearm sweating data do not represent whole body sweating, although, in a second set of experiments (unpublished observations) in which whole body sweating was measured, we again observed no differences between ES and placebo groups.

Skin temperature varies as a function of ambient temperature, and the effect of local skin temperature on the sudomotor (27) and vasomotor (43) responses to exercise are well known. In this study, the estradiol-treated group tended to have a lower $\overline{T}_{sk}$ throughout the experiment, but no significant difference was found between groups at any time (Fig. 1). Both groups showed a fall in $\overline{T}_{sk}$ at the onset of exercise followed by an increase as T_{es} rose. The reduction in $\overline{T}_{sk}$ is attributed to a transient active cutaneous vasoconstriction (19). Because T_{es} was not changed during rest by ES in the present study, the T_{es} threshold for initiation of cutaneous vasodilation and SR was not changed.

Effect of ES on HSP 70 synthesis. The HSP 70 family is essential for cellular survival of heat stress, and its induction by nonheat stress or by genetic manipulation is sufficient to protect cells from thermal injury (26). In the present study, 3 days of ES did not alter HSP 70 accumulation in blood mononuclear cells. This may be attributed to subject-to-subject variability in the degree of stress encountered under the exercise protocol, and thus in the changes in HSP seen, or to the tissue specificity of the HSP 70 response (26). Estradiol significantly elevated HSP 70 and HSP 90 protein concentrations in the ventromedial hypothalamus of gonadectomized adult female rats (28). It is possible that the leukocytes used in the present study were not tissue specific to induce HSP 70 synthesis by ES. Another factor to be considered is that HSP 72 is very sensitive to heat stress. Ryan et al. (33) showed that HSP 70 synthesis was only induced when T_{re} was elevated above 40°C in human subjects. Locke et al. (23) demonstrated that synthesis of HSP 72 was induced in lymphocytes, spleen cells, and soleus muscle after 20 min of exercise with T_{re} rising above 40°C. However, in the present study, T_{es} only reached 38.1°C, possibly an insufficient thermal stress to induce HSP 70 synthesis.

In conclusion, at the level of stress employed in this study (60% $\dot{V}o_{2max}$, ambient temperature = 25°C, 50% RH), 3 days of high circulating levels of estradiol (1,000 pg/ml) have no effect on core temperature or thermoregulatory responses in young women during the follicular phase of their menstrual cycles. These results are supported at the cellular level by a lack of change in HSP 70 concentration with ES in these subjects. In future studies, a greater thermoregulatory stress and a crossover design, in which each subject serves as her own control, would reduce variability and improve chances for identifying a significant effect of estrogen, if it exists. Although the estrogen dose employed in this study was developed and tested for safety in in vivo fertilization programs, a lower dose would be more physiological.

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EFFECTS OF ESTRADIOL ON THERMOTOLERANCE DURING EXERCISE AND HEAT STRESS IN FEMALE RATS. P.L. Moseley, H.E. Sandy, C.V. Gisolfi, and K.C. Kregel. University of Iowa, Iowa City, IA 52242 and University of New Mexico, Albuquerque, NM 87131.

Recent data suggest that estrogen replacement therapy improves thermoregulatory responses to exercise in the heat in post-menopausal women. In the present study, we investigated whether estrogen therapy in ovariectomized (OVX) rats is associated with enhanced thermotolerance to exertional heat stress. OVX Sprague-Dawley rats (220-270 g) received daily subcutaneous injections of either vehicle (sesame oil; n=18) or estradiol (E_2; 10 µg/100 g b.w.; n=18). Within each treatment group, three subgroups were utilized based upon the duration of injections: 1) 4-day, 2) 8-day, or 3) 12-day protocol. Four hours after the final daily injection, rats underwent an exertional heat tolerance test (HTT) consisting of continuous treadmill exercise at a velocity of 21.5 m/min (0% grade) and an ambient temperature of 35°C. Colonic temperature (T_c) was monitored via a thermistor probe and the HTT was terminated when a rat reached 40.4°C. Vehicle treatment had no effect on either the initial T_c, time to reach 40.4°C, or heating rate (per kg body mass) between the three treatment protocols. However, initial T_c values were reduced, heating rates were lower, and times to reach 40.4°C were increased in rats treated with E_2 for 8 or 12 days compared with 4-day treated group ($P<0.05$). In comparisons between treatment groups, both initial T_c and heating rate were lower and time to 40.4°C was higher in E_2- vs. vehicle-treated rats for both the 8- and 12-day protocols. These results demonstrate that administration of E_2 for 8 and 12 days increases thermotolerance in OVX rats exercising in warm ambient conditions.

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