Influence of hormone replacement therapy and aspirin on temperature regulation in postmenopausal women

E. M. BROOKS-ASPLUND, J. G. CANNON, AND W. L. KENNEY
Noll Physiological Research Center, Penn State University, University Park, Pennsylvania 16802-6900

Received 9 September 1999; accepted in final form 7 April 2000

Brooks-Asplund, E. M., J. G. Cannon, and W. L. Kenney. Influence of hormone replacement therapy and aspirin on temperature regulation in postmenopausal women. Am J Physiol Regulatory Integrative Comp Physiol 279: R839–R848, 2000.—Postmenopausal women receiving estrogen-replacement therapy (ERT) regulate body temperature (Tb) at a lower level than women not receiving hormone replacement therapy (untreated) and women using estrogen plus progesterone therapy (E + P), but it is not clear if reproductive hormones alter Tb by directly acting on central thermoregulatory centers or indirectly via a secondary mediator(s). The purpose of the present investigation was to examine the possible involvement of pyrogenic cytokines and cyclooxygenase (COX) products (e.g., prostaglandins) in the regulation of Tb in three groups of postmenopausal women (8 ERT, 7 E + P, and 8 untreated). We measured ex vivo secretion of cytokine agonists [tumor necrosis factor (TNF)-α and interleukin (IL)-1β and -6] and modifiers (IL-2 soluble receptor, IL-1 receptor antagonist, soluble TNF receptor type I, soluble TNF receptor type II, soluble IL-6 receptor, and soluble glycoprotein 130) from peripheral blood mononuclear cells and thermoregulatory responses at rest and during 1 h of passive whole body heating in the postmenopausal women before and after 3 days of placebo or aspirin (50 mg · day–1 · kg–1). With and without aspirin, the ERT group had a lower baseline rectal temperature (Tb; 0.44°C, P < 0.004) and a reduced Tb threshold for cutaneous vasodilation (0.29°C and 0.38°C, P < 0.01) compared with the untreated and E + P groups, respectively. In the placebo condition, waking morning oral temperature (Tm; ) correlated with ex vivo secretion of the proteins associated with IL-6 bioactivity. Aspirin caused significant reductions in waking Tb in the E + P group and in baseline Tb in the untreated group. However, the difference in thermoregulation brought about by steroid hormone treatment could not be explained by these relatively modest apparent influences by cytokines and COX products. Therefore, the altered thermoregulation induced by reproductive steroid therapy appears to occur via a mechanism distinct from a classic infection-induced fever.

skin blood flow; cytokines; cyclooxygenase; reproductive hormones; estrogen; progesterone

REPRODUCTIVE STEROID HORMONES, including estrogen and progesterone, alter thermoregulation in animals and humans (1, 4, 7, 29, 32). During a normal menstrual cycle in eumenorrheic young women, cyclic oscillations in body core temperature (Tc) correspond to fluctuations in the circulating ratio of progesterone-to-estrogen concentration (7, 29). Furthermore, the administration of exogenous steroid hormones in the form of oral contraceptives or hormone-replacement therapy (HRT) alter body temperature regulation in premenopausal and postmenopausal women, respectively (4, 8, 9, 32). Acute (2–3 wk) and chronic (≥2 yr) estrogen-replacement therapy (ERT) reduce Tc at rest and throughout exercise in postmenopausal women (4, 32) via an earlier activation of cutaneous vasodilation and sweating. The addition of progestins to HRT blocks the temperature-lowering effects of estrogen by independently raising the regulated level of Tc (4).

Although previous in vitro studies support a non-genomic central role (i.e., direct hypothalamic site of action) for reproductive steroid hormones on thermoregulatory control (23, 25, 34), the mechanism(s) by which these hormones act in humans is not clear. Direct application of estradiol (0.1 nM ≈ 30 pg/ml) increases the firing rate of warm-sensitive neurons from rat preoptic anterior hypothalamic (PO/AH) tissue slices within 2–3 min (25). Neurons in the PO/AH are sensitive to local changes in temperature and receive neural input from spinal cord and skin (3). Changing the preoptic temperature with implanted thermodes in conscious rhesus monkeys leads to the simultaneous changes in thermoregulatory responses (27). These results are consistent with the temperature-lowering effects of estrogen observed in humans (32) and animals (1). Opposite to the effects of estrogen on thermosensitive neurons, an intravenous injection (5 mg/kg) and direct application of progesterone (3 and 30 ng/ml) inhibited the firing rate of warm-sensitive neurons and stimulated cold-sensitive neurons in the PO/AH of the rabbit with an average time latency of 6–20 min (23, 34). Once again, findings from these studies performed in situ and in vitro are in agreement with the thermogenic effects of progesterone in humans and animals. Data from these investigations support a direct action by reproductive steroid hormones, because changes in firing rates of these thermosensitive neurons occurred too rapidly to be genomic.
in nature. Additionally, androgen, estrogen, and progesterone receptors have been characterized and mapped in the brain (21).

Secondary mediators, such as cytokines or endogenous antipyretics, may be involved in the thermoregulatory alterations associated with varying concentrations of estrogen and progesterone. Cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6, are "endogenous pyrogens" involved in the febrile response during infection or inflammation. It is believed that fever occurs as a result of cytokine-stimulated PG E₂ production in or near PO/AH or organum vasculosum of the lamina terminalis (OVLT) (12) or through a vagal-mediated release of PG E₂ near the OVLT (2). In contrast to the endogenous pyrogens, arginine vasopressin (AVP) is an endogenous antipyretic (19). Acting through V₁ receptors in the ventral septal area of the brain, the Tₚ-reducing effects of AVP are similar to those of aspirin and nonsteroidal antiinflammatory drugs (NSAIDs) (19).

Estrogen and progesterone can influence the secretion of cytokines and AVP. For example, progesterone and estrogen alter cytokine secretion from peripheral blood mononuclear cells (PBMCs) and placental macrophages in a dose-dependent and biphasic manner (14). In concordance with these in vitro data, IL-1 activity increases during the luteal phase (high circulating progesterone-to-estrogen ratio) compared with the follicular phase (low progesterone-to-estrogen ratio) of the menstrual cycle in young eumenorrheic women (6). Endogenous estrogen and acute exogenous estrogen administration increase circulating serum concentrations of AVP in the rat (26), in premenopausal women during the periovulatory phase of the menstrual cycle (15), and in postmenopausal women (28). If these aforementioned findings are causal, then one would expect ERT to reduce Tₚ in postmenopausal women by altering the secretion of pyrogenic cytokines, cytokine antagonists, or soluble receptors from PBMCs and/or increasing peripheral or central production of AVP. Likewise, because progesterone blocks the temperature-lowering effects of estrogen, one would expect that the addition of progesterone to HRT would have the opposite effect of estrogen on cytokine and AVP production.

Therefore, the purpose of the present investigation was to examine the involvement of cytokines and cyclooxygenase (COX)-dependent products (i.e., PG E₂) in the regulation of Tₚ in three groups of postmenopausal women (ERT, E + P, and women not using HRT (untreated)) at rest and during whole body heat stress. It was hypothesized that 1) blockade of prostaglandin production by administration of a COX inhibitor (aspirin) would lower the regulated Tₚ in E + P and untreated groups, 2) hormone replacement would alter cytokine agonist (IL-1β, IL-6, TNF-α), antagonist (IL-1-receptor antagonist), and soluble receptor (IL-1 type II soluble receptor, soluble TNF receptor type I, soluble TNF receptor type II, soluble IL-6 receptor (sIL-6R), soluble glycoprotein 130 (sgp130)) secretion from PBMCs, and 3) circulating AVP concentration would be greater in women using ERT compared with E + P and untreated groups.

**METHODS**

**Subjects.** The present investigation was approved in advance by the Institutional Review Board at the Pennsylvania State University. After a detailed explanation of the procedures, 23 postmenopausal women were recruited (8 not taking HRT, 8 on oral ERT, and 7 women on oral E + P). Women not receiving HRT were defined as postmenopausal by the following criteria: 1) complete cessation of menses for ≥1 yr following a history of eumenorrhea, 2) serum estradiol ≤20 pg/ml, and 3) serum follicle-stimulating hormone (FSH) ≤25 mIU/ml. In ERT and E + P groups, women had complete cessation of menses for ≥1 yr before receiving HRT from their personal physicians. In the ERT group, six women had undergone both an oophorectomy and hysterectomy and two had undergone a hysterectomy only. One woman from the untreated group had undergone a hysterectomy and oophorectomy. All women using ERT received 0.625 mg of Premarin, (Wyeth-Ayerst Laboratories, Philadelphia, PA) on a daily basis. In the E + P group, six women were receiving Prempro (Wyeth-Ayerst Laboratories), which included 0.625 mg of conjugated estrogens and 2.5 mg of medroxyprogesterone acetate. The other woman in the E + P group received 0.625 mg of Premarin and 5 mg of Provera (Pharmacia and Upjohn).

All subjects were screened by a physician on their initial visit to the laboratory (Table 1). A resting electrocardiogram, height, and weight were recorded for each subject. Body fat was estimated from skin-fold thickness measurements at seven sites (pectoral, triceps, midaxillary, abdomen, thigh, suprailiac, and subscapular). On the basis of activity within a 1-mo period of time near the experiment, vigorous physical activity (based on activity within 1 mo of the trial) was estimated using a validated questionnaire (13). Each woman reported the day of her last period or date of oophorectomy. With the use of these data, an estimate for the years since the onset of menopause for each woman was calculated as the elapsed time between the reported oophorectomy or last period and the time of the initial visit. A venous blood sample was collected for analysis of serum 17β-estradiol, estrone, and FSH concentrations (Table 1). Criteria for exclusion included 1) hypertension (resting systolic pressure >140 mmHg and a diastolic pressure >90 mmHg), 2) smoking, 3) hypertension (resting systolic pressure >140 mmHg and a diastolic pressure >90 mmHg), and 2) smoking.

**Table 1. Physical characteristics, activity level, and serum 17β-estradiol, estrone, and FSH concentrations by group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated (n = 8)</th>
<th>ERT (n = 8)</th>
<th>E + P (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>64 ± 2</td>
<td>60 ± 2</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>Years since menopause</td>
<td>14 ± 2</td>
<td>11 ± 1</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>Height, cm</td>
<td>163 ± 2.0</td>
<td>162.8 ± 1.8</td>
<td>161.1 ± 4.3</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69.1 ± 4.5</td>
<td>70.7 ± 3.2</td>
<td>68.8 ± 4.7</td>
</tr>
<tr>
<td>Activity</td>
<td>28 ± 7</td>
<td>29 ± 7</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>34 ± 3</td>
<td>36 ± 2</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Estrone, pg/ml</td>
<td>7.2 ± 1.2</td>
<td>33.2 ± 5.7*</td>
<td>24.2 ± 4.1*</td>
</tr>
<tr>
<td>FSH, mIU/ml</td>
<td>19.1 ± 4.5</td>
<td>169.3 ± 42.1*</td>
<td>80.8 ± 17.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Untreated, no hormone replacement therapy; ERT, estrogen replacement therapy; E + P, estrogen plus progesterone therapy; n, no. of subjects; FSH, follicle-stimulating hormone. Measurement of activity is based on a validated questionnaire [DiPietro et al. (13)]. *Significantly different from untreated group; †significantly different from ERT group.

**Partial thermoregulatory responses**

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated (n = 8)</th>
<th>ERT (n = 8)</th>
<th>E + P (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height, mmHg</td>
<td>140 ± 140</td>
<td>140 ± 140</td>
<td>140 ± 140</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>69.1 ± 4.5</td>
<td>70.7 ± 3.2</td>
<td>68.8 ± 4.7</td>
</tr>
<tr>
<td>Activity</td>
<td>28 ± 7</td>
<td>29 ± 7</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>34 ± 3</td>
<td>36 ± 2</td>
<td>37 ± 2</td>
</tr>
<tr>
<td>Estrone, pg/ml</td>
<td>7.2 ± 1.2</td>
<td>33.2 ± 5.7*</td>
<td>24.2 ± 4.1*</td>
</tr>
<tr>
<td>FSH, mIU/ml</td>
<td>19.1 ± 4.5</td>
<td>169.3 ± 42.1*</td>
<td>80.8 ± 17.0*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Untreated, no hormone replacement therapy; ERT, estrogen replacement therapy; E + P, estrogen plus progesterone therapy; n, no. of subjects; FSH, follicle-stimulating hormone. Measurement of activity is based on a validated questionnaire [DiPietro et al. (13)]. *Significantly different from untreated group; †significantly different from ERT group.
any diagnosed metabolic or cardiovascular disease, or 4) taking of any medication with the potential to influence thermoregulatory or cardiovascular variables of interest. One woman in the ERT group dropped out of the study and did not complete the heat trial with aspirin. Another woman in the ERT group was running a fever during her placebo trial, so her data were excluded from the thermoregulatory calculations for the ERT group with placebo.

Preexperimental procedures. Subjects were tested in random order between the months of August and March with no effort to artificially acclimate the subjects to heat. The study required five visits to the laboratory, including the initial physical screening. Randomization of testing order minimized the potential for any systematic seasonal effect. Pretest instructions included 1) no alcohol for 48 h, 2) no caffeine for 12 h, 3) no strenuous exercise for 12 h, and 4) consumption of an extra liter of water during the 24 h preceding the test.

Experimental procedures. Each woman underwent two experimental heat trials (described below) after receiving aspirin (Bufferin enteric-coated aspirin or placebo [lactose powder] blindly [single]) for 3 days before the day of the heat trial and one pill the morning of the heat trial. As in previous studies (10), dosage of aspirin was based on each individual’s body weight (50 mg·day⁻¹·kg body wt⁻¹) to account for variations in body mass and to prevent a smaller individual from becoming salicylate intoxicated. Daily dosage did not exceed 4,000 mg/day (maximal over-the-counter dosage). Aspirin (within minutes) acetylates and irreversibly inhibits COX-1 than COX-2 (22), aspirin consistently inhibits the COX enzyme (36). Although aspirin more effectively inhibits COX-1 than COX-2 (22), aspirin consistently reduces T in febrile patients (10). By inhibiting COX activity and prostaglandin production, aspirin blocks the central thermoregulatory effects of prostaglandins. Plasma salicylate concentration was measured spectrophotometrically at a wavelength of 540 nm (Sigma Chemical, St. Louis, MO) from samples obtained after 2 days of placebo or aspirin administration. The experimental heat trials were separated by a period of 7–8 wk to allow for adequate washout of the aspirin. During this time, subjects were asked to refrain from all NSAIDs.

In the morning (between 0600 and 0900), 1 day before the experimental heat trial, each woman reported to the laboratory for blood collection. Three 10-ml heparinized vacutainers and two 5-ml EDTA vacutainers of venous blood were collected for mononuclear cell isolation and measurement of plasma salicylate, osmolality, and AVP. The two 5-ml vacutainers were centrifuged for 15 min, and the plasma was frozen and later assayed in duplicate by radioimmunoassay for AVP. The three 10-ml vacutainers of blood were immediately centrifuged for 7 min at 800 rpm to obtain plasma samples. Plasma was frozen and later assayed for salicylate and osmolality (Advanced DigiMatic Osmometer, model 3D2, Advanced Instruments, Needham Heights, MA). PBMCs were immediately isolated from the venous blood (less the plasma) by density gradient centrifugation with Ficoll-Hypaque (Histopaque, Sigma). The cells were washed three times with 0.9% NaCl and resuspended in phenol red-free RPMI-1077 medium (Sigma) supplemented with 2 mM of l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), 2% (by volume) totally cellular human serum-replacement (Celox Laboratories, St. Paul, MN), and 1% M HEPES buffer (Sigma). Cells were incubated with and without 2 ng/ml lipopolysaccharide (LPS) from Escherichia coli (Sigma). Cells were incubated at a density of 2.5 × 10⁶ cells/ml in 24-well polystyrene plates (Corning Glass Works, Corning, NY) for ~26 h at 37°C in a humidified 5% CO₂ atmosphere. After incubation, cell supernatants were centrifuged at 10,000 rpm for 3 min then aliquoted in 1.5-ml microcentrifuge tubes and frozen at −70°C until assayed for cytokine agonists, antagonists, and soluble receptors.

On the day of the heat trial, each subject reported to the laboratory early in the morning (0700 and 0900). On arrival at the laboratory, the subject dressed in a sports bra and shorts, inserted a rectal thermistor (Yellow Springs Instruments, series 400), and was fitted in a water-perfused suit covering the entire body except for the hands, feet, forearms, and head. Subjects rested in a supine position in a thermoneutral environmental chamber (dry bulb temperature = 24°C and wet bulb temperature = 15°C) while measurement devices were attached. After measurement devices were attached, heart rate (HR), rectal temperature (T<sub>re</sub>), mean skin temperature (T<sub>sk</sub>), and laser-Doppler flux (LDF) were measured continuously throughout a 15-min baseline period in the thermoneutral condition. Mean arterial pressure (MAP) and forearm blood flow (FBB) measurements were collected every 5 min during this period.

At the end of the 15-min baseline period, infusion of warm water into the suit was initiated and two cotton blankets were placed on top of the subject to minimize heat loss. The temperature of the water perfusing the suit was increased in 2°C increments from 40 to 48°C over the first 8 min of whole body heating and was maintained at 48°C for the remainder of the heating period. During the heating period, continuous measurements of HR, T<sub>re</sub>, T<sub>sk</sub>, and LDF were collected. FBB and MAP readings were collected every 2 and 4 min, respectively, and MAP was always obtained after the FBB measurements. Whole body heating was performed for 60 min. The LDF and FBF versus mean body temperature (T<sub>b</sub>) thresholds for vasodilation were used as surrogate measures of central thermoregulatory control. In several cases, heating was performed an additional 10–20 min to ensure that a threshold for vasodilation could be determined.

After the whole body heating procedure, the temperature of the water perfusing the suit was decreased to 35°C and local heating (42–42.5°C) of the skin at the area around the laser-Doppler flow probe was initiated using a thermostatically controlled heater (33). This procedure was performed for 45 min while the subject rested in a supine position. MAP measurements were made every 5 min during this local-heating period. Maximal LDF was verified by performing a postocclusion reactive hyperemia maneuver (18).

Measurements. Each subject monitored and recorded her oral temperature (T<sub>or</sub>) on waking for the 3 days before the heat trial and on the morning of the heat trial using an over-the-counter mercury thermometer (III-6 Tem-Com Basal, Sunmark and II-7 Ovulindex Basal F/M). T<sub>or</sub> was measured using a YSI series 400 rectal thermistor inserted 10 cm past the anal sphincter. T<sub>sk</sub> was calculated as the unweighted average of temperatures recorded by thermocouples (Type T, Omega Engineering, Stamford, CT) affixed to eight skin sites (right chest, left chest, upper arm, upper back, lower back, abdomen, thigh, calf). T<sub>sk</sub> was calculated as T<sub>sk</sub> = 0.8 T<sub>or</sub> + 0.2 T<sub>re</sub> (30). MAP was measured by brachial auscultation on the left arm. HR was continuously measured with a Finapres cuff (Finapres blood pressure monitor, model 2300, Ohmeda, Louisville, CO) attached to the middle finger of the right hand.

FBB, a quantitative index of skin blood flow (SkBF), was measured by venous occlusion plethysmography on the left forearm using a mercury-in-Silastic strain gauge (EC4 Plethysmograph, Hokanson, Bellevue, WA). The quantitative level of SkBF can be used to estimate quantity of heat transfer. Although the pattern of SkBF may be similar be-
tween two women, the quantitative level of SkBF may be different. During whole body heating, increases in FBF are due exclusively to increases in blood flow to the skin rather than muscle, in which blood flow remains constant or decreases slightly (11). An occlusion cuff (Hokanson) around the wrist was inflated to a suprasystolic (200 mmHg) pressure to occlude hand blood flow while an upper arm cuff was cycled between 10 s of inflation (40–60 mmHg) and 5 s of deflation (E20 rapid cuff inflator, Hokanson). The mean FBF at each time point was calculated from three to five individual waveforms and used to calculate forearm vascular conductance (FVC = FBF/MAP).

Qualitative changes in SkBF were measured continuously throughout the experiments using laser-Doppler flowmetry (Periflux laser-Doppler flowmeter PF2B, Perimed, Stockholm, Sweden). The laser-Doppler flow probe was attached to the subject's right forearm using a thermostatically controlled heater. Cutaneous vascular conductance (CVC) was calculated as LDF/MAP. Because LDF readings are variable between sites within a given individual and between different individuals, CVC values at a given skin site were standardized by expressing CVC as a percentage of the maximal CVC (%CVCmax) at that skin site obtained during a 45-min period of local heating of the skin at 42–42.5°C (33). The combination of venous occlusion plethysmography and laser-Doppler flowmetry provides quantitative and continuous data, respectively. T_w, individual T_isk, and HR data were collected at a rate of five data points per second, averaged over 1-min intervals using a SuperScope II (GW Instruments, Somerville, MA) data-acquisition system, and stored on a dedicated computer (Macintosh, Apple Computer, Cupertino, CA). LDF data were recorded at a rate of one data point per second, and a mean was calculated for 1-min intervals.

Hormone and cytokine assays. Venous blood samples were collected from all women on their initial visit. Serum samples were stored on ice, centrifuged, and later analyzed for 17β-estradiol, estrone, and FSH concentrations. Circulating 17β-estradiol and estrone concentrations were analyzed by an 125I-labeled double-antibody radioimmunoassay (ICN Biomedicals, Costa Mesa, CA). The sensitivity of the 17β-estradiol assay was 9 pg/ml, and inter- and intra-assay coefficients of variation were <12% and <11%, respectively, for an estradiol range of 28–38 pg/ml. Sensitivity of the estrone assay was 1.2 pg/ml, and inter- and intra-assay precision coefficients of variation were <12% and <10% for an estrone range of 90–900 and 100–940 pg/ml, respectively. FSH was analyzed using a "two-step" sandwich-type ELISA procedure (Diagnostic Systems Laboratory, Webster, TX). Sensitivity of the FSH ELISA was 0.10 mIU/ml, and inter- and intra-assay coefficients of variation were <6% and <5% for an FSH range of 5–103 and 5–700 mIU/ml, respectively. Extracted plasma AVP concentrations were measured for each woman for both aspirin and placebo trials using a radioimmunoassay procedure (Diagnostic Systems Laboratory). Sensitivity of the AVP assay was 0.5 pg/ml, inter- and intra-coefficients of variation were <7% and <9%, respectively, for an AVP range of 6–16 pg/ml.

Supernatants were assayed in duplicate for cytokine-related proteins using ELISAs constructed with antibodies purchased from R&D Systems (Minneapolis, MN). Monoclonal mouse anti-human primary (capture) antibodies were coated on 96-well polystyrene plates (Corning). The standard curves consisted of serial dilutions of recombinant human cytokines (IL-6-PeproTech, Rock Hill, NJ; IL-1α, Cistron, NJ; all others, R&D Systems) ranging from 0.010 to 10 ng/ml. Detection was carried out by sequential incubation with biotinylated goat anti-human polyclonal secondary (detection) antibodies, streptavidin-conjugated horseradish peroxidase (Pierce, Rockford, IL), and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; Sigma). Absorbance at 405 nm was measured with a Labsystems Multiskan MCC/340 plate reader (Needham Heights, MA).

Analysis of data. Data are presented as means ± SE. SAS Statistical Software (Cary, NC) and SuperANOVA software (Abacus Concepts, Calabasas, CA) were used to perform all analyses, and the criterion for statistical significance of factors and their interactions was set at α = 0.05. Preplanned comparisons included group and drug comparisons in baseline T_re, T_b, and T_or and thresholds for FVC, FBF, and %CVC_max versus T_b curves. For significant factors in the ANOVA models, unplanned pairwise mean comparisons were performed using a Bonferroni correction.

A one-way ANOVA was performed to examine group differences in subject characteristics and hormone concentrations. Differences between groups (untreated, ERT, and E + P) and drugs (placebo and aspirin) for cell supernatant cytokine, plasma salicylate, plasma osmolality, and plasma AVP concentrations were analyzed using a two-way repeated-measures ANOVA. A three-way repeated-measures ANOVA was used to examine group, day, and drug differences in waking T_or. The correlation between waking T_or and baseline T_re was calculated by simple linear regression.

The independent variable baseline and heating time was partitioned into three regularly spaced time bins of 7-min width to make comparisons among the dependent variables of T_re, T_b, MAP, and HR at baseline (~7 min), midheating (25 min), and end heating (55 min). A three-way repeated-measures ANOVA model was fit to the data to examine group and drug differences in the independent variables over binned time.

Descriptive plots of FBF, FVC, and %CVC_max versus T_b curves were generated for each subject under placebo and aspirin. The curves were coded by an investigator, and four independent raters, blinded to the identity or drug of the subject, identified two features of the plot: a baseline and threshold. An average value for the baseline and threshold for each curve was obtained from the estimates of the four raters, and the curves had an intrarater reliability of 0.99 and 0.96 for the baseline and threshold, respectively. The baseline and threshold data were fit to a two-way repeated-measures ANOVA to examine group and drug differences in baseline and threshold values.

Simple linear regression analysis and analysis of covariance were performed to examine the relationship between serum estradiol concentration, baseline T_re, and T_b, thresholds for vasodilation (FVC and %CVC_max curves). Relationships between cytokine concentrations and baseline temperatures and thresholds for cutaneous vasodilation were determined by simple and multiple linear regression.

RESULTS
No significant differences existed among the three groups for age, years since menopause, height, weight, amount of regular vigorous physical activity, or body fat (Table 1). Serum 17β-estradiol concentrations were significantly greater (P < 0.05) in the ERT and E + P groups compared with the untreated group (Table 1). Serum estrone concentration was significantly greater in the ERT group compared with the untreated (P = 0.001) and E + P (P = 0.04) groups (Table 1). Plasma FSH was significantly greater in the untreated group.
compared with the other two groups ($P < 0.05$; Table 1).

Without aspirin, plasma osmolality, AVP concentration, and supernatant cytokine concentrations were not significantly different among the three hormone therapy groups with the exception of TNF-α, as shown in Table 2. Plasma salicylate concentrations were undetectable or negligible with placebo in all three groups ($1.0 \pm 0.1, 0.7 \pm 0.1$, and $0.8 \pm 0.2$ mg/dl for untreated, ERT, and E + P groups, respectively) and increased to a similar extent in all three groups with aspirin ($20.4 \pm 2.9, 19.5 \pm 0.9$, and $17.6 \pm 1.4$ mg/dl for untreated, ERT, and E + P groups, respectively; $P < 0.05$). The administration of aspirin had no significant influence on any of these variables with the exception of TNF-α, which was increased ~95% and 35% in the untreated and E + P groups, respectively ($P < 0.05$, data not shown). Only LPS-stimulated cytokine results without aspirin are presented, because many cytokines were undetectable in supernatants from unstimulated cells.

Significant hormone, aspirin, and hormone-by-aspirin interaction effects were noted for waking T$_{re}$. T$_{re}$ were measured consecutively over a 4-day period, averaged for each subject, and subsequently used to calculate the mean T$_{re}$ for each group for both placebo and aspirin (Fig. 1A), because day of measurement did not affect waking T$_{re}$. Daily waking T$_{re}$ in the E + P group was significantly higher than the untreated group without aspirin. Aspirin significantly reduced the mean daily waking T$_{re}$ in the E + P group ($P = 0.0001$).

Baseline T$_{re}$ and T$_{b}$ are plotted for the three groups of women with and without aspirin, respectively (Fig. 1, B and C). Without aspirin, baseline T$_{re}$ and T$_{b}$ were significantly lower in the ERT group compared with the other two groups. However, aspirin significantly increased resting T$_{re}$ and T$_{b}$ in the ERT and E + P groups and reduced resting T$_{re}$ in the untreated group such that with aspirin, baseline T$_{re}$ and T$_{b}$ for the ERT group remained significantly lower than the E + P group. Throughout heating, T$_{re}$ and T$_{b}$ remained lower in the ERT group compared with untreated and E + P groups. However, T$_{re}$ and T$_{b}$ for all three groups increased and began to merge after 1 h of heating. After 55 min, the only significant difference was T$_{re}$ between ERT and E + P groups with placebo. Simple linear regression revealed a highly significant relationship between log-normalized serum estradiol concentration and the change in T$_{re}$ with aspirin (Fig. 2; $r = 0.605$, $P = 0.0047$).

Baseline HR was similar among the three groups with and without aspirin but tended to be higher in the E + P group. Without aspirin, the HR response during whole body heating in the E + P group was significantly greater than the ERT and untreated groups at 25 and 55 min (70–77 beats/min for untreated and ERT groups vs. 81–90 beats/min for the E + P group, $P < 0.05$). The rise in HR over time was not affected by aspirin, but the HR values of the ERT and E + P groups were slightly reduced with aspirin such that no difference existed among the three groups.

MAP tended to be slightly lower in the ERT and untreated groups compared with the E + P group without aspirin, but it did not attain statistical significance. Aspirin ingestion did not affect MAP, but MAP significantly decreased over time with whole body heating in the three groups for both placebo and aspirin trials ($P < 0.05$).

Thresholds for cutaneous vasodilation were systematically calculated as an objective measure of thermo-regulatory control. We found significant differences among the three groups for cutaneous vasodilation (Fig. 3). The T$_{b}$ threshold for vasodilation (CVC curves) in the ERT group was always significantly lower than the E + P group with and without aspirin. Without aspirin, the thresholds for vasodilation (CVC curves) in the ERT group were significantly lower than the untreated group. For FVC-T$_{b}$ curves, aspirin significantly shifted the threshold for vasodilation rightward in the ERT group such that the threshold difference between ERT and untreated groups was no longer statistically significant (placebo: 37.18 ± 0.08, 36.67 ± 0.19, and 37.08 ± 0.04; aspirin: 37.14 ± 0.07, 36.94 ± 0.11, and 37.26 ± 0.07 for untreated, ERT, and E + P groups, respectively). A rightward shift in the %CVC$_{max}$-T$_{b}$ curve did not occur in the ERT group with aspirin, but the threshold tended to be higher. Although it was not statistically significant ($P = 0.076$), the threshold for vasodilation in the E + P group also tended to be higher with aspirin drug. Therefore, with aspirin, the T$_{b}$ threshold for vasodilation (both CVC and FVC curves) was lower in the ERT group compared with untreated and E + P groups, but this difference was only significant between ERT and E + P groups. These results correspond to results observed for T$_{re}$ and T$_{b}$ data. Resting FBF, FVC, and %CVC$_{max}$ were similar among the three groups at baseline for both placebo and aspirin.

Waking T$_{re}$ during placebo treatment was not related to IL-1β or TNF-α secretion by simple regression nor multiple regressions involving relevant antago-

| Table 2: AVP concentrations, plasma osmolality, and cytokine concentrations of LPS-stimulated cell culture supernatants (ng/ml) with placebo |
|-----------------|-----------------|-----------------|
|                 | Untreated | ERT | E + P |
| AVP, pg/ml      | 5.4 ± 1.8 | 4.5 ± 2.2 | 4.5 ± 1.3 |
| Osmolality, mOsmol/kgH₂O | 286 ± 3 | 286 ± 2 | 284 ± 3 |
| IL-1β           | 1.75 ± 0.24 | 2.30 ± 0.47 | 1.88 ± 0.31 |
| IL-1Ra          | 12.3 ± 1.6 | 12.9 ± 3.3 | 15.1 ± 2.9 |
| sIL-1RII        | 0.053 ± 0.010 | 0.054 ± 0.009 | 0.041 ± 0.007 |
| IL-6            | 26.6 ± 4.6 | 26.2 ± 5.4 | 39.2 ± 4.7 |
| sIL-6R          | 0.13 ± 0.02 | 0.15 ± 0.02 | 0.18 ± 0.03 |
| sTNFRI          | 0.05 ± 0.01 | 0.06 ± 0.01 | 0.06 ± 0.02 |
| sTNFR-II        | 0.65 ± 0.14 | 0.74 ± 0.15 | 0.67 ± 0.22 |

Values are means ± SE, AVP, arginine vasopressin; LPS, lipopolysaccharide; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; sIL-1RII, IL-1 type II soluble receptor; sIL-6R, soluble IL-6 receptor; sGP130, soluble glycoprotein 130; TNF, tumor necrosis factor; sTNFRI, soluble TNF receptor type I; sTNFR-II, soluble TNF receptor type II. *Significantly different from untreated.
nists and soluble receptors. A simple regression between waking Tor and IL-6 secretion was not significant ($r = 0.375, P = 0.103$), but incorporating sIL-6R and sGP130 concentrations into a multiple regression yielded a significant relationship ($r = 0.675, P = 0.032$, Fig. 4).

Baseline Tre measured on arrival at the laboratory were 0.44 ± 0.13°C higher ($P = 0.0014$) than waking Tor. These two measures were not significantly correlated ($P > 0.25$).

Secretion of IL-6 and IL-1β (log-normalized) was positively correlated with baseline Tre and Tb thresholds for vasodilation (CVC) by simple regression. For all four comparisons, the calculated coefficients of multiple correlations ($r$) were 0.44 and $P$ values were <0.05. Multiple regressions involving relevant antagonists improved the correlation for IL-6, but not IL-1. However, these statistical relationships disappeared when data from one woman with an abnormally low Tb (35.52°C) were excluded [this woman had a normal waking Tor (36.8°C)].

**DISCUSSION**

Three lines of evidence support the hypothesis that COX products and pyrogenic cytokines are involved in temperature regulation after menopause; however, the contribution of these factors appears to be minor relative to the overall changes brought about by steroid hormone therapy. First, waking Tor correlated with a composite estimate of IL-6 activity (secreted IL-6, sIL-6R, and sGP130). The positive regression coefficient for sIL-6R was in accord with other reports that this receptor subunit stabilizes IL-6 in the extracellular fluid yet allows interaction with cell-associated glycoprotein 130 subunits and subsequent signal transduction (16). Second, waking Tor in the E + P group was significantly reduced with aspirin. Third, baseline Tre in the
untreated group was significantly reduced by aspirin treatment compared with placebo.

Nevertheless, most of the evidence indicates that reproductive steroid-induced changes in thermoregulation are largely independent of COX products or pyrogenic cytokines. First, aspirin did not prevent the progesterone-mediated elevation in \( T_c \) in the E + P group. Second, baseline \( T_r \), and the \( T_b \) threshold for cutaneous vasodilation for the ERT group remained below both untreated and E + P groups.

Fig. 3. Cutaneous vascular conductance (CVC), expressed as a percentage of maximal CVC (%CVC\(_{\text{max}}\)), is plotted against \( T_b \) for placebo (A) and aspirin (B). The \( T_b \) threshold for the initiation of cutaneous vasodilation was significantly lower in the ERT group compared with both E + P and untreated groups. Without aspirin, \( T_b \) threshold was lower in the ERT group compared with both E + P and untreated groups. With aspirin, the CVC threshold for the ERT was only significantly lower than the E + P group. (*ERT vs. E + P and untreated groups, \( P < 0.05 \); †ERT vs. E + P, \( P < 0.05 \)).

Fig. 4. Statistical relationship between oral temperature and a composite estimate of interleukin (IL)-6 activity based on lipopolysaccharide-induced secretion of IL-6, soluble IL-6 receptor (sIL-6R), and soluble glycoprotein 130 (sGP130). \( f(\text{IL-6}) = (0.011)\text{IL-6} + (1.592)\text{sIL-6R} - (0.244)\text{sGP130}. r = 0.675, P = 0.032. \)

This result is consistent with findings from a recent study by Charkoudian and Johnson (9), who examined reflex control of the cutaneous circulation and sweating responses to whole body heating in premenopausal women at hormonally distinct points of their menstrual or oral contraceptive cycles with and without administration of ibuprofen. Ibuprofen did not affect the shift in thermoregulatory control accompanied by increased plasma progesterone concentrations.

Although we did not measure regional sweating responses in the present investigation, we did not see differences in whole body sweating among untreated, ERT, and E + P groups of women in response to exercise in the heat in our previous study (4). In young women (8, 29) and postmenopausal women (32), reproductive steroids alter thermoregulatory control similarly to cutaneous vasodilation. With respect to the effect of NSAIDs on sweating, Charkoudian and Johnson (9) demonstrated that ibuprofen had no effect on the onset of sweating in young women during the low-hormone or high-hormone phase. Aspirin administration increased the onset of sweating in older women during heat stress (31) and increased evaporative water loss (35) in rodents. Aspirin toxicity is characterized by increased sweating, but women in our study were not salicylate intoxicated as indicated by plasma salicylate concentrations. If aspirin did increase the onset of sweating or sweat output, then aspirin should lead to a decrease in \( T_c \) rather than an increase. Therefore, we do not believe that the increase in regulated body temperature with aspirin administration in the E + P group was a result of reduced sweating.

TNF-\( \alpha \) secretion from LPS-stimulated PBMCs was significantly greater in ERT and E + P groups without aspirin, implying that estrogen and progesterone may alter physiological and/or thermoregulatory systems by affecting cellular responsiveness to exogenous pyro-
gens. The role of TNF-α in thermoregulation is controversial (12, 20). For example, TNF-α is suspected to act as an endogenous cryogen rather than pyrogen (20), because an intraperitoneal injection of TNF-α antiserum before an intramuscular injection of LPS in rats resulted in a significantly greater fever compared with the control rats (LPS alone) after 3 h and up to 8 h after injection. If one considers the untreated and ERT groups, this cryogenic theory would coincide nicely with our findings, because TNF-α from LPS-stimulated cells was approximately two times greater in the ERT group compared with the untreated group. However, if this theory were true, how does one explain the higher regulated Tc in the E + P group coincident with elevated concentrations of TNF-α? TNF-α may well be a cryogen, but it did not appear to be produced in greater amounts in the ERT compared with the E + P group.

Waking Tore, baseline Tore, and Tb thresholds for dilation (CVC) correlated with secretion of IL-6 (and associated soluble receptors) and IL-1β. However, the correlations observed in the laboratory disappeared after the influence of one extreme data point was eliminated. It is possible that basal temperature at waking is influenced significantly by IL-6, but by the time the subjects reached the laboratory, other activity- or stress-related factors have overwhelmed the more subtle cytokine-mediated influences. Once again, these findings imply that shifts in the regulated level of body temperature associated with HRT administration occur primarily by direct actions of the reproductive steroid hormones rather than through cytokines or prostaglandins.

A higher circulating concentration of AVP likewise does not explain the reduced regulated Tc in the ERT group, because plasma AVP concentration and osmolality were not significantly different among the three groups of postmenopausal women (Table 2). The increase in baseline plasma AVP was associated with a reduction in hematocrit, indicating an expansion of plasma volume. Findings from Stachenfeld (28) and Tankersley et al. (32) previously noted an expansion of plasma volume that accompanied the acute administration of oral and percutaneous ERT (2–3 wk) to a group of postmenopausal women. In contrast, plasma volume was not different among ERT, E + P, and untreated groups with chronic (≥2 yr) administration of oral HRT (4). Although AVP concentration may increase with acute administration of estrogen and contribute to an expansion of plasma volume, circulating AVP concentration apparently returns to baseline, as does plasma volume, with chronic exogenous estrogen administration. These observations imply that the reduced Tc in the ERT group is not due to a higher circulating concentration of AVP.

Our findings suggest that aspirin blocks a cryogenic factor that is exclusively stimulated by exogenous estrogen, because women not receiving HRT (low circulating levels of estrogen and progesterone) responded with no change or a slight decrease in Tore. This corresponds to the highly significant relationship between serum estradiol concentration and the change in Tore with estradiol (r = 0.605, P = 0.0047). Recently, it has been noted that in vitro aspirin can alter oxygenase activity of COX-2 and increase formation of 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) (22). 15-HETE has been reported to block the production of proinflammatory leukotrienes (LTB4, LTC4, and LTD4), but the biological effects of 15-HETE on temperature regulation and potential interactions with reproductive hormones are not known. Another explanation is that aspirin’s influence on afebrile individuals may be dependent on an individual’s baseline Tc and hormonal status. In contrast to our findings, Charkoudian and Johnson (9) did not find a change in thermoregulatory responses in the low- and high-hormone groups with ibuprofen. However, these investigators administered an acute dose of ibuprofen (800 mg). Recent studies performed in vitro have described the differential actions of aspirin and other NSAIDs on the two types of COX isoenzymes, COX-1 and COX-2 (22). COX-2 is the inducible form of COX and is increased 10- to 80-fold during inflammation. Subtle differences in thermoregulatory responses to anti-inflammatory and antipyretic medications may be due to their differential biochemical properties and kinetics. Additional studies with a larger sample size are warranted to verify these unexpected findings.

In summary, the reduced Tore and earlier activation of cutaneous vasodilation in the ERT group was not accompanied by an altered ex vivo secretion of cytokines from PBMCs compared with E + P and untreated groups. These findings correspond to recent findings in young women (17, 24). More importantly, the following relationship for thermoregulatory control of body temperature existed with and without aspirin: E + P > untreated > ERT. These data indicate that the higher body temperature and Tb threshold for activation of cutaneous vasodilation observed in postmenopausal women receiving exogenous E + P occur independently of COX-dependent products and through a different mechanism than the increase in Tc associated with infection and inflammation. Because waking Tore correlated with IL-6 and aspirin reduced waking Tore or baseline Tore in isolated groups, cytokines or COX products may play a limited role in thermoregulatory control. Given that steroids can readily pass through the blood-brain barrier to alter firing rates of thermosensitive neurons, a direct action by estrogens and progestins to act on neurons located in thermoregulatory centers seems plausible. Within 20–40 min of a single injection of progesterone (5 mg/kg), Nakayama and Suzuki (23) observed changes in the firing rate of thermosensitive neurons in rabbits. This thermoregulatory response occurred too rapidly to be genomic in nature. The current observations and results from previous studies demonstrating a lack of an influence of HRT on resting SkBF, maximal SkBF (5), and the sensitivity of the skin to reflex-induced cutaneous vasodilation (4) indicate that the dominant influence of estrogen and progesterone on thermoregulation occurs...
by direct actions on thermosensitive neurons located in hypothalamic thermoregulatory centers rather than by modifying the peripheral response to thermoregulatory effenter systems.

Perspectives

HRT is now widely prescribed to millions of postmenopausal women in the United States and other countries. HRT effectively ameliorates climacteric symptoms (e.g., hotflashes), and there is substantial evidence that HRT reduces a woman’s risk for cardiovascular disease development and osteoporosis. In addition to these effects, our findings demonstrate the importance of HRT in yet another physiological system, thermoregulation. These findings indicate that ERT, but not E + P, may increase a woman’s tolerance to heat stress and exercise by reducing the regulated level of Tc through an earlier activation of the cutaneous vasodilator system. Although reproductive hormones altered thermoregulation independently of changes in ex vivo cytokine synthesis, reproductive steroid hormones likely alter cellular responsiveness to cytokines and other signaling factors. In future studies of reproductive steroid hormones on physiological function, it will be important to consider alterations in target tissue receptors and signaling in addition to changes in ligand synthesis to fully understand the system.

The authors thank the participants for time and effort in the present investigation. The assistance provided by Stacey (Wladkowski) Dunhar, Mark Dunhar, Duna Zaleman, Heather Harr, Rick Ball, Jane Daun, Fred Weyandt, Marlin Druckenmiller, and the General Clinical Research Center nursing staff is greatly appreciated.

This research was supported by the National Institutes of Health Grants ROI-AG-07004-09 and MO1-RR-10732 and an American College of Sports Medicine Foundation Grant.

REFERENCES


