Androgens influence microvascular dilation in PCOS through ET-A and ET-B receptors

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Wenner MM, Taylor HS, Stachenfeld NS. Androgens influence microvascular dilation in PCOS through ET-A and ET-B receptors. Am J Physiol Endocrinol Metab 305: E818–E825, 2013. First published August 6, 2013; doi:10.1152/ajpendo.00343.2013.—Hyperandrogenism and vascular dysfunction often coexist in women with polycystic ovary syndrome (PCOS). We hypothesized that testosterone compromises cutaneous microvascular dilation in women with PCOS via the endothelin-1 ET-B subtype receptor. To control and isolate testosterone’s effects on microvascular dilation, we administered a gonadotropin-releasing hormone antagonist (GnRHant) for 11 days in obese, otherwise healthy women [controls, 22.0 (4) yr, 36.0 (3.2) kg/m2] or women with PCOS [23 (4) yr, 35.4 (1.3) kg/m2], adding testosterone (T; 2.5 mg/day) on day 4 of administration. At baseline (BL), ET-A receptor inhibition enhanced heat-inhibited cutaneous microvascular dilation in both groups [ΔCVC, 2.03 (0.65), PCOS 2.10 (0.25), AU/mmHg, P < 0.05]; ET-B receptor inhibition reduced vasodilation in controls only [ΔCVC, 0.98 (0.39), 1.41 (0.45) AU/mmHg for controls, PCOS] compared with saline [ΔCVC controls, 1.27 (0.48), PCOS 1.31 (0.13) AU/mmHg]. GnRHant enhanced vasodilation in PCOS [saline ΔCVC 1.69 (0.23) AU/mmHg vs. BL, P < 0.05] and abolished the ET-A effect in both groups, a response reasserted with T in controls. ET-B receptor inhibition reduced heat-induced vasodilation in both groups during GnRHant and T [ΔCVC, controls: 0.95 (0.21) vs. 0.51 (0.13); PCOS: 1.27 (0.23) vs. 0.84 (0.27); for GnRHant vs. T, P < 0.05]. These data demonstrate that androgen suppression improves microvascular dilation in PCOS via ET-A and ET-B receptors.

polycystic ovary syndrome; endothelin; vasoconstriction; vasodilation; testosterone; aldosterone

POLYCYSTIC OVARY SYNDROME (PCOS) is the most common reproductive endocrinopathy in young women (5), affecting 6–10% of women of reproductive age (41). PCOS is a primary cause of infertility and is associated with increased risk for cardiovascular disease. Although not present in every woman with PCOS, both insulin resistance and obesity are hallmark characteristics of PCOS, and it is likely that both contribute to their greater cardiovascular risk. Although the factors contributing to cardiovascular disease in women with PCOS are not completely clear, PCOS is associated with endothelial dysfunction, which precedes hypertension. For example, both flow-mediated and nitrate-mediated vasodilation are attenuated in women with PCOS compared with healthy women of similar age (11, 28). Moreover, total and free testosterone (T; the primary androgen elevated in PCOS), along with insulin resistance, are independent predictors of the impaired vasodilation (28).

Vascular dysfunction in the microcirculation impacts both vascular resistance and insulin-mediated glucose disposal in the periphery, thereby contributing to hypertension and insulin resistance. Thus peripheral microvascular dysfunction is a key feature of cardiovascular disease risk associated with these chronic conditions (22). Androgen exposure attenuates microvascular circulatory responses in androgen-treated hypogonadal men (6), most likely by altering endothelial function (11, 13, 28).

Although hyperandrogenism and endothelial dysfunction often coexist in women with PCOS, a causal relationship has not been established. T may influence microcirculatory regulation with effects on both vascular smooth muscle and the endothelium through actions on substances in the blood such as endothelin-1 (ET-1), a vasoactive substance involved in regulating endothelial function that is commonly chronically increased in women with PCOS. ET-1 binds two receptor subtypes, endothelin A (ET-A) and endothelin B (ET-B). Both ET-A (3, 31) and ET-B (15) receptors are found in vascular smooth muscle and mediate vasoconstriction. However, ET-B receptors are also located in the endothelium, where they mediate vasodilation (14, 16, 21). Importantly, sex and sex hormones may direct ET-1 receptor subtypes (26, 44).

In humans, the skin is an excellent model organ to test hypotheses relating to endothelial control of the microcirculation (18, 26, 44). Cutaneous local microvascular responsiveness during local heating is mediated to a large extent by endothelial nitric oxide synthase (24). The control of microcirculatory responsiveness is independent of functional sympathetic nerves (36), so it can be used to evaluate peripheral microvascular endothelial function (1, 23, 25–27, 33, 39). Recently, we demonstrated lower ET-B receptor-mediated vasodilation in the skin in women with PCOS compared with healthy control women, although skin microvasculature responded similarly between the two groups to ET-A receptor blockade (44). We interpreted these findings to suggest that women with PCOS may be less sensitive to ET-B-mediated vasodilation and propose that chronically elevated androgens are a contributing factor. In the present investigation, we studied T effects on cutaneous endothelium-mediated vasodilation in the microcirculation in women with PCOS. We hypothesized that T suppression in women with PCOS would enhance vasoconstrictive responsiveness to local endothelium-mediated cutaneous heating, whereas T administration would inhibit these vasodilatory responses. We further hypothe-
esized that T effects are mediated by ET-1 via the ET-B subtype receptor.

PCOS, concomitant with insulin resistance and obesity, is also associated with inflammatory responses and elevated aldosterone levels (4). Aldosterone plays an important role in cardiovascular disease, likely through its mineralocorticoid receptor, because inhibition of this receptor decreases atherosclerosis by reducing oxidative stress (37). Observational studies have implicated an androgen contribution to the increased aldosterone and renin production that are implicated in pathogenesis of PCOS and its cardiovascular sequelae (4). Therefore, we tested the hypothesis that T exposure contributes to the greater serum aldosterone concentration (S[ALD]) and plasma renin activity (PRA) in women with PCOS.

**METHODS**

**Subjects**

We recruited six women with PCOS and seven women without PCOS (control). All of the women were nonsmoking and had a BMI of >30 kg/m². We recognize that this represents a small number of subjects, but our power analysis (see Data Analysis and Statistics) demonstrates that we recruited an adequate number of subjects to test our hypothesis. Women with PCOS are difficult to recruit for such invasive studies. The women were interviewed to obtain their medical history and indicated good health other than PCOS. Control women reported regular menstrual cycles (26–32 days) with no gynecological abnormalities. The vast majority of women with PCOS in our outreach area (New Haven, CT) were obese, so we recruited obese women for our controls to reduce the influence of obesity on our findings. All subjects gave written informed consent to participate in the study, which conformed to the guidelines contained in the Declaration of Helsinki and had prior approval by the Human Investigation Committee of the Yale School of Medicine.

Women in both groups underwent transvaginal ultrasound to either confirm the diagnosis of PCOS or exclude PCOS and polycystic appearing ovaries in the controls. Potential controls were also excluded if they had any of the symptoms or signs of PCOS (see below). For the diagnosis of PCOS, in addition to androgen excess, at least one of the two following criteria were present: oligo/anovulation, defined as an intermenstrual period of ≥45 days or a total of ≤8 menses/yr, and/or having polycystic ovaries. Polycystic ovaries were defined by the morphological appearance of 12 small follicles in the range of 3–9 mm mean diameter in the ovary on day 3, as determined by transvaginal ultrasound. We also excluded other disorders of the ovaries, adrenal and pituitary. Criteria were evaluated by an obstetrician/gynecologist with more than 18 years of experience in this area (H. S. Taylor). No subject in either group was taking any medications.

**Oral Glucose Tolerance Test**

A 3-h oral glucose tolerance test (OGTT) was conducted to determine glucose tolerance in all women within 2 wk of the start of the study. With the subjects in the seated position, we inserted an intravenous catheter into an antecubital vein. After a 30-min resting period, we drew a baseline blood sample. The subjects then drank a 75-g glucose beverage (Orangedex; Custom Laboratories, Baltimore, MD), with blood sampled every 30 min for a 180-min period following drinking. Plasma concentration of glucose and serum concentration of insulin were measured at each of these time points and used to determine an area under the curve for both substances as an indicator of glucose tolerance and insulin resistance.

**Experimental Protocol**

Each subject participated in three experimental sessions to assess microvascular dilation, occurring in the following order: baseline (BL) during the first 5 days of a normal menstrual cycle, gonadotropin-releasing hormone (GnRH) antagonist administration alone, and finally, while taking GnRH antagonist with T. None of the women with PCOS were menstruating, so they participated in the BL test at their convenience, but prior to the other testing days. The GnRH antagonist was used to minimize fluctuations in ovarian hormones in control women and to suppress ovarian T production in women with PCOS (see below).

To assess microvascular dilation, we measured red blood cell flux [index of skin blood flow (SKBF)] using laser Doppler flowmetry (Doppler Monitor, PF 5020 LDPM Unit; Perimed, Stockholm, Sweden) during microdialysis infusions of ET-1 receptor antagonists, followed by local heating of the skin. Local heating of the skin induces rapid endothelial-dependent vasodilation (Fig. 1) (19, 25, 26, 33). Measurements of SKBF coupled with cutaneous microdialysis are commonly used to study the mechanisms involved in vascular dysfunction. Moreover, because the responses are local, microdialysis substantially minimizes the risks associated with venous infusion of drugs.

**GnRH antagonist (ganirelex acetate).** Ganirelix acetate is a synthetic decapeptide that competes with naturally occurring GnRH for receptor binding, so it functions as a competitive receptor antagonist. Ganirelix acetate is derived from native GnRH with substitutions at positions 1, 2, 3, 6, 8, and 10. Ganirelix acetate competitively blocks the GnRH receptors on the pituitary gonadotroph and subsequent transductions pathway and induces a rapid, reversible suppression of gonadotropin secretion (34, 35). In young women with regular menstrual cycles, continued administration of ganirelix acetate prevents the rise in estrogens and progesterone; the hypothalamic-pituitary-ovarian axis is reversed upon cessation of drug therapy (34, 35).

To compare hormone effects on microvascular dilation, all subjects received the GnRH antagonist ganirelix acetate (250 μg/day, Antagon; Organon, West Orange, NJ) every day for 11 days. In controls, the GnRH antagonist administration began on days 25–28 of their menstrual cycle to reduce the risk of endometrial bleeding and other potential side effects. Women with PCOS who participated in this study were not menstruating, so they began the GnRH antagonist administration at their convenience. The subjects self-administered daily subcutaneous injections of the GnRH antagonist after training by qualified study personnel. This method of GnRH antagonist administration is easily discontinued in the event of uncomfortable side effects such as headaches, vaginal bleeding, and vasomotor symptoms. Women were tested on day 4 of GnRH antagonist administration.

Beginning on the 8th day of GnRH antagonist administration, the women received methyl T at an oral dose of 2.5 mg/day (Compounded Solutions, Monroe, CT) for the final 4 days of GnRH antagonist treatment, at which time they completed the final experimental session. We chose methyl T in this study because it is less rapidly metabolized due to its 17-methyl group and not aromatized to estradiol. This design permitted within- and between-subject comparisons concerning T effects on changes in SKBF regulation and on sodium-regulating hormones. Utilizing this hormone intervention eliminated other potential confounders, such as GnRH and the gonadotropins, as well as other ovarian products. Thus, this experimental design enabled us to isolate and directly examine T effects on microvascular dilation.

**SKBF and microdialysis protocol.** SKBF tests were conducted in an environmental chamber (T = 28°C). Subjects ate a diet controlled for water and sodium the night before and the morning of the SKBF test under each experimental condition (~13 kcal/kg body wt). Upon arrival, hydration state was immediately assessed from urine-specific gravity, which was between 1.003 and 1.026 in all subjects. Following the urine sample, the subject was weighed to the nearest 10 g on a beam balance and positioned in a semirecumbent position in a dental chair modified to support the forearm. During a 60-min resting period, we inserted an intravenous catheter into an antecubital vein, and subjects were instrumented for the measurement of beat-to-beat arte-
rrial blood pressure (Pinaz method, Finometer; Finapres Medical Systems, Amsterdam, The Netherlands) and skin microdialysis (see below). After 1 h of seated rest, a blood sample was drawn to measure serum concentrations of 17β-estradiol (S[E2]), progesterone (S[P4]), testosterone (S[T]), S[ALD]), and plasma endothelin-1 (P[ET-1]; 1–23) concentration and PRA.

For microdialysis studies, under sterile conditions, four 27-gauge needles were inserted intradermally on the dorsal aspect of the forearm. The entrance and exit sites were 2 cm apart, and the needles were ±2 cm apart. Microdialysis probes were threaded through the lumen of the needle, after which the needle was removed, leaving the hollow fiber portion of the microdialysis probe in place under the skin. Laser Doppler probes were placed on the surface of the skin above each microdialysis site to measure SkBF. The Doppler probes both measure SkBF and control local skin temperature. All four microdialysis probes were infused with 0.9% saline (2 μl/min; Harvard microinfusion pump) for 120 min after placement to allow for recovery from the microdialysis probe placement (2, 10, 17).

After the 2-h waiting period, we continued the isotonic saline infusion in the first probe. In the second, third, and fourth probes, we began infusion of the ET-A receptor antagonist (BQ-123, 500 nM) (30, 44), the ET-B receptor antagonist (BQ-788, nM 300 nM) (44), and the combined infusion of the ET-A and ET-B receptor antagonists (i.e., BQ-123 and BQ-788), respectively. The infusion rate for all probes was 5.0 μl/min for 45 min. The optimal concentrations of the antagonists were determined in an earlier study, with doses ranging from 75 to 750 nM; for each antagonist, we chose the dose after which we did not see any further changes in SkBF, indicating complete inhibition of these receptors (44).

After the 45-min infusion of the blocking agents, resting SkBF measurements at all sites were measured for 5 min, and all four local heating devices were raised to 42°C (32, 38). Under these conditions, we expected to see a rapid rise in SkBF, followed by a plateau of ~35–45 min (See Fig. 1). After this plateau in SkBF was achieved, we maintained this temperature for 5 min while continuously measuring SkBF and beat-to-beat blood pressure. To maintain ET-A and B receptor inhibition during the heating period, the blocking agents were infused continuously through the second, third, and fourth probes, whereas saline was infused continuously through the first probe. Each series of infusions was followed by a slight increase in probe temperature (43°C) and an infusion of sodium nitroprusside (SNP; 28 mM, 10 μl/min) to determine maximal SkBF.

Blood analysis. Immediately following blood collection, an aliquot was transferred to a into a tube without anticoagulant for the determination of S[ALD], S[E2], S[P4], serum insulin, and total and free S[T]. All other aliquots were immediately placed in chilled tubes containing EDTA and were analyzed for P[ET-1]. The samples were centrifuged, frozen immediately, and stored at −80°C until analysis. Intra- and interassay coefficients of variation for the midrange standard (127 ± 9.8 pg/ml) were 2.3 and 2.4% for S[E2] (Siemens Diagnostic, Los Angeles, CA), 2.6 and 23.0% for S[P4] (3.5 ± 0.23 ng/ml) (Siemens), 2.0 and 2.3% for S[T total] (397 ± 30 nmol/l), 3.0 and 3.6% for free serum S[T free] (8.4 ± 0.97 pg/ml; Siemens), and 5.6 and 5.9% for serum sex hormone binding globulin (S[SHBG]); 50 ± 13 nmol/l) (IBL America, Minneapolis, MN). Intra- and interassay coefficients of variation for the midrange standard were 2.6 and 2.1% for S[ALD] (155 ± 15.5 pg/ml; Siemens), 6.0 and 5.9% for P[ET-1] (1.5 ± 0.5 fmol/l; Phoenix Pharmaceuticals, Belmont, CA), 1.8 and 2.6% for PRA (5.0 ± 1.5 ng·ml−1·h−1; Diasorin, Stillwater, MN), and 2.3 and 3.5% for P[Ins] (43 ± 4 μU/ml; Siemens).

Data Analysis and Statistics

Laser Doppler flowmetry data were recorded at 1,000 Hz using Powerlab (ADInstruments, Bella Vista, New South Wales, Australia). After a plateau was attained with each probe, a mean of 2 min of SkBF and mean arterial blood pressure (MAP) at each antagonist dose were used for analysis. Change from resting cutaneous vascular conductance (CVC; SkBF/MAP) during local heating was our indicator of peripheral microcirculatory responsiveness and is expressed as ΔCVC. We chose ΔCVC because we found baseline variability across skin sites within and across women (45). We found little effect of further heating and nitroprusside to normalize the values to %CVC max. To determine effects on ΔCVC with the ET receptor subtype blocking, group comparisons were made using two-way repeated ANOVA with PASW Statistics 19 (IBM SPSS, Chicago, IL), followed by post hoc testing to determine specific differences. Differences were considered statistically significant when P < 0.05 after post hoc testing. All data are presented as means ± SE in graphs and mean (SD) in tables.

Sample size calculation. Sample size calculations were based on our primary outcome variable of interest: ΔCVC. The desired statistical test is two-sided, and we assumed an α-level of 0.01 to account for multiple comparisons. Kellogg et al. (26) reported a 6 (3%) difference between men and women in resting SkBF, using BQ-788 during microdialysis with laser Doppler techniques; our pilot data indicated similar effect size and error terms within subjects using ΔCVC. Given six per group and an α-level of 0.01, this effect size allowed us >80% power (0.883) for ANOVA to differentiate these changes from chance (G-Power 3.1, Faul F, Erdfelder E, Lang AG, and Buchner A, 2006, 2009, Heinrich-Heine-University, Düsseldorf Germany).

RESULTS

General subject characteristics were similar between control and PCOS groups, as measured from the baseline blood sample on the OGTT testing day (Table 1). Results from the OGTT (Table 1) are consistent with normal glucose and insulin responses in the control women and with poor glucose tolerance and insulin resistance in women with PCOS. Finally, height, weight, and BMI indicated a similar level of obesity in both groups.

Hormone Responses During Experimental Protocol

Across all testing days, S[E2] and S[P4] were low and similar between groups, as expected from the GnRH antagonist, and BL testing in controls was in the early follicular phase (Table 2).
Table 1. Subject characteristics in control women and women with PCOS taken at rest on the day of the OGTT prior to any hormone intervention

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PCOS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>24 (1)</td>
<td>23 (4)</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>95.1 (11.3)</td>
<td>95.5 (11.2)</td>
</tr>
<tr>
<td>Height, cm</td>
<td>1.63 (0.07)</td>
<td>1.64 (0.07)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>36.0 (3.2)</td>
<td>35.4 (1.3)</td>
</tr>
<tr>
<td>P(20), nm/l</td>
<td>5.14 (0.55)</td>
<td>4.91 (0.65)</td>
</tr>
<tr>
<td>P(100), pmol/l</td>
<td>59.1 (32.1)</td>
<td>62.5 (20.2)</td>
</tr>
<tr>
<td>Glucose, nm/l</td>
<td>18,682 (2,902)</td>
<td>25,276 (2,903)*</td>
</tr>
<tr>
<td>Insulin, m/l</td>
<td>6,978 (3,021)</td>
<td>12,897 (4,979)*</td>
</tr>
</tbody>
</table>

Data are presented as means (SD). PCOS, polycystic ovary syndrome; OGTT, oral glucose tolerance test; AUC, area under the curve. BMI, serum concentrations of glucose (P(20)) and insulin (P(100)), and AUC during the OGTT. *Different from control. Differences were considered significant at P < 0.05.

Although the lack of a further decrease in S(ET,1) was somewhat unexpected with GnRH antagonist, it is likely the result of initially low levels of S(ET,1) in both groups (floor effect). At BL and during T administration, S(T total) was similar between women with and without PCOS, but S(T free) was higher in the women with PCOS, concomitant with lower S(SHBG) in PCOS under both conditions (Table 2). Administration of the GnRH antagonist suppressed both total and free serum T concentrations similarly in both groups.

Across all testing days S(ET,1), S(ALD), and PRA were higher in women with PCOS compared with controls (Table 2). GnRH antagonist administration did not impact S(ALD) or PRA in the control group. In contrast, in women with PCOS, GnRH antagonist was associated with lower S(ALD), which was partially restored with T.

Microvascular Responses

Resting SkBF. Resting CVC was similar between the groups at baseline and was unaffected by the GnRH antagonist administration: 0.09 (0.02) and 0.07 (0.04) AU/mmHg, collapsed CVC mean for BL and GnRH antagonist conditions for control and PCOS, respectively. Maximum CVC induced by SNP infusion was also unaffected by group, hormone administration, or type of perfusion [collapsed means for all conditions were 1.51 (0.89) and 1.89 (0.81) and AU/mmHg, CVC for control and PCOS, respectively]. Mean arterial pressure as determined by our beat-to-beat blood pressure monitor (Finometer) was similar across hormone conditions and between groups; collapsed means for all conditions in the groups were 67 (9) and 66 (15) mmHg for control and PCOS, respectively.

Cutaneous microvascular response to local heating. Preheating blood flow was unaffected by either ET-A or ET-B infusion. Prior to hormone intervention (BL), ET-A receptor inhibition enhanced microvascular responses (ΔCVC) to local cutaneous heating compared with saline in both the control and PCOS groups (Fig. 2), whereas ET-B receptor inhibition attenuated ΔCVC in control women only (Fig. 2). In controls, the GnRH antagonist had minimal effects on ΔCVC with saline, and ET-B receptor blockade attenuated ΔCVC (Fig. 3A). GnRH antagonist administration enhanced ΔCVC in women with PCOS compared with BL during saline infusion (P < 0.05; Figs. 2 and 3B). In contrast, ET-B receptor blockade with and without ET-A blockade reduced ΔCVC response to local skin warming in women with PCOS (Fig. 3A). In controls, T administration did not impact ΔCVC with heating during saline infusion (Fig. 3A); however, ET-A receptor blockade enhanced ΔCVC, and ET-B receptor blockade lowered ΔCVC relative to saline (P < 0.05; Fig. 3A). During T administration, ΔCVC was elevated compared with BL during saline infusion in women with PCOS (Figs. 2 and 3B). ET-B receptor inhibition groups.

Table 2. Pretest hormone responses on the 3 experimental testing days in control women and women with PCOS

<table>
<thead>
<tr>
<th></th>
<th>BL</th>
<th>BL</th>
<th>BL</th>
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<tbody>
<tr>
<td>S(E2), pg/ml</td>
<td>50.1 (34.9)</td>
<td>38.6 (34.9)</td>
<td>38.6 (34.9)</td>
</tr>
<tr>
<td>S(P4), ng/ml</td>
<td>0.8 (0.3)</td>
<td>0.8 (0.2)</td>
<td>0.8 (0.2)</td>
</tr>
<tr>
<td>S(T total), nmol/l</td>
<td>37.4 (2.2)</td>
<td>46.1 (2.9)</td>
<td>46.1 (2.9)</td>
</tr>
<tr>
<td>S(T free), nmol/l</td>
<td>0.4 (0.2)</td>
<td>1.6 (0.2)*</td>
<td>1.6 (0.2)*</td>
</tr>
<tr>
<td>S(SHBG), nmol/ml</td>
<td>40.8 (3.5)</td>
<td>15.2 (6.4)*</td>
<td>15.2 (6.4)*</td>
</tr>
<tr>
<td>P(ET-1), fmol/ml</td>
<td>0.7 (0.4)</td>
<td>1.5 (1.4)*</td>
<td>1.5 (1.4)*</td>
</tr>
<tr>
<td>S(ALD), pg/ml</td>
<td>42.1 (31.6)</td>
<td>130.2 (63.1)*</td>
<td>130.2 (63.1)*</td>
</tr>
<tr>
<td>PRA ng/ml ANG/h</td>
<td>0.5 (0.3)</td>
<td>1.4 (0.3)*</td>
<td>1.4 (0.3)*</td>
</tr>
</tbody>
</table>

Data are presented as means (SD). Concentrations of serum 17β-estradiol (S(E2)), progesterone (S(P4)), total testosterone (S(T total)), free testosterone (S(T free)), sex hormone-binding globulin (S(SHBG)), and serum aldosterone (S(ALD)) and plasma concentration of endothelin-1 (P(ET-1)) and plasma renin activity (PRA). BL, baseline; GnRH, gonadotropin-releasing hormone; T, testosterone; ANG, angiotensin. *Different from control; †different from BL. Differences were considered significant at P < 0.05.

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reduced ΔCVC compared with saline infusion, whereas ET-A inhibition alone had little impact on ΔCVC compared with saline (P < 0.05; Fig. 3B).

DISCUSSION

In this investigation, we demonstrated that suppressing the chronic elevations of free testosterone in women with PCOS improved microvascular dilation. This improved microvascular response suggests that elevations in testosterone contribute to microvascular dysfunction in PCOS. These findings are of particular importance because we have demonstrated that changes in hormone exposure are seen in end organ cardiovascular effects in women, as measured by peripheral microvascular responsiveness. Furthermore, we demonstrated that the testosterone effects are mediated via both ET-A and ET-B receptors. In both men and women, ET-A and ET-B receptors are found on the vascular smooth muscle, where they mediate vasoconstriction, whereas only ET-B receptors are found on the endothelium, where they mediate vasodilation. Our data demonstrate that microvascular responses to local cutaneous warming were greater during ET-A inhibition compared with saline in both PCOS and control, indicating that ET-A receptors mediate vasoconstriction in the skin microcirculation as in other organs. However, ET-B receptor inhibition decreased blood flow in control women only (Fig. 3A), supporting our earlier findings demonstrating that women with PCOS exhibit lower ET-B receptor-mediated vasodilation (44) and indicating differential control of ET-1 in women with and without PCOS (44). We propose that the endothelial dysfunction observed in women with PCOS is related to altered responsiveness of the ET-B receptor subtype in the peripheral microcirculation. In contrast, it is clear that the ET-A receptor subtype plays an important role in ET-1-mediated peripheral vasoconstriction in both groups of women. Although testosterone suppression and administration did not impact blood concentration of ET-1, testosterone appeared to mediate blood flow through subtype receptors in the regulation of the cutaneous microcirculation. Interestingly, our data also demonstrate that testosterone effects on ET-1 regulation of the microcirculation are unaffected by short-term, mild testosterone administration. These findings have special significance for women with PCOS with chronic endogenous androgen exposure.

Microvascular Function

In this investigation, perfusion of cutaneous microdialysis probes with the ET-A receptor antagonist (BQ-123) enhanced heat-related vascular responsiveness in women in both groups similarly prior to hormone intervention, suggesting normal vascular smooth muscle function. However, ET-B receptor inhibition was associated with attenuated vasodilation in healthy controls but not in women with PCOS. These distinct findings indicate that PCOS alters ET-1 subtype receptor activity within the endothelin system, which in turn controls vascular tone. Our findings compliment earlier data demonstrating reduced vasodilation in the skin during acetylcholine administration in women with PCOS, indicating microvascular endothelial dysfunction in women with PCOS (29). We propose that downregulated ET-B receptors, or their sensitivity, on the endothelium contributes to ET-1-mediated changes in endothelial function in women with PCOS.

Prior to hormone administration, resting cutaneous vascular conductance was similar between the groups, suggesting that, despite differences in insulin resistance between the groups, resting basal microvascular dilation was similar between controls and PCOS. During local skin warming, the GnRH antagonist administration enhanced vasodilation in women with PCOS relative to baseline in our saline probe, suggesting that testosterone suppression may increase microvascular responsiveness in women with PCOS. Expressed another way, the chronically high levels of free testosterone in women with PCOS may contribute to their risk of endothelial dysfunction. Moreover, the GnRH suppression of testosterone eliminated the impact of ET-A receptor inhibition and enhanced that of ET-B receptor inhibition in both groups of women. Thus, testosterone exposure likely influences the ET-1 receptor subtype response. Importantly, estradiol exposure is also important to ET-1 vasomotor tone in the microvasculature, so there may be a number of other factors influencing the ET-1 effects we observed during GnRH antagonist administration, including changes in sex hormone binding globulin concentration. It is interesting to note that when reproductive hormones were controlled with the GnRH antagonist, responses to both ET-1 subtype receptors were similar between the two groups, emphasizing the importance of the hormonal milieu in influencing cardiovascular risk within women.
Short-term testosterone administration restored the increased vasodilation during ET-A receptor inhibition in controls but had little impact on warming-induced vasodilation during ET-A receptor inhibition in women with PCOS. In contrast, testosterone administration increased ET-B receptor-mediated vasodilation in both groups. Thus, microvascular responsiveness during local skin warming was preserved in PCOS during short-term testosterone administration and appeared to be mediated by ET-B receptors. Taken together, testosterone exposure effects on ET-1 endothelial regulation through the ET-B receptor result in vasodilation in the microcirculation, as suggested by earlier investigations (11, 13, 28).

Interestingly, the women with PCOS in our study were all insulin resistant, and testosterone exposure is fundamental to the development of endothelial dysfunction and hypertension in insulin-resistant rats (42, 43). We believe our findings demonstrate that testosterone plays a key role in the development of microvascular dysfunction in women with PCOS. The relationship between androgens and insulin resistance is a core component of PCOS, and serum free testosterone concentration was elevated in our PCOS subjects compared with controls.

### Fluid Regulatory Hormones

Our findings contribute to the accumulating evidence connecting PCOS with increased risk of cardiovascular disease (7, 9, 20). Consistent with previous findings, serum aldosterone concentration and plasma renin activity are enhanced in women with PCOS compared with controls, and these cardiovascular and sodium-regulating hormones may also contribute to greater cardiovascular risk in women with PCOS (4, 9).

Endothelial dysfunction can be reversed with anti-inflammatory drugs such as spironolactone, which improves flow-mediated vasodilation in women with PCOS (40), and can reduce inflammatory markers even in healthy patients (4). Spironolactone also blocks the proinflammatory actions of aldosterone and is also an androgen receptor antagonist (4). Progesterone and aldosterone show a strong predictive relation in the luteal phase of the menstrual cycle in women without PCOS and in women with PCOS who have a luteal phase, concomitant with a strong relation between androgens and aldosterone (4). Our study confirmed these findings with our GnRH antagonist model demonstrating that changes in serum aldosterone concentration and plasma renin activity were sensitive to the changes in hormone exposure in the women with PCOS. Moreover, testosterone administration was also associated with increased serum aldosterone concentration, suggesting a causal relation between androgens and aldosterone in women with PCOS. Thus, the combination of the proinflammatory actions of aldosterone with androgen effects on hormonal sodium regulation adds yet another dimension for hypertension risk for women with PCOS, even in women who are currently normotensive and still have normal endothelial function. Importantly, the changes in serum aldosterone concentration were independent of changes in progesterone, so they were not a response to progesterone-mediated diuresis.

In an earlier study, 25% of PCOS patients had high serum aldosterone concentration (>850 nmol/l) and a normal serum aldosterone/renin ratio, essentially excluding primary aldosteronism (4). The exact cause for the consistent finding of elevated serum aldosterone concentration in PCOS is not yet known, but others have hypothesized a genetic component (4), a result of adrenocorticotropic hormone actions (4), or greater aldosterone sensitivity to progesterone. A primary hypothesis is that the greater serum aldosterone is related to the insulin resistance associated with PCOS. Earlier studies have demonstrated direct relations between resting serum insulin and serum aldosterone concentration in women with PCOS (9), and both serum aldosterone concentration and plasma renin activity are reduced with metformin (12).

### Limitations

The limitations of our study include the relatively small number of subjects, which may have limited the power to examine all hormonal effects on the microcirculation. However, our sample size is similar to that of other published studies on women with PCOS; women with PCOS who are not taking hormonal or metabolic medications are challenging to recruit, and our power calculations indicate that the small sample can be used because of the low error in our outcome variables. As discussed earlier, PCOS is often associated with insulin resistance, and the subjects in our study with PCOS were also insulin resistant and obese. Thus, we cannot completely separate our findings from those of insulin resistance. However, although obesity and insulin resistance can contribute to cardiovascular disease, endothelial dysfunction in women with PCOS can also be independent of obesity and insulin resistance (8, 28), and there was a clear difference in serum free testosterone exposure between the control and PCOS groups. Both our control and PCOS groups were obese, suggesting that the present findings are not a sole function of obesity. Somewhat surprisingly, serum total testosterone concentration was not elevated in all of our PCOS subjects, and the serum total testosterone concentration change was mild during testosterone administration. Our data clearly demonstrated changes in sex hormone binding globulin and serum free testosterone concentrations as well as cutaneous microcirculatory changes related to our hormone suppression administration protocol. Thus serum total testosterone concentration may not be an adequate marker for changes in testosterone exposure when comparing young women with and without PCOS.

### Conclusions

Our study demonstrated that suppressing free testosterone improved microvascular dilation in women with PCOS and that transient testosterone administration did not diminish microvascular dilation. We also demonstrated that testosterone directs the actions of ET-1 on microvascular endothelial responsiveness in women with PCOS through both ET-A and ET-B receptors. Moreover, we have confirmed and strengthened previous findings regarding the important relationship between androgens and the proinflammatory effects of aldosterone in women with PCOS.

### Perspectives

Women with PCOS have a unique hormonal milieu that is often associated with infertility but also has important implications for metabolic and cardiovascular health. Women with PCOS are often insulin resistant, increasing their risk for developing cardiovascular disease, including endothelial dysfunction and hypertension. Our data demonstrate that andro-
gens contribute to their impaired vascular function because suppressing androgens in women with PCOS increased microvascular vasodilatory responses to heating via an ET-B receptor mechanism. These findings illustrate the important interaction between ovarian hormones, testosterone, and the endothelin system on cardiovascular function in women and identify a potential new target for treatment in women with PCOS.

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DISCLOSURES

The authors report no conflicts of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

M.M.W. performed the experiments; M.M.W. and N.S.S. analyzed the data; M.M.W. and N.S.S. interpreted the results of the experiments; M.M.W. drafted the manuscript; M.M.W., H.S.T., and N.S.S. edited and revised the manuscript; M.M.W., H.S.T., and N.S.S. approved the final version of the manuscript; M.M.W., H.S.T., and N.S.S. prepared the figures.

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