Gender Differences in the Renal Response to Renin-Angiotensin System Blockade

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Evidence suggests that gender differences exist in renin-angiotensin system (RAS) function. It was hypothesized that women may differ also in their response to RAS blockade. The renal and peripheral hemodynamic responses to incremental dosages of an angiotensin receptor blocker and the degree of angiotensin II (AngII) insensitivity achieved during 8 wk were examined in men and women. Participants were 30 young healthy men (n = 15; mean age 27 ± 2) and women (n = 15; mean age 28 ± 2) who were on a controlled sodium and protein diet for 1 wk before each study. The humoral, renal, and systemic response to incremental dosages of irbesartan (75 mg for 4 wk, then 150 mg for 4 wk) was assessed, as was the pressor response to AngII (3 ng/kg per min), at 2-wk intervals. AngII type 1 receptor expression in skin biopsies was assessed at baseline and after 8 wk by a real-time PCR protocol. Men and women both exhibited significant declines in BP. Women achieved significantly reduced AngII sensitivity compared with men at lower dosages, showing no pressor response at 4 wk of 75 mg/d irbesartan, whereas men continued to exhibit a pressor response at 4 wk of 150 mg/d. Receptor expression at baseline did not differ between men and women but by 8 wk was significantly decreased in women and unchanged in men. Our findings indicate that men may require larger dosages of angiotensin receptor blocker than do women and that the BP response cannot be used as a surrogate marker for adequate RAS blockade of the renal microvasculature.


Clinical and epidemiologic evidence suggests that women with renal disease progress more slowly to end stage in comparison with men (1). The mechanisms underlying this difference remain unknown, but evidence indicates that at least one explanation for this phenomenon is a physiologic gender-based difference in the function of the renin-angiotensin system (RAS). This system is an important mediator of renal and cardiovascular physiology and pathophysiology (2,3), and angiotensin II (AngII) is its principal effector. Studies from our laboratory have demonstrated distinct gender differences in RAS function (4–7). In vivo findings in the canine have shown that estrogen replacement after ovariectomy reduces AngII type 1 (AT1) receptor number in several tissues, including kidney (8). Studies in mouse models of vascular disease have suggested that estrogen and AngII receptor blockers (ARB) act synergistically on several outcomes, including atherosclerotic changes (9) and neointimal thickening after vascular injury (10).

Taken together, these experiments in the animal and human models suggest a possible synergistic effect between ARB and estrogen. We therefore examined AT1 receptor blockade by ARB in young, healthy men and women. It was hypothesized that such a synergistic effect would manifest in gender differences in the extent to which ARB administration results in decreased AngII sensitivity and reduced receptor expression (11,12). These experiments were conducted in young, healthy individuals who were on a controlled sodium and protein diet. Renal hemodynamic function was assessed using classic inulin and para-aminohippurate (PAH) clearance techniques. The extent of AT1 receptor blockade was assessed by three independent means: The response to an AngII challenge during treatment with irbesartan, an ARB that is used commonly in clinical practice; reactive changes in circulating levels of renin and AngII with irbesartan treatment; and the change in tissue AT1 receptor expression gleaned from skin biopsy tissue (13). Identical dosages were used in both men and women because these medications are not marketed as requiring dosage adjustment on the basis of body size or gender. The study was conducted during an 8-wk period, during which each participant was studied on five occasions.
Materials and Methods

Thirty normal, healthy men and women were recruited to participate in this study (15 men and 15 women). Their mean age was 27 ± 2 yr. Each participant underwent a detailed history and physical examination by a qualified internist. All were normotensive, nonobese, and nonsmokers. No participant was ingesting any regular medications, including oral contraceptives. The study was performed with the approval of the University Health Network Research Ethics Board and with the informed written consent of each participant.

All participants were counseled to adhere to a diet that maintained their normal caloric intake, their sodium intake to >2 mmol/kg per 24 h, and their protein intake to 1 to 1.5 g/kg per 24 h for 7 d before each phase of the study. A 24-h urine sample was collected to determine compliance with the diet, and data from participants were used only when the excretion rate of sodium was >2 mmol/kg per d and the urea excretion rate was between 3 and 6 mmol/kg per d. No data were excluded on this basis. Protein intake was estimated from the urine urea concentration using standard equations and was corrected for body weight. The participants were advised not to ingest any caffeine for 12 h before the study. On each day of the testing, the participants reported to the Renal Physiology Laboratory at the Toronto General Hospital. All studies were conducted at 8:30 a.m. with the participants lying supine in a warm, quiet room.

Study Protocol

On each study day, an 18-G peripheral venous cannula was inserted into an antecubital vein for infusions of inulin, PAH, and AngII, and another cannula was placed in the opposite arm for blood sampling. Each participant voided spontaneously before starting the study. Hemodynamic parameters (mean arterial pressure [MAP] and heart rate) were measured throughout the study by an automated sphygmomanometer (Dinamap, Criticon, Tampa, FL) and were recorded once in each half hour of the protocol. Renal hemodynamic function was measured using inulin and PAH clearance techniques. After collection of blood for inulin and PAH blank, hematocrit (HCT), 17β-estradiol (in women only), plasma renin concentration (PRC), and plasma AngII, a priming infusion that contained 25% inulin (60 mg/kg) and 20% PAH (8 mg/kg) was administered. Thereafter, inulin and PAH were infused continuously at a rate that was calculated to maintain their respective plasma concentrations constant at 20 and 1.5 mg/dl. Participants remained supine at all times. After a 90-min equilibration period and in each half hour for 90 min, blood was collected for inulin, PAH, and HCT. GFR and effective renal plasma flow (ERPF) were estimated by steady-state infusion of inulin and PAH according to the calculation method described by Schnurr et al. (14).

A solution of AngII (51.2 µg/vial; Clinalfa, Läufelfingen, Switzerland) was prepared by dissolving the diluent in 1 ml of normal saline to produce a concentration of 50 µg/ml. A total of 99 ml of normal saline then was added to 1 ml of AngII to produce a concentration of 500 ng/ml. AngII was infused at 3 ng/kg per min for 30 min. Participants remained supine. Blood was collected once at the end of the AngII infusion period for HCT, inulin, and PAH. An additional collection of blood for HCT, inulin, and PAH was obtained at the end of a 30-min recovery period.

Participants then were initiated on irbesartan, 75 mg once daily for a total of 4 wk. The experiments were repeated after 2 and 4 wk on this dosage. They then were initiated on irbesartan 150 mg once daily for a total of 4 wk. They were studied similarly at 2 and 4 wk on the increased dosage. In women, the baseline AngII infusion was administered during the luteal (high estrogen) phase of the menstrual cycle. Skin biopsies were obtained from 10 participants in each group on the first study day and on the last study day, under sterile conditions, after subcutaneous installation of local anesthetic with Xylocaine, as described previously (6).

Sample Collection and Analytic Methods

Blood samples that were collected for inulin and PAH determinations were centrifuged immediately at 3000 rpm for 15 min at 4°C. Plasma was separated, placed on ice, and then stored at –70°C before the assay. Inulin concentrations were measured by a modified method of Walser et al. (15), and PAH concentration was measured by a spectrophotometric method according to Brun (16). The mean of the two pre-AngII clearance periods represented baseline GFR and ERPF, expressed per 1.73 m². Filtration fraction (FF) represented the ratio of GFR to ERPF. Renal blood flow (RBF) was calculated by dividing the ERPF by (1 – HCT). Renal vascular resistance (RVR) was derived by dividing MAP by the RBF.

AngII was measured by RIA. Blood was collected into prechilled tubes that contained EDTA and angiotensinase inhibitor (0.1 ml of Bestatin Solution; Buhlmann Laboratories, Schonembuch, Switzerland). After centrifugation, plasma samples were stored at –70°C until analysis. On the day of analysis, plasma samples were extracted on phenylsilylsilica columns. A competitive RIA kit supplied by Buhlmann Laboratories was used to measure the extracted AngII. The detection limit of the assay was approximately 2.0 pg/ml, with a precision of 8.3% intra-assay variation and 9.0% interassay variation. PRC was measured by two-site immunoradiometric assay in which two mAb to human active renin are used. One antibody was coupled to biotin, and the second was radiolabeled for detection. The sample that contained active renin was incubated simultaneously with both antibodies to form a complex. The radioactivity of this complex was directly proportional to the amount of immunoreactive renin present in the sample (17). Plasma 17β-estradiol was determined by RIA using the DPC kit (Drug Products Corp., Los Angeles, CA).

Skin biopsies were stored in liquid nitrogen at –70°C before processing. AT₁ mRNA levels were assessed by a PCR protocol. RNA was isolated using an RNaseasy kit (Qiagen, Mississauga, ON, Canada), and 25 to 50 ng was used per reaction. Reverse transcriptase–PCR was performed using a One-Step RT-PCR kit (Applied Biosystems, Foster City, CA). One-step PCR was performed under the following conditions: 48°C for 30 min and 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s, using a real-time PCR protocol, the ABI PRISM 7000 Sequence Detection System. The human AT₁ receptor forward primer was 5′-AAATGGCTGGTTTATCTGAAT-3′, and the reverse primer was 5′-TTTTGATCACCTGGGTCGAATT-3′, corresponding to bp 67 to 90 and 152 to 173 of the human AT₁ receptor cDNA sequence, respectively. The final concentration of AT₁ primers was 0.5 µM. The human AT₁ probe was 5′-ACTCATGATGCATC-CCAGAAAATCG-3′, corresponding to bp 92 to 119 of the human cDNA sequence. The AT₁ receptor probe used a FAM dye with a TAMRA quencher at a final concentration of 0.2 µM. For internal control, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used, supplied by the manufacturer (TaqMan GAPDH Control reagents; Applied Biosystems), at a final concentration of 0.1 µM. The final concentration of human GAPDH probe was 0.05 µM. The GAPDH probe used a JOE dye and a TAMRA quencher, as per the manufacturer. Standard curves were developed with RNA that was obtained from human kidney cortex (0.4 to 50 ng). Experiments were performed in duplicate or triplicate and repeated at least once. Data are presented as the ratio of human AT₁ receptor mRNA/GAPDH mRNA, in arbitrary units (6).
Statistical Analyses

Results are presented as mean ± SE. Between-group baseline differences were determined using nonparametric methods (Wilcoxon rank sums). Within-subject and between-group differences in the longitudinal responses to irbesartan and the acute responses to AngII infusion were determined by repeated measures ANOVA and Bonferroni correction. Within-subject and between-group differences in AT₁ receptor expression were assessed using nonparametric methods. All statistical analyses were performed using the statistical package SAS (SAS Institute Inc., Cary, NC). *P ≤ 0.05 was designated to represent statistical significance.

Results

Baseline Parameters

Participants were similar in age, body mass index (BMI), urine sodium excretion, protein intake per kilogram per day, plasma AngII, and PRC (Table 1). A baseline significant difference was detected in body surface area (BSA), weight (Table 1), MAP, and RVR (Table 2).

Response to ARB

At 75 mg of irbesartan, women received 1.14 ± 0.04 mg/kg and men received 0.96 ± 0.05 mg/kg (P = 0.004); women received 43 ± 1 mg/m², and men received 38 ± 1 mg/m² (P = 0.005); women received 3 ± 0.1 mg/kg per m² and men received 3 ± 0.1 mg/kg per m² (P = 0.7). At 150 mg of irbesartan, women received 2.3 ± 0.08 mg/kg and men received 1.9 ± 0.08 mg/kg (P = 0.004); women received 87 ± 2 mg/m², and men received 76 ± 2 mg/m² (P = 0.0005); women received 6 ± 0.2 mg/kg per m² and men received 6 ± 0.2 mg/kg per m² (P = 0.7).

During the course of the study, MAP (Figure 1) and RVR declined significantly in men and women with no significant gender differences in the responses. There were no significant changes detected in GFR, ERPF, RBF, or FF over time (Table 2).

Evidence of AT₁ receptor blockade was provided by renin and AngII circulating levels (Figures 2 and 3). In women, baseline renin levels were 15 ± 5 in women (P = 0.03 versus baseline) and 50 ± 17 in men (P = 0.03 versus baseline and P = 0.3 versus response in women); AngII levels were 24 ± 5 in women (P = 0.02 versus baseline) and 18 ± 4 in men (P = 0.01 versus baseline and P = 0.5 versus response in women). After 4 wk of treatment with 75 mg, renin levels were 105 ± 34 in women (P = 0.002 versus baseline) and 47 ± 12 in men (P = 0.03 versus baseline and P = 0.02 versus response in women); AngII levels were 28 ± 4 in women (P = 0.02 versus baseline) and 15 ± 2 in men (P = 0.03 versus baseline and P = 0.04 versus response in women). After 2 wk of treatment with 150 mg, renin levels were 93 ± 30 in women (P = 0.001 versus baseline) and 55 ± 10 in men (P = 0.01 versus baseline and P = 0.02 versus response in women); AngII levels were 33 ± 7 in women (P = 0.003 versus baseline) and 18 ± 2 in men (P = 0.02 versus baseline and P = 0.02 versus response in women). After 4 wk of treatment with 150 mg, renin levels were 104 ± 28 in women (P = 0.001 versus baseline) and 100 ± 18 in men (P = 0.001 versus baseline and P = 0.5 versus response in women); AngII levels were 38 ± 8 in women (P = 0.003 versus baseline and 28 ± 4 in men (P = 0.001 versus baseline and P = 0.4 versus response in women).

Data from skin biopsy tissue are presented as the ratio of human AT₁ receptor mRNA/GAPDH mRNA, in arbitrary units. At baseline, women exhibited mRNA corrected for GAPDH signal equal to men at baseline (0.77 ± 0.16 versus 0.71 ± 0.15; P = 0.9). After 8 wk of treatment with irbesartan, women exhibited a significant decline in AT₁ receptor mRNA (0.26 ± 0.16; P = 0.04), whereas men exhibited no change (0.71 ± 0.14; P = 0.9; Figure 4).

Response to AngII during ARB Treatment

In the untreated phase of the study, no significant differences were detected between men and women in the response to AngII in MAP, GFR, ERPF, RBF, or FF over time (Table 3). After 2 wk of treatment with 75 mg of irbesartan, in response to AngII, men exhibited a significant increase in MAP (P = 0.04), whereas women exhibited no change (P = 0.87). Both groups demonstrated significant declines in the ERPF, RBF, and FF responses to AngII, although the responses were blunted significantly in women compared with men. After 4 wk of treatment with 75 mg of irbesartan, women exhibited a significantly blunted response to AngII, whereas men continued to demonstrate significant AngII-mediated changes in MAP, ERPF, RBF, and FF. After 2 wk of treatment with 150 mg of irbesartan, the response to AngII was abolished in women but not in men, who continued to demonstrate significant responses after 4 wk of treatment with 150 mg of irbesartan. The difference in response to AngII was significant at 4 wk of treatment with 75 mg of irbesartan and at 2 wk of treatment with 150 mg of irbesartan.

Discussion

Studies from this laboratory previously demonstrated that gender-mediated differences exist in RAS function between men and women, with women exhibiting a significantly blunted response to AngII (7), and between women who are users and nonusers of oral contraceptive medications (5,6). Emerging epidemiologic evi-
Evidence suggests that women and men also may differ in the disease-modifying response to angiotensin-converting enzyme (ACE) inhibition (18). The aim of this study was to examine whether the physiologic and molecular responses to RAS blockade differ between men and women and to assess whether any gender differences exist in the degree of AT1 receptor blockade proffered by ARB as estimated by three independent methods: (1) MAP and renal response to infused AngII, (2) the reactive increase in circulating RAS components (this phenomenon is caused by interruption of the negative feedback exerted by AngII on renin secretion and represents a valid method of assessing the intensity and the duration of AT1 receptor blockade [19–23]), and (3) the decline in AT1 receptor expression.

Our major finding was that the response to AngII infusion was abolished in women at low dosages of irbesartan after 4 wk, whereas AngII insensitivity never was achieved in men, even after 4 wk of 150 mg. Similarly, in women, renin and AngII plasma levels were elevated maximally by 4 wk of low-dosage irbesartan, whereas in men, levels approached those in women only at 4 wk of 150 mg/d irbesartan. Furthermore, AT1 receptor mRNA expression showed no gender difference at baseline, but after 8 wk of irbesartan therapy, expression levels were significantly decreased in women but unchanged in men (Figure 4).

Reasons for this gender difference in susceptibility to AT1 receptor blockade are not readily obvious from this protocol. It seems unlikely that this phenomenon could have occurred by

### Table 2. Renal response to ARB over timea

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>2 wk 75 mg</th>
<th>4 wk 75 mg</th>
<th>2 wk 150 mg</th>
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<tr>
<td><strong>MAP (mmHg)</strong></td>
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<td>80 ± 1</td>
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<td>76 ± 2b</td>
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<td>74 ± 1b</td>
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<td>124 ± 6</td>
<td>116 ± 5</td>
<td>118 ± 5</td>
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<td>108 ± 8</td>
<td>118 ± 7</td>
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<td><strong>ERPF (ml/min per 1.73 m²)</strong></td>
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<td>743 ± 48</td>
<td>759 ± 57</td>
<td>718 ± 37</td>
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<td>623 ± 37</td>
<td>649 ± 52</td>
<td>670 ± 55</td>
<td>684 ± 47</td>
<td>628 ± 32</td>
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<td><strong>RBF (ml/min per 1.73 m²)</strong></td>
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<tr>
<td>male</td>
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<td>1233 ± 79</td>
<td>1247 ± 88</td>
<td>1172 ± 55</td>
<td>1184 ± 74</td>
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<td>1005 ± 82</td>
<td>1032 ± 88</td>
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*aARB, AngII receptor blocker; ERPF, effective renal plasma flow; FF, filtration fraction; MAP, mean arterial pressure; RBF, renal blood flow; RVR, renal vascular resistance.

bP < 0.05 versus baseline value.

cP < 0.05 versus baseline value in men.

Figure 1. Mean arterial pressure at baseline and in response to incremental angiotensin II (AngII) receptor blockers (ARB) in men and women at 75 mg at 2 and 4 wk and at 150 mg at 2 and 4 wk. *P < 0.05 versus baseline.

Figure 2. Plasma renin concentration at baseline and in response to incremental ARB in men and women at 75 mg at 2 and 4 wk and at 150 mg at 2 and 4 wk. *P < 0.05 versus baseline; #P < 0.05 versus response in men.
chance, given the degree of significance achieved and the parallel results provided by the three independent methods that were used to assess blockade. Lack of compliance in men also seems unlikely because the longitudinal MAP response was appropriate in both men and women, and circulating RAS components did eventually rise in men, although only at larger dosages. The gender difference cannot be attributed to variations in sodium or protein intake, because these parameters were similar in men and women after correction for body weight. It is evident that women received higher dosages than did men when corrected for BSA and weight but not BMI. However, further examination reveals that AngII insensitivity was achieved in women at dosages that were significantly lower than those required by men, when factored by BSA and weight, suggesting that body size alone cannot explain the results. It is important to reiterate that these medications are not marketed to be dosed by body weight, BSA, or BMI.

Sex hormones may explain this phenomenon (24). Studies in animal models have demonstrated that estrogen and RAS blockade may act synergistically to downregulate the AT1 receptor. In a set of experiments in a polyethylene cuff-induced vascular injury mouse model, Liu et al. (10) studied male, female intact, and female ovariectomized mice that were treated with estrogen alone, olmesartan alone, and estrogen and olmesartan in combination. Neointimal thickening was significantly less in intact female than in male mice, differences that were abolished by ovariectomy. Low-dosage olmesartan inhibited neointimal thickening only in female mice. Co-administration of estrogen and olmesartan to ovariectomized mice synergistically inhibited neointima formation and DNA synthesis. Similar results were obtained by Tsuda et al. (9) in ovariec-
tomized mice that were fed a high-cholesterol diet, wherein co-administration of estrogen and olmesartan improved atherosclerosis without changing plasma cholesterol levels. This gender difference also seems to be relevant to humans who receive ACE inhibition. In the Ramipril Efficacy in Nephropathy (REIN) study, the investigators noted a significantly augmented renal survival and antiproteinuric impact of ramipril in women compared with men (18).

The AT1 receptor expression results deserve comment. It has been shown in experimental models that ARB therapy can reduce AT1 receptor mRNA expression (11,12). The mechanism in unclear but could be due to ARB-mediated increases in circulating AngII augmenting the formation of Ang 1 to 7 (25) or activating AT2 receptors (26). Although this study cannot clarify the mechanism, it is clear that, similar to other measures of adequacy of RAS blockade, women exhibited an augmented response. It is plausible that RAS blockade and estrogen acted synergistically to decrease mRNA receptor expression. In an in vivo study by Owonikoko et al. (8) in an ovariec-
tomized canine model, the effect of estrogen on AT1 receptor expression was assessed with noninvasive positron emission tomography imaging techniques. This revealed that estrogen replacement after ovariectomy resulted in decreased AT1 receptor ligand binding in the renal cortex and the adrenal gland compared with the estrogen-depleted state. Therefore, estrogen alone may have affected AT1 receptor expression; however, this is unlikely because at the time of both skin biopsies, women were in the luteal phase, and estrogen levels did not differ. In this set of experiments, the AT1 receptor expression results correlate well with the physiologic responses, as in a previous study from this laboratory (6). Taken together, these two observations suggest that AT1 receptor mRNA levels are associated with responsiveness to AngII and AngII blockade.

Another finding from our study deserves comment. Previous studies (27,28) revealed that there were no clinically significant differences in irbesartan pharmacokinetics between men and women, and, indeed, during the course of the study, the irbe-
sartan-mediated reduction in MAP was similar in both groups. However, the striking differences in AngII sensitivity between ARB-treated men and women indicate that the MAP response to ARB cannot be used as an indication of blockade of the renal microvasculature. The mechanism for the further decline in BP values cannot be determined from this protocol but may relate to the ongoing natriuretic effect of ARB therapy, resulting in a negative sodium balance.

This study has several limitations. First, the study sample was not from a target patient population in that participants were selected for normal arterial pressure, renal function, and urinalysis. By studying a healthy sample, we hoped to examine the impact of gender, without confounding factors. Because this study provides physiologic evidence for gender differences in the response to RAS blockade, it could serve as a starting point for a similar study in patients with renal disease. A second limitation was the study length. Although evidence exists to suggest that ARB achieve blood levels rapidly and exert their antiproteinuric and vasodilatory effects within the time frame of this study (29), it is possible that further blockade of the RAS would have occurred if the study duration were longer. A third limitation was the dosage of irbesartan used. In a study by Ribstein et al. (30), the systolic BP response to AngII infusion was assessed before and after treatment with varying dosages of irbesartan. The authors found that the pressor response was blocked completely at peak drug levels with dosages of 150 and 300 mg and that the pressor inhibitory effect of irbesartan was dosage dependent. This suggests that if higher dosages had
been used in the men in our study, then full blockade may have been achieved. Importantly, enrollment in the Ribstein study was restricted to normotensive, lean, white men who were between 18 and 35 yr of age. The possibility that women may require smaller dosages to achieve full blockade was not addressed. Last, the sample size of the study was small as a result of the practical difficulties of recruiting and studying healthy individuals in such a detailed and complex manner. We attempted to minimize the effect of the small sample size by having homogeneous study groups. We ensured that participants were ingesting similar amounts of salt and protein to minimize RAS activation, that no female participant was ingesting oral contraceptive medications, and that all female participants were studied in the same phase of the menstrual cycle at each point in the protocol. Perhaps because of this careful prestudy preparation, the sample size was large enough to show significant findings that are biologically plausible and in keeping with previous animal and human data.

**Conclusion**

Normotensive men and women with normal renal function respond differently to RAS blockade with irbesartan, at the level of both the peripheral vasculature and the renal microvasculature. Our findings also indicate that men may require larger dosages of ARB than do women and that the BP response cannot be used as a surrogate marker for adequate RAS blockade of the renal microvasculature. Although mechanisms that are responsible for the protective effect of female gender on progression of renal disease were not addressed, increased sensitivity to modulation of the RAS as observed in these studies could account in part for the different rates of progression in women.

**Acknowledgments**

This work was supported by a New Emerging Team grant from the Canadian Institutes of Health Research (to J.A.M., D.C.C., and J.W.S.) and grants from the Physicians’ Services Foundation Inc. (to J.A.M. and D.C.C.) and the Heart and Stroke Foundation of Canada (to J.A.M.; grant NA 5008). D.Z.C. received funding from the KRESCENT program and the University of Toronto Clinician Scientist Program.

We acknowledge Clinalfa for providing AngII and PAH for these experiments.

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