CHAPTER 2 | PART ONE

GENDER DIFFERENCES IN RESPONSE TO ACUTE AND CHRONIC ANGIOTENSIN II INFUSION: A TRANSLATIONAL APPROACH

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ABSTRACT

Women with renal disease progress to end stage at a slower rate than men do. As angiotensin II has known hemodynamic and direct renal effects, we hypothesized that the female protection may result from sex differences in responses to angiotensin II. Therefore, we studied sex differences in the response to angiotensin II, during acute (human) and chronic (rats) and ex-vivo angiotensin II administration.

In young healthy men (n=18) and women (n=18) we studied the responses of renal hemodynamics ($^{125}$I-iothalamate and $^{131}$I-Hippuran) and blood pressure to graded angiotensin II infusion (0.3, 1.0, and 3.0 ng/kg/min for 1h). Men showed increased responses of diastolic blood pressure (p=0.01), mean arterial pressure (p=0.05), and a more pronounced decrease in effective renal plasma flow (p=0.009) than women.

We also studied the effect of chronic angiotensin II administration (200ng/kg/min for 3 weeks) on the kidney in Wistar rats. Males had an increased response of proteinuria to chronic angiotensin II infusion than females (GEE-analysis, p=0.001). Male, but not female, angiotensin II treated rats showed an increased numbers of renal interstitial macrophages as compared to sham treated rats (p<0.001). In vascular reactivity measurements in aortic rings, females showed an enhanced angiotensin II type 2 receptor-mediated relaxation compared with males (p=0.03).

In conclusion, sex differences are present in the response to acute and chronic infusion of angiotensin II. Thus chronic infusion of angiotensin II results in an increased blood pressure and proteinuria response in male healthy rats. This difference in angiotensin II sensitivity could play a role in sex differences in progression of renal disease.
INTRODUCTION

Women generally have a lower risk for developing cardiovascular disease (CVD) and chronic kidney disease (CKD) than men. They also progress slower to end stage renal disease (ESRD) after a renal insult. The renin-angiotensin aldosterone system (RAAS), with angiotensin II (ang II) as a principal effector, is an important mediator of renal and cardiovascular physiology and pathophysiology with both hemodynamic effects and pro-inflammatory and pro-fibrotic effects. Thus, differences in responsiveness to ang II might provide a possible explanation for the sex difference in progression to renal disease. Indeed, in the last few years, sex differences in the components and regulation of the RAAS have been recognized to be of functional importance.

Elucidation of such sex differences is important, since this may translate into sex specific treatment and subsequently to better outcomes in men and women. This suggestion is in accordance with the evidence suggesting sex differences in the efficacy and effects of anti-hypertensive medication in general and more specifically in RAAS blockade. In cardiovascular studies, women appear to be less responsive to angiotensin-converting enzyme inhibition than men (ACE-i), while proteinuric women responded better to treatment with ACE-i than men. Men require larger doses of ang II type 1 receptor (AT1-R) blocker to achieve the same blood pressuring lowering effect compared with women. These results illustrate the importance of a better understanding of the effect of sex differences in the RAAS.

Human sex differences in RAAS function have also been observed in responsiveness to ang II infusion. Two studies in healthy normotensive men and women showed differences in the renal responsiveness to graded ang II infusion, whereas blood pressure responses were similar, pointing towards specific renal differences between men and women. Both studies, however, did not study women at a fixed time-point of the menstrual period. It is well known that this may be of great influence on both the RAAS and renal hemodynamics. Indeed, in their paper, Miller et al. showed that baseline characteristics for blood pressure, effective renal plasma flow (ERPF), renal blood flow (RBF), filtration fraction (FF) and renal vascular resistance (RVR) are affected by 17ß-estradiol concentrations. Moreover, they also showed that 17ß-estradiol concentrations affected the response of ERPF and aldosterone to ang II infusion. Therefore, it is important to standardize the time-point of the menstrual cycle in small physiological studies.

To substantiate, however, the relevance of differences in the RAAS for the susceptibility to chronic renal damage, it is crucial to demonstrate differences in responsiveness to chronic ang II administration as well. This is cumbersome to study in humans. Available animal data on ang II infusion so far are concordant with the acute human data, showing a greater increase in blood pressure response to ang II infusion in males compared with females. Whether, the renal effects of chronic ang II infusion are also sex-dependent has not been studied.

Therefore, in the present translational study, we combined human and animal experiments to investigate sex differences in the response to ang II. In healthy young men and women, we studied the responses of blood pressure and renal hemodynamics to acute graded ang II infusion, on standardized sodium intake and measurements.
during the mid-follicular phase. The chronic renal effects of ang II were studied in healthy young male and female rats. We measured blood pressure, proteinuria and renal morphological markers of pro-inflammatory pathways and fibrosis after three weeks of chronic ang II infusion. Finally, to dissect the role of the different ang II receptor subtypes, in vitro vascular ang II responses were tested in isolated aortic rings of healthy male and female Wistar rats.

**MATERIALS AND METHODS**

**Human experiments**

**Study population**

The study population was recruited from the ongoing Groningen Renal Hemodynamic Cohorts (GRECO) program. The GRECO program hosts and harmonizes studies on renal hemodynamics at the renal function unit of the University Medical Center Groningen, by standardization of methodology, including harmonization of study protocols and calibration over time. This allows combined analyses in subjects from different sub studies. The current study population consisted of 36 healthy, Caucasian subjects (women, n=18; men, n=18). Female subjects were studied in the RETAP project and we compared these women with male subjects from the Gene-Environment project. For the parameters used in the present study, the protocols were prospectively designed to be identical in design and measurements. All subjects were non-smokers and normotensive, having a sitting systolic blood pressure < 140 mmHg and diastolic blood pressure < 90 mmHg measured by an automatic sphygmomanometer (Dinamap, GE Medical Systems, Milwaukee, Wisconsin, USA) and were not treated with antihypertensive medication. Their medical history revealed no significant diseases. Subjects with obesity (BMI>30 kg/m² at screening) were excluded. Physical examination and electrocardiography did not reveal any abnormalities. None of the women were users of oral contraceptive medication. Both studies were approved by the local medical ethical committee, (METc number: RETAP study 2010/294, Gene-Environment study 2001/012) and all subjects gave written informed consent in accordance with the Declaration of Helsinki. The RETAP study was registered in the Netherlands National Trial Register (www.trialregister.nl; trial registration number: 2635) as RESpome To Angiotensin II in formerly Preeclamptomatic women (RETAP) study.

**Study protocol**

The study protocol consisted of a 7-day period on a standardized sodium diet (aim: 200 mmol Na⁺/day). For assessment of dietary compliance and the achievement of a stable sodium balance, 24 h urine was collected at day 3 and day 6. As both renal hemodynamics and ang II responsiveness are greatly influenced by female sex hormones, all measurements in females were performed during the mid-follicular phase (day 7±2 of menstrual cycle). At day 7, the subjects reported at the research unit at 8am after an overnight fast. Body weight, length and waist-to-hip ratio were
measured at the start of this day. Intravenous cannulas were inserted into both forearms, one for drawing blood samples, the other for infusion of ang II. Subjects received standardized meals and fluids during the day, with sodium intake adjusted to the prescribed diet. To ensure sufficient urine output, infusion of 250 mL/h of 5% glucose was administered and every hour 250 mL of oral fluids were provided.

Glomerular filtration rate (GFR) and ERPF were measured from the clearance of constantly infused radio-labeled tracers, $^{125}$I-iothalamate and $^{131}$I-Hippuran, respectively, in semi-supine position in a quiet room. After a blank blood sample was drawn, a priming solution containing 0.04 ml/kg body weight (0.04 MBq of $^{125}$I-iothalamate and 0.03 MBq of $^{131}$I-Hippuran per milliliter saline) plus an extra bolus of 0.06 MBq of $^{125}$I-iothalamate was given, followed by constant infusion at 12 mL/h of the same solutions. After a 2h stabilization period, stable plasma concentrations of both tracers were attained, and the clearance periods started. The clearances were calculated using the formula $U*V/P$ for GFR and $I*V/P$ for ERPF. $U*V$ represents the urinary excretion of the tracer, $I*V$ represents the infusion rate of the tracer, and $P$ represents the tracer value in plasma at the end of each clearance period. The urinary clearance of $^{125}$I-iothalamate was multiplied by the ratio of plasma-to-urinary clearance of $^{131}$I-hippuran to correct for voiding errors.

GFR and ERPF were indexed for body surface area (BSA), by dividing the raw sample by BSA and multiplying it with 1.73 m$^2$. BSA was calculated according to the DuBois-DuBois formula.

Blood pressure and heart rate were measured by using an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA). The appropriate blood pressure cuff was determined on the basis of arm circumference. Mean arterial pressure (MAP) was calculated as diastolic pressure plus one-third of the pulse pressure.

Baseline values for blood pressure, GFR and ERPF were obtained from 10am to 12pm at 15 min intervals. Between 12pm and 3pm ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously, at a constant rate in doses of 0.3, 1 and 3 ng/kg/min each during 1h. During these ang II infusions blood pressure was measured at 5-min intervals.

Urine samples were drawn from the 24h-urine collected by all subjects. The level of urinary sodium, potassium and urea were assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel).

Animal experiments

**Experimental set up**

Experiments were conducted under protocols approved by the Animal Ethical Committee of the University of Groningen. Four months old female (n=25) and male (n=19) Wistar rats (Harlan Inc, Horst, the Netherlands) were kept in a 12-hour light-dark cycle and constant room temperature, with food and water freely available in the home cages. Both female and male rats were divided in two groups: one sham-pump treated group (control; female, n=14; male, n=10), and one group receiving ang II infusion continuously via an osmotic minipump (female, n=11; male, n=9). Amounts of ang II infusion were similar between males and females, i.e. we infused...
ang II with a rate of 200μg/min/kg (ang II in saline containing 0.01 N acetic acid). For minipump implantation rats were anesthetized with isoflurane and oxygen and minipumps were implanted intraperitoneally (Alzet, Cupertino, CA, model 2004). In all rats, blood pressure and proteinuria were measured at baseline (i.e. before minipump implantation) and at weekly intervals before and after pump implantation (day -1 (i.e. day before pump implantation), 7, 14, and 21). Subsequently, after three weeks of infusion (day 21), rats were sacrificed and left kidney and the thoracic aorta were collected. Parts of the kidney were harvested and were fixed in 4% paraformaldehyde in PBS or snap frozen.

**Measurement of blood pressure**

Blood pressure was measured using an indirect tail-cuff plethysmographic method with a blood pressure monitor (Apollo 179; IITC Life Science, Woodland Hills, California, USA). All rats were conscious during the measurements. In order to reduce spontaneous variation in blood pressure, rats were extensively trained for a period of four weeks on a daily basis to get used to the tail-cuff method. Prior to blood pressure readings, rats were optimally warmed using a warmth lamp to induce vasodilation of the tail vein. Readings were repeated ten times and after excluding the lowest value, the average of the lowest three values for systolic blood pressure were used for further analysis.

**Blood and urine sampling and analysis**

Twenty-four-hour urine samples were collected from all rats. Therefore, rats were placed in metabolic cages on days -1, 7, 14 and 21. Urinary concentration of protein (Pyrogallol Red – Molybdate Complex) was determined as previously described and 24-hour excretion rates were calculated. A blood sample was taken at sacrifice in a pre-cooled EDTA tube (day 21) and immediately centrifuged at 4°C, 956g for 10 min. Plasma was subsequently stored at -80°C until analysis. Urinary and plasma creatinine concentrations were determined (CREA plus, cobas, Roche Modular, Basel) from samples collected on day 21 and creatinine clearance was calculated according to the standard formula ((urinary creatinine (mmol)*1000)/plasma creatinine (µmol)*(urine volume/1440)).

**Immunohistochemistry**

After PFA fixation of renal tissue for 24 hr, and 70% alcohol for at least 24 hr, tissue was processed for paraffin embedding according to standard methods. For immunohistochemistry, 2 μm sections were cut. Total macrophages/monocytes (ED-1; 1:100 diluted, AbD Serotec, Düsseldorf, Germany) and CD206-positive macrophages (representing type 1 macrophages; 1:1000 diluted; Abcam, Cambridge, UK) as previously described. Numbers of interstitial ED-1 and CD206 positive cells were determined by manually analysing 30 randomly selected cortical fields per kidney (40x magnification), excluding fields with glomeruli. For each cortical field, the number of positive cells was counted.

The pre-fibrotic marker for myofibroblast transformation, alpha-smooth muscle actin (α-SMA), was detected using a murine monoclonal antibody (α-SMA; clone 1A4; Sigma) as previously described. Sections stained for α-SMA were scanned
using an Aperio ScanScope CS and analyzed with Aperio ImageScope v10.2.2.2319 (Aperio, Vista, CA, USA). The ‘Positive pixel Count V9’ algorithm was used to analyse α-SMA-positive pixels in 30 randomly selected cortical fields per kidney after excluding vessels and glomeruli. The positive surface area (number of positive pixels), was divided by the total area of the field measured, providing a number of α-SMA positive pixels corrected for the area analyzed. Researchers were blinded for group allocation of the rats while analysing the kidney slides.

**Gene-expression analysis**

Total kidney RNA from homogenized renal cortex and homogenized aorta was isolated with Trizol Reagent (Invitrogen) following manufacturer’s instructions. Total RNA was quantified using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). cDNA synthesis was performed as described before. Real time RT-PCR was performed using Lightcycler 480 (Roche, Applied Science) and Applied Biosystems reagents according to the manufacturer’s instructions. Expression levels were normalized to those of 18S ribosomal RNA, which was analysed in separate runs. Primers and probes for the angiotensin II type 1 (AT1-R) and the angiotensin II type 2 (AT2-R) receptors were obtained from Applied Biosystems (TaqMan Gene Expression Assays, AT1-R: Rn00578456_m1 and AT2-R: Rn00560677_m1). The sequence for 18S (M11188) (sense primer, antisense primer, and probe, respectively; all from 5’ to 3’) was: CGGCTACCACATCCAAGGA, CCAATTACAGGGCCTCGAAA, CGCGCAAATTACCCACTCCGA.

**Aortic ring contraction experiment**

Immediately following sacrifice, the thoracic aortas of the sham treated males and females were used to measure the ex-vivo sensitivity to ang II using standard isotonic contraction experiments as previously described. Aortic rings (2mm) from the rats were kept in Krebs solution (at 37°C) and aerated with 95% CO₂ and 5% O₂. Krebs buffer was freshly made before the start of each experiment and contained in mmol/L: 120 NaCl, 5.9 potassium chloride (KCl), 25.2 NaHCO₃, 1.2 NaH₂PO₄, 10.4 glucose, 1.21 MgCl₂·6H₂O, and 2.52 CaCl₂. All Krebs ingredients were purchased from E. Merck (Darmstadt, Germany). The aortic rings were equilibrated for 30 minutes before they were primed and checked for viability by evoking a contraction with KCl (60mM) for 10 minutes.

Six rings from each rat were used to study 1: ang II induced contraction (2 rings), 2: ang II induced contraction via the AT1-R (2 rings) and 3: ang II induced relaxation via the AT2-R (2 rings) according to standard methods.

1: To study the ang II sensitivity per se, a cumulative ang II (Bachem AG, Bubendorf, Switzerland) concentration-response curve (10⁻⁸M-10⁻⁶M) was obtained. 10⁻⁵M phenylephrine (Sigma, St. Louis, MO, USA) was added after completing the ang II concentration-response curve to assess total aortic ring contraction. The ang II-mediated contraction was then expressed as a percentage of the maximum contraction after 10⁻⁵M phenylephrine.

2: The functional response of the AT1-R to ang II was studied after incubation with 10⁻⁶M PD-123319 (AT2-R antagonist; Park-Davis, Michigan USA) and the selective nitric oxide (NO) synthase inhibitor N⁶-nitro-L-arginine methyl ester (L-NMMA) (10⁻⁴M; Calbiochem Brand of EMD Biosciences, Inc., La Jolla) to prevent any
confounding effects by the basal release of NO. Both compounds were present during the entire experiment. Then, a cumulative ang II concentration-response curve ($10^{-10}$M-$10^{-6}$M) was obtained, followed by $10^{-5}$M phenylephrine, as described above. The ang II-mediated contraction was then expressed as a percentage of the maximum contraction after $10^{-5}$M phenylephrine.

3: The functional response of the AT2-R to ang II was studied after incubation with $10^{-5}$M losartan (AT1-R antagonist; Merck Research laboratories, Rahway, USA). After pre-contraction with $10^{-6}$M phenylephrine, the cumulative ang II concentration-response curve was obtained. The ang II-mediated relaxation was then expressed as a percentage of the maximum pre-contraction with phenylephrine.

Data analysis and power analysis

Statistical analysis was performed using SPSS for Windows (Version 20.0). Parametric data are presented as mean ± standard deviation (SD) or mean ± standard error of the mean (SEM) in text, table and figures and analyzed using Student $t$-test. Nonparametric data are presented as median (25$^{th}$-75$^{th}$ percentile) and analyzed using Mann Whitney-U test or Kruskal Wallis test.

The responses to ang II infusion in humans (blood pressure and renal hemodynamics) and rats (blood pressure and proteinuria) were analyzed by generalized estimating equations (GEE) analysis. Variables with a skewed distribution were log-transformed to fulfill criteria for GEE analysis. Statistical significance was accepted at $p \leq 0.05$.

RESULTS

Human study

Baseline characteristics

Baseline characteristics of the female and male subjects are shown in table 1. Age was not different between both groups. Men had a significantly higher height and body weight compared to women. Also BMI and waist-to-hip ratio were not different between both groups. No significant differences were found in urinary sodium, potassium or urea excretion between both groups, reflecting equal sodium, potassium and protein intake between the groups during the dietary week. At baseline, men had a significantly higher systolic blood pressure than women, but no differences were found in diastolic blood pressure and MAP between both groups. Men had a significantly lower heart rate compared to women. With regards to renal hemodynamics, at baseline, no differences in GFR were found between both groups, but men had a significantly higher ERPF compared with women.
Table 1. Baseline characteristics of human subjects

<table>
<thead>
<tr>
<th></th>
<th>Female (n = 18)</th>
<th>Male (n = 18)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>36 ± 5</td>
<td>31 ± 11</td>
<td>0.092</td>
</tr>
<tr>
<td>Height, m</td>
<td>171 ± 5</td>
<td>184 ± 6</td>
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</tr>
<tr>
<td>Body weight, kg</td>
<td>68.0 ± 8.4</td>
<td>78.7 ± 8.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.2 ± 2.7</td>
<td>23.2 ± 2.2</td>
<td>0.969</td>
</tr>
<tr>
<td>Waist/Hip ratio</td>
<td>0.83 ± 0.04</td>
<td>0.85 ± 0.08</td>
<td>0.397</td>
</tr>
<tr>
<td>Urinary sodium, mmol/24h</td>
<td>221 ± 63</td>
<td>200 ± 69</td>
<td>0.356</td>
</tr>
<tr>
<td>Urinary potassium, mmol/24h</td>
<td>80 ± 34</td>
<td>68 ± 22</td>
<td>0.267</td>
</tr>
<tr>
<td>Urinary urea, mmol/24h</td>
<td>339 ± 89</td>
<td>383 ± 82</td>
<td>0.132</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>115 ± 8</td>
<td>124 ± 12</td>
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</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>71 ± 8</td>
<td>73 ± 8</td>
<td>0.403</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>85 ± 8</td>
<td>90 ± 8</td>
<td>0.098</td>
</tr>
<tr>
<td>Heart rate, p/min</td>
<td>67 ± 8</td>
<td>57 ± 8</td>
<td>0.001</td>
</tr>
<tr>
<td>GFR, mL/min/1.73m²</td>
<td>109 ± 15</td>
<td>111 ± 14</td>
<td>0.693</td>
</tr>
<tr>
<td>ERPF, mL/min/1.73m²</td>
<td>380 ± 69</td>
<td>493 ± 78</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index; GFR, glomerular filtration rate; ERPF, effective renal plasma flow. Data are expressed as mean ± SD or median (25th-75th percentile).

Figure 1: Mean (± SD) systolic blood pressure (A), diastolic blood pressure (B) and mean arterial pressure during (C) acute angiotensin II infusion in females (open squares) and males (closed squares). Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. * significantly different from baseline (student t-test), p < 0.05. # significantly different from females after infusion of the same dose of angiotensin II (student t-test), p < 0.05. ** curves of males and females significantly different (GEE analysis), p < 0.05.

Blood pressure response to acute ang II infusion in men and women

The responses of blood pressure to increasing doses of ang II infusion in men and women are shown in figure 1. It is shown that the lowest dose, 0.3 ng/kg/min was a non-pressor dose, with even a significant decrease in diastolic blood pressure compared to baseline in women. Significant dose-dependent increases occurred during 1.0 and 3.0 ng/kg/min in both groups. However, during several infusion doses, men had
higher systolic blood pressure (baseline, 0.3, 1.0 and 3.0 ng/kg/min), diastolic blood pressure (0.3 and 1.0 ng/kg/min) and MAP (0.3, 1.0 and 3.0 ng/kg/min) compared with women. Analyzing the dose response curve as a whole using GEE analysis indicated that the responses, albeit small, were statistically significant different for diastolic blood pressure (p=0.01) and MAP (p=0.05). After correction for baseline value, sex difference in MAP responses became borderline significant (p=0.08), however, a significant sex difference in diastolic blood pressure response to ang II was still present (p=0.01).

**Renal hemodynamic response to acute ang II infusion in men and women**

Figure 2 shows the responses of GFR (panel A) and ERPF (panel B) during ang II infusion in men and women. Women responded to ang II infusion by a significant decline in GFR during the 1.0 ng/kg/min and 3.0 ng/kg/min infusion rate, while men responded to ang II by maintaining GFR at all infusion doses. At dose 3.0 ng/kg/min, GFR was significantly lower in women than in men (p=0.001). Analyzing the dose response curves as a whole using GEE analysis indicated that the response was significantly different between males and females for GFR (p=0.01; corrected for baseline values).

ERPF significantly and progressively decreased during increasing doses of ang II in men and women. Analyzing the dose response curve as a whole using GEE analysis, indicated that the response was significantly different between males and females for ERPF (p=0.009; corrected for baseline value).

**Figure 2**: Mean (± SD) glomerular filtration rate (A) and effective renal plasma flow (B) during acute angiotensin II infusion in females (open squares) and males (closed squares). Abbreviations: GFR, glomerular filtration rate; ERPF, effective renal plasma flow; ang II, angiotensin II.* significantly different from baseline (student t-test), p<0.05. # significantly different from females after infusion of the same dose of angiotensin II (student t-test), p<0.05.

** curves of males and females significantly different (GEE analysis), p<0.05.
Animals were randomly divided into two groups: untreated control group (sham) and ang II treated group. The body weight was recorded at the beginning of the study and at weekly intervals. At the end of the study, the rats were sacrificed under isoflurane anesthesia, and the kidneys were removed and weighed. The right kidney was used for measuring proteinuria, while the left kidney was used for further analysis.

Analysis of kidney function showed a significant increase in albuminuria in the ang II treated group compared to the sham group (p<0.05). Moreover, the systolic blood pressure was significantly higher in the ang II treated group compared to the sham group (p<0.01). There was no significant difference in creatinine clearance between the two groups (p>0.05).

Histological analysis revealed a significant increase in the number of macrophages in the kidney sections of the ang II treated group compared to the sham group (p<0.01). The expression of α-SMA, a marker of interstitial fibrosis, was also significantly increased in the ang II treated group (p<0.01).

In conclusion, the administration of ang II in rats resulted in a significant increase in albuminuria, systolic blood pressure, and histological evidence of interstitial fibrosis. These findings suggest that ang II plays a significant role in the pathogenesis of renal damage in rats.

Reference:
II infusion was shown as compared to the sham treated groups. However, after ang II infusion no differences were found between female and male rats. There was a strong relationship between αSMA and blood pressure at 3 weeks ($R^2=0.282$ of beta=0.531, p=0.013).

**Figure 3:** Median (with 25th-75th percentile) systolic blood pressure (A) and proteinuria (B) during chronic angiotensin II infusion in female (open squares) and male (black squares) rats; * significantly different from baseline (Mann-Whitney U test), p≤0.05. # significantly different from females (Mann-Whitney U test), p≤0.05. ** curves of males and females significantly different (GEE analysis: interaction between angiotensin II proteinuria response and sex).

**Figure 4:** Median (interquartile range) numbers of ED-1 positive cells (A) and α-smooth muscle actin expression (B) in the interstitium of the kidney of female rats and male rats without chronic angiotensin II infusion (white bars) and with chronic angiotensin II infusion (black bars). Abbreviations: Ang II, angiotensin II. * p≤0.05 (Mann-Whitney U test).
**AT1 and AT2-receptor mRNA**

AT1-R and AT2-R expression in kidney and aorta were evaluated (figure 5). No difference in mRNA expression for AT1-R in the kidney was found between sham treated female and male rats, nor did we observe an effect of ang II treatment on AT1-R expression in the kidney. The mRNA expression for AT2-R in the kidney, however, is higher in female rats compared with male rats, with again no effect of ang II treatment. No differences in mRNA expression for AT1-R and AT2-R in the aorta were found between sham treated female and male rats, nor did we observe an effect of ang II treatment on vascular AT1- and AT2-R expression.

**Figure 5:** Median (with 25th and 75th percentile) angiotensin 1 receptor and angiotensin 2 receptor mRNA expression in the kidney (A and B) and the aorta (C and D) of female and male rats without chronic angiotensin II infusion (white bars) and with chronic angiotensin II infusion (black bars). Abbreviations: ang II, angiotensin II; AT1-R, angiotensin II type 1 receptor; AT2-R, angiotensin II type 2 receptor. * p≤0.05 (Mann-Whitney U test).
Ex-vivo vasoreactivity to ang II in isolated aortic rings of rats

The above-mentioned results show differences in AT2-R expression between males and females and may suggest differences in vascular responses to ang II between males and females. We tested this hypothesis using the vasoactive response of the thoracic aorta to ang II in the presence or absence of an AT1-R blocker or an AT2-R blocker. Figure 6A-6C represent the ang II contraction curve (A), the ang II contraction curve mediated by the AT1-R (B) and the ang II dilatation curve mediated by the AT2-R (C) for males and females. Figure 6D shows the area under the curve (AUC) of the cumulative concentration-response curves to ang II under the three conditions. No differences were found in ang II induced contraction per se and ang II induced contraction via the AT1-R between males and females. However, we found AT2-R mediated relaxation upon ang II in females, but not in males.

Figure 6: Ex-vivo angiotensin II responses in aortic vessels of female and male rats. Angiotensin II contraction (A and D), AT1-R-mediated contraction (B and D), AT2-R-mediated relaxation to ang II (C and D). Data are expressed as mean and SEM (A-D) and area under the curve (D). Abbreviations: Ang II: angiotensin II; AT1-R: angiotensin II type 1 receptor; AT2-R: angiotensin II type 2 receptor. * p<0.05 (Student t-test).
DISCUSSION

In this translational study, we found sex differences in the response to ang II infusion in both healthy human subjects and in healthy rats. In humans, men showed an increased blood pressure and ERPF response, and a slightly decreased GFR response to acute ang II infusion compared with women. We showed that similar chronic ang II infusion resulted in a higher blood pressure in males compared with females, which confirms previous studies. However, we also showed an increased proteinuria response to this chronic ang II infusion in male rats compared with female rats. This was previously only shown in spontaneous hypertensive rats (SHR). The increased ang II response in males was associated with more inflammation in the kidney compared with female rats and with a decreased vascular relaxation response upon the AT2-R in males. It may be speculated that our data point towards an increased ang II sensitivity in men, which may be involved in the higher risk for renal damage in males on the long term.

Our human data showed a higher blood pressure response to acute ang II infusion in men compared with women. These results differ from those from Miller et al and Gandhi et al, which might be due to differences in study protocol and patient selection. We feel that the main difference between our study and those of Miller and Gandhi is that we standardized measurements by phase of the menstrual cycle, as the latter can considerably affect RAAS-activity. We found in both men and women the expected renal hemodynamic response to ang II infusion, namely a decrease in ERPF. This response is due to renal vasoconstriction, mainly in the efferent arteriole. However, the ERPF response is more pronounced in men compared with women. A greater decrease in ERPF in males may reflect more available AT1-R due to an upregulation in a less active intrarenal RAAS system. For GFR, the response was more pronounced in women than in men, since women responded to ang II by showing a significant decline in GFR, whereas men responded by maintaining GFR. This finding is consistent with the results of Miller et al; it suggests that the afferent arteriole is more sensitive to ang II stimulation in women as compared with men. This could be a protective mechanism of the female glomerulus to prevent glomerular hypertension. It indicates a different balance between afferent and efferent vasoconstriction in response to ang II between men and women.

To evaluate the effect of chronic ang II infusion, we studied the renal damage and blood pressure response to three weeks of similar ang II infusion in male and female rats. In accordance with the acute effects of ang II on blood pressure in our human study and with results of other rat studies, we observed a more rapid increase of blood pressure to ang II infusion in male compared with female rats. Male rats also showed higher proteinuria at baseline and an increased proteinuria response after similar chronic ang II infusion compared with female rats. This suggests an increased sensitivity of the proteinuria response to ang II in males compared with females. To our knowledge, this is the first study showing this sex difference in proteinuria response to chronic infusion of ang II in healthy rats. Sullivan et al have shown this in SHR rats. Others have shown that transgenic rats bearing the mouse Ren2 gene have sex dependent differences in proteinuria, with higher protein excretion in males compared to females. This, however, appeared to be due to higher levels of ang II in
male transgenic rats compared with female transgenic rats, rather than with higher ang II sensitivity. Furthermore, a sex dependent effect on development of proteinuria has been shown in spontaneously hypertensive rats.\textsuperscript{28} Our data thus now show that the sex dependency of ang II induced proteinuria also occurs after chronic infusion of ang II in healthy rats. The reason for the sex difference in proteinuria is not completely understood. Several mechanisms could be involved: it seems likely that hemodynamic effects are involved, since the ang II-induced blood pressure increase was more severe in males compared with females in our study. This may cause an increased pressure-induced injury in males compared with females.\textsuperscript{32}

Also inflammatory processes and/or fibrosis may be involved in the sex differences in ang II-induced proteinuria.\textsuperscript{33} Interstitial macrophage influx differed between males and females after 3 weeks of ang II infusion; we observed increased numbers of macrophages 3 weeks after ang II infusion only in males. The increased numbers of interstitial macrophages could either be the cause or consequence of the increased protein excretion. Excessive tubular reabsorption of protein results in tubulo-interstitial infiltration of inflammatory cells, especially monocytes.\textsuperscript{33} On the other hand, macrophages themselves have also been shown to induce kidney damage.\textsuperscript{34} Thus our data suggest a possible ang II-induced vicious circle of renal damage, which might be more prominent in males.

Despite the higher proteinuria in males, there were little interstitial profibrotic changes in the kidney, as measured by αSMA. These changes also did not differ between males and females. In the present study, the small pro-fibrotic damage after 3 weeks of ang II infusion appeared to be blood pressure related since there was a strong relationship between αSMA and blood pressure. As there was no difference in blood pressure after 3 weeks ang II treatment between males and females, this may also explain why we did not detect a sex difference in pro-fibrotic kidney damage. However, we cannot exclude that longer infusion of ang II or higher dosis of ang II would result in increased sex differences in proteinuria and blood pressure and would also induce sex differences in pro-fibrotic markers. This should be subject of further research.

Differences in responsiveness to ang II between males and females could be due to difference in expression of the AT1-R and AT2-R and in the balance between the two. Although in the kidney the mRNA expression for the AT1-R did not differ between males and females, AT2-R mRNA was higher in female rats compared with male rats. Aortic mRNA levels of AT1-R and AT2-R did not show any differences. This increased expression of the AT2-R in females is in line with studies of the group of Denton et al, who suggested that the AT2-R plays a role in the decreased responsiveness to ang II of females.\textsuperscript{3,12,35-37} Indeed, the results of our ex-vivo aorta experiments confirmed this suggestion; we found that the relaxation response to ang II via the AT2-R was higher in females compared with males. The isolated aortic rings provide a well-established read-out for vascular pharmacological responses, the uncertainty of the generalizability to other vascular beds (i.e peripheral resistance vessels, or the renal vascular bed), is a limitation inherent to this set-up. It may be speculated that such changes in the AT2-R in the aorta may play a role in the regulation of blood pressure by influencing vascular smooth muscle tone and therefore aortic stiffness and thus central blood pressure. However, to further substantiate the role of the AT2-R in sex difference
in the blood pressure or renal response upon ang II, our data need to be confirmed in studies employing preparations of other vessels - such as small resistance arteries or vessels of the renal vascular bed.

Despite the increased response to ang II of the AT2-R in the aorta of females, and in contrast to the kidney, we did not find an increased AT2-R mRNA in the aorta. Differences in translation, function or the downstream pathway of the AT2-R could be involved. It should be noted here that mRNA expression of the AT1-R and AT2-R might not directly relate to changes in protein levels and receptor activity. Although it is recognized that the AT1-R is mainly regulated by post-transcriptional regulation, such as mRNA stability and alternative splicing, it has also been shown that the AT1-R can be internalized and shed from the surface. Similar posttranslational and transcriptional mechanisms may apply for the AT2-R, although this remains to be established.

As pointed out we infused ang II for only three weeks. This resulted in relatively little profibrotic damage and proteinuria, while we did not observe renal damage (as characterized by glomerular sclerosis - data not shown). The question arises whether prolonged infusion or a combination of ang II infusion with another hit (eg nephrectomy) does induce (differenced) renal damage in male and female rats. Moreover, the present experiments (both human and rats) were performed in young adults. Therefore, the differences observed in the RAAS in the present study may not be relevant to older individuals, such as older men and postmenopausal women.

In conclusion, in both humans and rats, males seem to have a higher vascular and renal sensitivity to ang II compared to females. In response to acute ang II infusion, men have a stronger rise in blood pressure and a stronger ERPF response, but a decreased GFR response than women. In rats, during chronic ang II infusion, pressor responses are also larger in males compared with females, and are associated with a higher response of proteinuria and intrarenal inflammation in males compared with females. This study showed that differences in function of the RAAS between males and females, including differences in presence and function of the AT2-R, could be an explanation for the sex differences in development of renal disease. Further research is needed to elucidate these sex differences in RAAS regulation, which might contribute to a better view on the differences in renal risk profile and treatment recommendations between men and women.

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REFERENCES


