Chapter 6

Angiotensin II sensitivity after preeclampsia: Translational data from early-onset human preeclampsia and experimental preeclampsia

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Submitted
ABSTRACT

Formerly preeclamptic women have an increased risk for cardiovascular and renal diseases later in life. Whether this is due to preeclampsia itself or co-morbid conditions is unknown. Our aim was to establish, in a translational approach, whether the increased angiotensin II sensitivity in preeclampsia persists postpartum and whether preeclampsia itself plays a role. In 18 formerly healthy early-onset preeclamptic and 18 formerly healthy pregnant women (controls) baseline blood pressure and responses to angiotensin II (0.3, 1.0, and 3.0ng/kg/min) were measured. Simultaneously, in never-pregnant, formerly healthy pregnant, and formerly experimental preeclamptic rats, angiotensin II sensitivity was studied; in-vivo by measuring changes in blood pressure and proteinuria during angiotensin II infusion with osmotic minipumps (200ng/kg/min), and ex-vivo by vascular reactivity measurements. In humans, no difference in baseline mean arterial blood pressure was seen (85 ± 8 vs 86 ± 9; p=0.71), while 1.0ng/kg/min angiotensin II showed a trend towards an increased response in formerly preeclamptic women vs controls (p=0.057). In rats, chronic infusion of angiotensin II showed a trend towards a larger rise in systolic blood pressure (69.4% vs 53.8%; p=0.068) and a significantly higher systolic blood pressure at termination in formerly preeclamptic vs never-pregnant rats (159.5 ± 29.5 vs 136.7 ± 16.8; p=0.046). In response to angiotensin II, there was a significant increase in proteinuria in formerly preeclamptic rats only. In conclusion, healthy formerly early-onset preeclamptic women and formerly experimental preeclamptic rats exhibit subtle persistent increased angiotensin II sensitivity. Together, these data support a role for preeclampsia itself in altered angiotensin II sensitivity postpartum.
INTRODUCTION

Preeclampsia is a pregnancy specific syndrome, clinically characterized by the presence of hypertension and proteinuria in the second half of pregnancy\textsuperscript{1}. It is a leading cause of maternal and perinatal mortality. Women that suffered from preeclampsia, especially early-onset preeclampsia, have an increased risk for cardiovascular- and renal diseases later on in life\textsuperscript{2-5}.

The common hypothesis is that pre-existing vascular and metabolic risk factors cause both preeclampsia and later cardiovascular and renal disease\textsuperscript{6}. Data, however, from the Hunt-cohort showed that only approximately 50\% of the increased risk after preeclampsia could be explained by common risk factors\textsuperscript{7}. Indeed, it has been demonstrated that preeclampsia itself could lead to kidney and endothelial damage since in a large cohort familial aggregation did not explain the increased risk for end-stage renal disease (ESRD)\textsuperscript{8}. These studies suggest that preeclampsia itself may also contribute to the increased risk to develop cardiovascular and renal damage after preeclampsia. However, studies above are epidemiological studies, which are not optimal to answer the question whether preeclampsia itself contributes to the increased vascular risk.

Mechanistic studies in formerly preeclamptic women have shown that the increased angiotensin II (ang II) sensitivity, which is present during preeclampsia\textsuperscript{9}, persisted after preeclampsia; albeit more subtle\textsuperscript{10,11}. However, the formerly preeclamptic women in these studies had multiple comorbidities, such as hypertension and obesity, which makes it difficult to discriminate between the effect of common risk factors and the effect of preeclampsia itself. Furthermore, sodium intake, menstrual cycle period and time-after pregnancy were not completely standardized in these studies and may have been of influence.

We hypothesized that preeclampsia itself may play a role in the persistent increased ang II sensitivity. Therefore, we investigated blood pressure response upon ang II infusion in healthy, normotensive, formerly early-onset preeclamptic women, selected for absence of comorbidity and without signs of underlying diseases. Furthermore, we performed a rat study; to test the hypothesis that preeclampsia itself may induce persistent increased ang II sensitivity. We used healthy rats in which we induced preeclampsia by infusion of low dose lipopolysaccharide (LPS)\textsuperscript{12} during pregnancy, and measured blood pressure and renal responses to chronic ang II infusion as well as ex-vivo ang II sensitivity in the aorta postpartum.

MATERIALS AND METHODS

Human study

Study population
The study was approved by the local medical ethical committee (METc-number: 2010/294) and all women gave written informed consent in accordance with the Declaration of Helsinki. The study was registered in the Netherlands National Trial Register (www.trialregister.nl; trial register number: 2635) as REsponse To Angiotensin II in formerly Preeclamptic women (RETAP) study.
Thirty-six healthy, normotensive postpartum Caucasian women were studied at the University Medical Center Groningen (UMCG); 18 healthy women with a history of early-onset preeclampsia (fPE-women) and 18 healthy women with a history of uncomplicated, normotensive pregnancy (fHP-women) were used as a control group. 264 women with a history of early-onset preeclampsia one to ten years ago were selected from an electronic delivery database of the UMCG Obstetrics department. Medical records were reviewed for accuracy of diagnosis of preeclampsia, which was defined according to the definition of the International Society for the Study of Hypertension in Pregnancy 13. Early-onset preeclampsia was defined as developing preeclampsia before 34 weeks of gestation. Women with a history of renal disease, using any antihypertensive medication, with a BMI>30 kg/m² at screening, with diabetes or a history of gestational diabetes were excluded. Also current pregnancy, current lactation, being post-menopausal, and use of oral contraception 14, 15 were used as exclusion criteria.

224 early-onset fPE-women were invited by mail to participate in the study. In total, 24 of these women were willing to participate and were invited for a screening visit to the UMCG. After the screening visit, one woman was excluded for using antihypertensive medication and one woman was using hormonal suppletion which could not be temporarily stopped, one woman was excluded because of hypertension measured during the screening visit, and 3 women were excluded for other reasons (pregnancy, time-consuming protocol, post-menopausal). Each of the remaining fPE-women was matched for age and year of index pregnancy (within one year) with a parous control whose pregnancy had been uncomplicated and normotensive. These fHP-women were recruited either through the department’s electronic delivery database or recruited amongst hospital/department employees and their family members. Their records were evaluated to confirm that their pregnancy was indeed uneventful. Exclusion criteria as described above for the cases were applied. All subjects were non-smokers and normotensive at screening, having a sitting systolic blood pressure (SBP) <140mmHg and diastolic blood pressure (DBP) <90mmHg measured by Dinamap (the average of three measurements was taken). Physical examination and electrocardiography did not reveal any abnormalities.

**Study protocol**

In the week prior to the measurements, women were asked to use a sodium standardized diet (aim: 200 mmol Na+/24-hour urine) starting from day one of their menstrual cycle. To assess dietary compliance, 24-hour urine was collected at day 3 and day 6 of the dietary week and results were discussed with all women. During the last day of the dietary week, blood pressure was measured during a period of 24-hours by ambulatory blood pressure measurement (ABPM; Spacelabs Healthcare). The cuff was placed around the non-dominant arm at the brachial level. The recorders were programmed to measure blood pressure at a 20-min interval during daytime and at an hourly interval during nighttime (10pm till 6am). Women were asked to fill out a diary during this 24-hour to differentiate between day- and nighttime measurements and to correct for intense exercise afterwards.
At the study day, women reported at the research unit at 8.00am after an overnight fast. Body weight was measured at the start of this day. An intravenous cannula was inserted into each forearm, one for drawing blood samples, the other for infusion of ang II. During the measurements, women were sitting in semi-supine position in a quiet room. All women received standardized meals and fluids during the day. Blood pressure and heart rate were measured by the use of an automated sphygmomanometer (Dinamap; GE Medical Systems, Milwaukee, Wisconsin, USA) at 15-min intervals. Mean arterial pressure (MAP) was calculated as 2-times DBP plus SBP divided by 3. Baseline values for blood pressure were obtained from 10.00am to 12.00pm. Between 12.00pm and 3.00pm ang II (Clinalfa, Merck Biosciences AG, Läufelfingen, Switzerland) was administered intravenously, at a constant rate in doses of 0.3, 1.0, and 3.0 ng/kg/min each during 1-hour. During the ang II infusions blood pressure was measured at 5-min intervals.

**Blood and urine sampling and analysis**

Fasting blood samples were drawn for analysis of hematocrit (Ht), glucose, glycated hemoglobin (HbA1C), insulin, and creatinine. Measurements were performed by the use of an automated clinical chemistry analyzer (Roche Modular; Sysmex hematology analyzer (for Ht) and Sysmex Tosoh G8 (for HbA1C)). Fasting serum insulin was determined by an automated immunoassay analyzer (Architect, Abbott). Homeostasis model assessment (HOMA) was calculated by: (glucose x insulin)/22.5. Blood samples for baseline assessments (non-fasting samples) were drawn at 10.00am. These plasma samples were collected in pre-cooled tubes and immediately centrifuged at 4°C, 956g for 10 min and subsequently stored at -80°C until analysis of aldosterone and plasma renin activity (PRA). Aldosterone was measured with a commercially available radioimmunoassay kit (coat a count RIA, Siemens). PRA was measured with a radioimmunoassay that detects the amount of angiotensin I produced per hour in the presence of excess endogenous angiotensigen (nanograms of angiotensin I produced per liter of plasma per hour; CisBio International, France). To analyze auto-antibodies against the angiotensin II type I receptor (AT1-AA) IgG fractions for the neonatal cardiac contraction assay were prepared and isolation and cultivation of the neonatal heart cells were performed as described previously 16. Using the chronotropic responses to AT1-R mediated stimulation of cultured neonatal rat cardiomyocytes coupled with receptor-specific antagonists the AT1-AA were detected 17.

Urine samples were drawn from the 24-hour urine collected at day 6 of the dietary week by all women. The levels of sodium, creatinine and albumin were assessed by the use of an automated clinical chemistry analyzer (Roche Modular Basel). The creatinine clearance was then calculated using the formula: (urinary creatinine/24h*1000)/plasma creatinine. The estimated glomerular filtration rate (eGFR) was calculated using the CKD-epi formula 18. This creatinine-based equation has been shown to accurately estimate the GFR in a healthy population. As some study subjects were still slightly menstruating during the 24-hour urine collections, these samples were not suitable for albuminuria measurement. Therefore, to test for albuminuria, a random morning urine sample was collected after completion of the study, at a point in time where subjects were certain not to menstruate to exclude confounding by admixture of blood.
Power analysis
This study was originally designed to assess both blood pressure and renal response to ang II after low and high sodium diet in formerly preeclamptic women compared to healthy controls. The cross-over design of the study with several main end-points resulted in a multivariate power calculation. In the multivariate power calculation 3 factors (response to ang II, low and high sodium diet and control group vs. formerly preeclamptic women) and 2 confounders were taken into account. We calculated the total number of participants by \( n = 10 \times 5 \) (3 factors and 2 confounders). Therefore, 25 subjects per group were needed. Due to the low incidence of early-onset preeclampsia and the intensiveness of the study protocol, we were not able to include 25 women per group in our hospital. However, after including 18 women per group and performing an interim analysis, we found a significant difference on our renal function end point between both groups (not shown in this paper). Therefore, we decided to stop including at \( n = 18 \) after an interim-analysis. Since we describe our secondary endpoint in this paper, as an explorative study, no separate power analysis was performed.

Data analysis
Statistical analysis was performed using SPSS for Windows (Version 20.0) on a standard computer. Parametric data are presented as mean ± standard deviation (SD) in text, table and figures, unless otherwise stated and analyzed using Student t-test. Nonparametric data are presented as median (25th-75th percentile) and analyzed using Mann-Whitney U. Chi-square was used to analyze the AT1-AA and Pearson Correlation to test the correlation of AT1-AA with time since index pregnancy. Univariate linear regression was performed to test whether BMI was of influence on the differences found. Blood pressure responses upon the different ang II infusion steps was analyzed using the general estimating equations (GEE-analyses; mixed model analysis). Baseline blood pressure, BMI and an interaction term (group (fHP and fPE) * ang II infusion step) were entered as covariates, and exchangeable was chosen as correlation matrix. GEE-analyses was also performed to test whether the presence of AT1-AA and “time since index pregnancy” was of influence on the ang II sensitivity. In all cases, differences were considered significant if \( p < 0.05 \).

Animal study

Animals
Experiments were conducted under protocols approved by the Animal Ethical Committee of the University of Groningen. 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. Until selection for experiments vaginal smears were taken daily. Rats were rendered pregnant by housing them on pro-oestrous with fertile males for one night. Day 0 of pregnancy was documented by the presence of spermatozoa in the vaginal smear. Subsequently, pregnant rats were randomly allocated into two groups; healthy pregnant rats (HP-rats, \( n = 19 \)) or experimental preeclamptic rats (PE-rats, \( n = 22 \)). As controls, rats that have never been pregnant were used (NP-rats, \( n = 25 \)).
All rats were equipped with a permanent cannula inserted into the right jugular vein under isoflurane/oxygen anesthesia on day 0 of pregnancy, according to standard methods. The permanent jugular vein cannula allows stress free infusion of lipopolysacharide (LPS; E-Coli, 0.55: BS, Whittaker MA Bioproducts, Walkerville, Md.) or saline.

**Experimental set up**

At day 14 of pregnancy, rats received a 1-hour low dose LPS infusion or a 1-hour saline infusion. Non-pregnant rats received saline infusion 2 weeks after cannulation. To study the long-term effects of experimental preeclampsia the following experimental set-up was chosen. Six weeks postpartum and nine weeks after cannulation in NP-rats, baseline values for blood pressure and proteinuria were assessed. Then, in each group of rats, 50% of the rats received ang II infusion continuously via an osmotic minipump, whereas the other 50% received a sham pump for three weeks. Blood pressure, proteinuria and creatinine clearance (only after 3 weeks) were measured weekly during this infusion period in all rats. Subsequently, after three weeks of infusion rats were sacrificed and kidneys and aortas were collected. Kidneys were checked for kidney inflammation (macrophages influx) and kidney damage (deposition of αSMA). To evaluate whether the in-vivo effect of ang II was due to increased vascular responsiveness to ang II, the aortas of the rats with sham pumps were used for aortic contraction experiments.

**Induction of experimental preeclampsia**

On day 14 of pregnancy pregnant rats randomized for the experimental preeclampsia group were infused with LPS (1µg/kg bw in 2 ml saline in 1-hour) according to standard methods; rats allocated to the healthy pregnant group were infused with only saline (2 ml in 1-hour). Non-pregnant rats received saline infusion according to the same protocol. After delivery, pups were immediately dissociated from their mothers to avoid an effect of lactation. The number of alive and dead pups was counted and weight and length of the pups that were alive, i.e. moving at time of measurements, were measured. Two weeks after delivery, the permanent jugular vein cannulas were surgically removed by a small incision under isoflurane/oxygen anesthesia.

**In-vivo ang II infusion and sacrifice of the rats**

Six weeks after delivery, the NP-rats, the fHP-rats, and the fPE-rats were further randomly divided in a control group (c-NP, n=12, c-fHP, n=10, and c-fPE, n=11) receiving sham pumpsintraperitoneally (ip) for 3 weeks and a group receiving an osmotic minipump with ang II ip (infusion rate: 200ng/min/kg in saline containing 0.01 N acetic acid; A-NP, n=13; A-fHP, n=9; and A-fPE, n=11; Alzet, Cupertino, CA, model 2004) for 3 weeks. Blood pressure and proteinuria were measured weekly. Three weeks after pump implantation, rats were anesthetized and aortic blood pressure (see below) was measured before termination by heart puncture. After termination, the left kidney was harvested and parts of the kidney were placed in 4% paraformaldehyde in PBS or snap frozen. The thoracic aorta was also harvested. Small parts of the thoracic aorta were fixed in 4% paraformaldehyde or snap frozen. Since we were interested in the effect of former preeclampsia on the ex-vivo vascular
reactivity, without confounding by prior chronic ang II infusion, we used the thoracic aortas of the rats treated with sham pumps for vascular reactivity experiments.

**Measurement of blood pressure**

Blood pressure was measured at baseline (i.e. 6 weeks after delivery) and weekly for 3 weeks during sham or ang II infusion using an indirect tail-cuff plethysmographic method with a rat tail 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. 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 remaining values for SBP was used for further analysis. In addition, blood pressure was measured at termination (i.e. after 21-days of infusion) using a bed-side monitor (Datex-Ohmeda, Cardiocap/5). Rats were anesthetized with isoflurane/oxygen and the abdominal cavity was opened (100% O₂, 0.8mL/min, 5% isoflurane for induction followed by 2%). A catheter was inserted in the abdominal aorta and the blood pressure was noted after 20 seconds of recording.

**Blood and urine sampling and analysis**

Rats from all groups were placed in metabolic cages for 24-hour urine collection prior to implantation of the osmotic minipumps, to assess baseline proteinuria. After pump implantation, proteinuria was measured weekly for 3 weeks. Urinary concentrations of protein (Pyrogallol Red – Molybdate Complex) were determined as previously described and 24-hour excretion rates were calculated. To determine creatinine, a blood sample was collected in a pre-cooled tube at termination (day 21) and immediately centrifuged at 4°C, 956g for 10min and 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 paraformaldehyde fixation, renal tissue was processed for paraffin embedding according to standard methods. For immunohistochemistry, 2µm sections were cut. Total macrophages/monocytes (ED-1) and M2-like macrophages were identified by staining for CD68 (1:100 diluted, clone ED1, AbD Serotec, Düsseldorf, Germany) and CD206 (1:1000 diluted; Abcam, Cambridge, UK) respectively according to standard methods 21. The pre-fibrotic marker for myofibroblast transformation alpha-smooth muscle actin (αSMA) was detected using a murine monoclonal antibody (αSMA; clone 1A4; Sigma) as previously described 22.
Morphometric analysis of immunohistochemical staining
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 in a view. For each cortical field, the number of positive cells was counted. Sections stained for αSMA 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 αSMA-positive pixels after excluding vessels. The total positive surface area of all fields, was divided by the total area of all fields measured, providing a number of αSMA positive pixels corrected for the area analyzed. Researchers were blinded for group allocation of the rats while analysing the kidney slides.

mRNA expression analysis
Total aortic and kidney RNA 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 23. Real time quantitative 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 AT1-R and AT2-R were obtained from Applied Biosystems (TaqMan Gene Expression Assays, AT1-R: Rn00578456_m1 and AT2-R: Rn00560677_m1). The sequences for 18S (M11188) (sense primer, antisense primer, and probe, respectively; all from 5’ to 3’) were: CGGCTACCACATCCAAGGA, CCAATTACAGGGCCTCGAAA, CGCGCAAATTACCCACTCCCGA.

Protein expression analysis
Kidney lysates (20 µg per lane) were run on 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidine difluoride membranes. Membranes were rinsed with tris buffered saline (TBS) containing 0.05% Tween 20 (TBST) then blocked with TBST containing 5% bovine serum albumin (BSA) for 1-hour at room temperature (RT). Blots were then incubated with primary antibodies (AT1-R: ab124734 (Abcam), AT2-R: Mab 3659 (R&D systems, Inc, Minneapolis)) diluted in TBST containing 1% BSA overnight at 4 ºC. The blots were then rinsed with TBST (3 times) for 5 min at RT and incubated with the appropriate secondary antibody (Polyclonal Goat anti Rabbit HRP; P0448 (Dako Netherlands bv) diluted in TBST containing 1% non-fat dry milk and 2% normal rat serum for 1-hour at RT. The blots were rinsed another 3 times for 5 min with TBST before visualization was done by enhanced chemiluminescence (ECL), according to standard procedures with a molecular imager g gelodi XR system from Bio-Rad (Bio-Rad Laboratories, Inc). After that, blots were stripped by incubating in 25 mM Glycine, 2% SDS, (pH 6.7) for 30 min at RT. Stripped blots were then rinsed extensively with TBST and reprobed as described above with a β Actin antibody (SC-47778 (Santa-Cruz Biotechnology, Inc.). Using the expression of β-Actin as reference, protein bands were quantified with Image-lab 4.0.1 from Bio-Rad. The ratio of AT1-R or AT2-R to β-Actin was calculated.
Chapter 6

Aortic ring contraction experiment

Drugs and chemicals

Krebs buffer was freshly made before the start of each experiment and contained in mmol/L: 120 natrium chloride (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 stock solutions for phenylephrine (Sigma, St. Louis, MO, USA), ang II (Bachem AG, Bubendorf, Switzerland), PD-123319 (Park-Davis), Losartan (Merck Research laboratories, Rahway, USA), and N⁵-nitro-L-arginine methyl ester (L-NMMA; Calbiochem Brand of EMD Biosciences, Inc., La Jolla) were prepared in saline (0.9%NaCl in distilled water).

Ex-vivo aortic reactivity to ang II

The ex-vivo sensitivity to ang II in aortic tissue was studied in isolated aortic rings of the c-NP, c-fHP, and c-fPE-rats, using standard isotonic contraction experiments as previously described 24. Aortic rings (2mm) from the rats were kept in Krebs solution (at 37°C) and aerated with 95% CO₂ and 5% O₂. 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 twice. To study the ang II sensitivity per se, a cumulative ang II concentration-response curve (10⁻¹⁰M-10⁻⁶M) was obtained. 10⁻⁵M phenylephrine 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. Response to ang II via the ang II type 1 (AT1-R) and/or type 2 receptor (AT2-R) were studied as described previously 24. In short functional response of the AT1-R to ang II was studied after incubation with 10⁻⁶M PD-123319 (AT2-R antagonist) and selective nitric oxide (NO) synthase inhibitor L-NMMA (10⁻⁴M) to prevent any confounding effects by the basal release of NO 25. Then, a cumulative ang II concentration-response curve (10⁻¹⁰M-10⁻⁶M) was obtained according to standard methods 26. 10⁻⁵M phenylephrine 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. The functional response of the AT2-R to ang II was studied after incubation with 10⁻⁵M losartan (AT1-R antagonist). After pre-contraction with 10⁻⁴M phenylephrine, the cumulative ang II concentration-response curve (10⁻¹⁰M-10⁻⁴M) was obtained. The ang II-mediated relaxation was then expressed as a percentage of the maximum pre-contraction with phenylephrine.

Data analysis

Statistical analysis was performed using SPSS for Windows (Version 20.0) and area under the curve (AUC) was calculated using GraphPad Prism 5, on a standard computer. Parametric data are presented as mean ± standard deviation (SD) in text, table and figures, unless otherwise stated and analyzed using Student t-test. Nonparametric data are presented as median (25th-75th percentile) and analyzed using Mann-Whitney U. For multiple testing, One-way ANOVA followed by LSD post-hoc analysis was used. The increase in systolic blood pressure and proteinuria was calculated as the percentage increase three weeks after minipump implantation as compared to baseline systolic
blood pressure and proteinuria. To test for significant correlations, the Spearman’s rho correlation test was used. In all cases, differences were considered significant if \( p < 0.05 \).

**RESULTS**

**Human study**

**Baseline characteristics of the women**

There were no statistically significant differences in most of the characteristics between the two groups (Table 1). However, fPE-women had a significantly higher BMI and office blood pressure at intake, all within the healthy range. Importantly, 24-hour ABPM and baseline blood pressure at the study day were not different between the groups. Analysis of blood parameters revealed no differences (Table 2).

**Table 1. Baseline characteristics**

<table>
<thead>
<tr>
<th></th>
<th>fHP-women (n = 18)</th>
<th>fPE-women (n = 18)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36 ± 5</td>
<td>36 ± 5</td>
<td>0.951</td>
</tr>
<tr>
<td>Gravidity</td>
<td>2.5 ± 1.3</td>
<td>2.6 ± 1.1</td>
<td>0.951</td>
</tr>
<tr>
<td>Parity</td>
<td>2.0 ± 0.7</td>
<td>2.2 ± 1.0</td>
<td>0.589</td>
</tr>
<tr>
<td>Elapsed time since index pregnancy (years)</td>
<td>4.2 ± 2.6</td>
<td>5.3 ± 3.0</td>
<td>0.243</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>23.2 ± 2.7</td>
<td>25.9 ± 3.5</td>
<td>0.015</td>
</tr>
<tr>
<td>Waist/Hip ratio</td>
<td>0.83 ± 0.04</td>
<td>0.84 ± 0.06</td>
<td>0.443</td>
</tr>
<tr>
<td>Urinary sodium (mmol/24h)</td>
<td>221 ± 64</td>
<td>258 ± 86</td>
<td>0.145</td>
</tr>
<tr>
<td>Urinary albumin/creatinine</td>
<td>0.6 ± 0.3</td>
<td>0.5 ± 0.4</td>
<td>0.212</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Screening visit(^1)</td>
<td>90 ± 7</td>
<td>97 ± 8</td>
<td>0.01</td>
</tr>
<tr>
<td>24-hour(^2)</td>
<td>90 ± 7</td>
<td>90 ± 8</td>
<td>0.997</td>
</tr>
<tr>
<td>Baseline(^3)</td>
<td>85 ± 8</td>
<td>86 ± 9</td>
<td>0.714</td>
</tr>
</tbody>
</table>

*fHP-women, formerly healthy pregnant women; fPE-women, formerly early-onset preeclamptic women; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. 1Average of three blood pressure measurements assessed by the Dinamap during the screening visit in the hospital; 2blood pressure measured using ambulatory blood pressure measurement for 24-hours at the end of the dietary week; 3baseline blood pressure measured at study day, measured for 2 hours with a 15-min interval. Data are presented as mean ± SD.
Table 2. Blood parameters

<table>
<thead>
<tr>
<th></th>
<th>fHP-women (n = 18)</th>
<th>fPE-women (n = 18)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>0.38 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.634</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.0 ± 0.5</td>
<td>5.0 ± 0.3</td>
<td>0.605</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>35 (32.75-37.25)</td>
<td>34 (30.75-35.25)</td>
<td>0.203</td>
</tr>
<tr>
<td>Insulin (uU/mL)</td>
<td>7.1 (4.7-9.3)</td>
<td>7.65 (4.6-10.8)</td>
<td>0.525</td>
</tr>
<tr>
<td>HOMA(^1)</td>
<td>1.55 (0.96-2.21)</td>
<td>1.69 (1.08-2.37)</td>
<td>0.369</td>
</tr>
<tr>
<td>Creatinin clearance (mL/min)(^2)</td>
<td>113 ± 24</td>
<td>123 ± 25</td>
<td>0.220</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m(^3))(^3)</td>
<td>109 ± 10</td>
<td>103 ± 15</td>
<td>0.117</td>
</tr>
<tr>
<td>PRA (nmol Ang I/L/h)</td>
<td>0.20 (0.10-0.50)</td>
<td>0.20 (0.09-0.30)</td>
<td>0.584</td>
</tr>
<tr>
<td>Aldosteron (pmol/L)</td>
<td>71 (29-93)</td>
<td>59 (35-96)</td>
<td>0.839</td>
</tr>
</tbody>
</table>

\(^1\)Calculated using the formula: \((\text{glucose}*\text{insulin})/22.5\); \(^2\)calculated using the formula: \(((\text{urinary creatinine excretion}/24h)*1000)/\text{plasma creatinine}\); \(^3\)calculated using the CKD-epi formula 18. Data are presented as mean ± SD as median (25th-75th percentile).

Increased blood pressure response to ang II infusion in fPE-women

In response to 1.0 ng/kg/min ang II infusion, a trend towards an increased DBP response upon ang II in fPE-women as compared to fHP-women was present. DBP after 0.3 and 3.0 ng/kg/min ang II was not different between both groups (Figure 1B). Similar results were found for MAP responses upon ang II infusion (Figure 1C). No significant differences in SBP response were observed between the two groups during the subsequent ang II infusion doses (Figure 1A). Heart rate was not significantly different after ang II infusion between the two groups (data not shown). Studying the AT1-AA showed that 44.4% of the fPE-women and 16.7% of the fHP-women were positive for AT1-AA (8 vs 3 respectively; \(p=0.070\)). Serum from fPE-women increased the overall cardiomyocyte-beating rate, although not significant compared to fHP-women (median change in bpm: fPE-women: 6.4 (0.5-16.7) vs fHP-women: 0.68 (0.5-4); \(p=0.139\)). No significant correlation was found between the presence of AT1-AA and the degree ang II sensitivity or time after index pregnancy (data not shown).
Angiotensin II sensitivity after preeclampsia

Figure 1. Blood pressure response upon angiotensin II infusion in women.
The mean (±SD) of the systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (C) at baseline and after 0.3, 1.0, and 3.0 angiotensin II (ng/kg/min) respectively as compared to baseline in fHP-women (circle) and in fPE-women (square, dotted line). Data were analyzed using the general estimating equations and corrected for baseline blood pressure and BMI. fPE: formerly preeclamptic women; fHP: formerly healthy pregnant women. P-values are presenting the difference between the two groups at 1.0ng/kg/min angiotensin II.

Animal study
Rat characteristics
At the end of pregnancy, HP-rats weighed significantly more compared to the PE-rats (Table 3A). The total number and weight of pups was not significantly different, however, the length of the pups was significantly decreased in PE-rats as compared to HP-rats. Moreover, one pup from one PE-rat and eight pups from another PE-rat were born dead (Table 3B). Baseline SBP, i.e. six weeks after delivery, was slightly but significantly lower in the fPE-rats as compared to the NP-rats. No difference in baseline proteinuria was found between the three groups.

Increased blood pressure response upon ang II infusion in fPE-rats
In the control rats, i.e. rats treated with sham pumps, no significant changes in SBP were observed in the three weeks of treatment (not shown). However, SBP increased in all groups of rats following three weeks of continuous ang II infusion (Figure 2A), with the highest increase in A-fPE-rats. The percentage increase in SBP in A-fPE-rats was higher (borderline significant), as compared with A-NP-rats (Figure 2A). SBP increase after one and two weeks of ang II infusion revealed no differences between the three groups of rats (not shown). SBP at time of termination (under anesthesia) was significantly higher in A-fPE-rats as compared to A-NP-rats (Figure 2B), with no significant differences between the three groups of sham treated rats (not shown).
Table 3. Rat characteristics during pregnancy (A) and postpartum (B)

<table>
<thead>
<tr>
<th></th>
<th>HP-rats (n = 19)</th>
<th>PE-rats (n = 22)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A - PREGNANCY CHARACTERISTICS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat maternal weight(^1) (g)</td>
<td>383 ± 24</td>
<td>361 ± 26</td>
<td>0.013</td>
</tr>
<tr>
<td>Number of pups(^2)</td>
<td>12.4 ± 3.4</td>
<td>10.7 ± 3.9</td>
<td>0.150</td>
</tr>
<tr>
<td>Length of pups(^2) (mm)</td>
<td>50.6 ± 1.4</td>
<td>49.2 ± 2.7</td>
<td>0.034</td>
</tr>
<tr>
<td>Weight of pups(^2) (g)</td>
<td>6.1 ± 0.3</td>
<td>6.0 ± 0.8</td>
<td>0.556</td>
</tr>
<tr>
<td><strong>B - POSTPARTUM CHARACTERISTICS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat maternal weight(^3) (g)</td>
<td>269.7 ± 17.9</td>
<td>274.9 ± 17.8</td>
<td>276.4 ± 12.8</td>
</tr>
<tr>
<td>Baseline SBP(^4) (mmHg)</td>
<td>129.9 ± 9.5</td>
<td>127.5 ± 9.8</td>
<td>122.9 ± 9.1*</td>
</tr>
<tr>
<td>Baseline proteinuria(^4) (mg/24h)</td>
<td>4.3 ± 1.2</td>
<td>4.2 ± 2.3</td>
<td>3.3 ± 1.8</td>
</tr>
</tbody>
</table>

HP, healthy pregnant; PE, experimental preeclampsia; fHP, formerly healthy pregnant; fPE, formerly experimental preeclampsia; NP, never pregnant; SBP, systolic blood pressure. \(^1\)Weight at the day before delivery; \(^2\)only pups that were moving were measured; \(^3\)weight at the time of osmotic minipump implantation (six weeks postpartum); \(^4\)baseline values measured six weeks postpartum (fHP- and fPE-rats) or at the same time-interval for the NP-rats. Data are presented as mean (± SD). \(*: p<0.05\) vs NP-rats.
Angiotensin II sensitivity after preeclampsia

Figure 2. Blood pressure response upon chronic angiotensin II infusion in rats.
(A) The mean ± SEM of the percentage increase in systolic blood pressure (SBP) after three weeks of 200ng/kg/min angiotensin II (ang II) compared to baseline SBP in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Blood pressure was measured by the use of a tail cuff device. Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. (B) The mean ± SEM of the SBP at termination after three weeks of 200ng/kg/min ang II in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis.

Changes in sensitivity of the thoracic aorta to ang II: aortic ring contraction experiment
Ex-vivo ang II sensitivity was studied in aortic rings in the sham treated rats. Although the area under the curve (AUC) showed no significant differences between the three groups (Figure 3 A1 and A2), logEC50 was significantly lower in the c-fHP-rats as compared with c-fPE-rats (Table 4). No significant difference in AT1-R mediated vasoconstriction was observed between the three groups of rats (Figure 3 B1 and B2). It can be seen that AT2-R mediated relaxation, as measured by AUC (area of the positive peaks) was significantly impaired in the c-fPE-rats as compared to the c-NP-rats (Figure 3 C2).

Significant increase in proteinuria following ang II infusion in fPE-rats
No significant increase in proteinuria over the three weeks of treatment was seen in rats that received a sham osmotic minipump (not shown). After three weeks of continuous ang II infusion, proteinuria increased in all three groups, with a trend towards increased proteinuria in fPE vs fHP-rats (data not shown). At this time point, the percentage increase in proteinuria as compared to baseline was significantly higher in A-fPE as compared to A-fNP and A-fHP (Figure 4A). Creatinine clearance (mL/min) was not significantly different between the rats implanted with a sham osmotic minipump (cNP 2.5 ± 0.5 vs c-fHP 2.8 ± 0.7 vs c-fPE 2.5 ± 0.7; p = 0.408). Also no difference was seen between the rats receiving ang II (A-NP 2.2 ± 0.6 vs A-fHP 2.3 ± 0.5 vs A-fPE 2.3 ± 0.4; p = 0.859).
Chapter 6

Figure 3. Ang II dose-response curves in the thoracic aorta.

The mean ± SEM of the cumulative angiotensin II (ang II) dose-response curves in the thoracic aorta from never pregnant rats (NP; circle; n=12), formerly healthy pregnant rats (fHP; square; n=10), and formerly experimental preeclampsia rats (fPE; pyramid upward; n=11). (A) ang II was added with no inhibitor present; (B) ang II was added after incubation with the AT2-R antagonist PD123319 and L-NMMA; (C) ang II was added after incubation with the AT1-R antagonist losartan. (A2, B2, C2): mean ± SEM AUC of the cumulative ang II dose-response curves in never pregnant rats (NP; white bar), formerly healthy pregnant rats (fHP; black bar), and formerly experimental preeclampsia rats (fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. *: p<0.05

Table 4. logEC50 and Emax dose response curves

<table>
<thead>
<tr>
<th></th>
<th>c-NP-rats (12)</th>
<th>c-fHP-rats (10)</th>
<th>c-fPE-rats (11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>log EC50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>-7.29 (± 0.18)</td>
<td>-7.45 (± 0.23)</td>
<td>-7.25 (± 0.24)*</td>
</tr>
<tr>
<td>Ang-II contraction</td>
<td>-7.29 (± 0.30)</td>
<td>-7.42 (± 0.24)</td>
<td>-7.29 (± 0.28)</td>
</tr>
<tr>
<td>Ang-II relaxation¹</td>
<td>-8.11 (± 1.04)</td>
<td>-8.66 (± 0.62)</td>
<td>-8.13 (±1.14)</td>
</tr>
<tr>
<td>Emax</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ang-II</td>
<td>26.06 (± 13.59)</td>
<td>25.81 (±9.62)</td>
<td>23.93 (± 13.09)</td>
</tr>
<tr>
<td>Ang-II contraction</td>
<td>22.14 (± 10.54)</td>
<td>18.64 (± 8.27)</td>
<td>17.84 (± 7.08)</td>
</tr>
<tr>
<td>Ang-II relaxation¹</td>
<td>4.60 (± 5.00)</td>
<td>6.31 (± 9.77)</td>
<td>4.80 (±10.11)</td>
</tr>
</tbody>
</table>

c-NP, control never pregnant rats; c-fHP, control formerly healthy pregnant rats; c-fPE, control formerly experimental preeclampsia rats. ¹for fPE group only 7 rats were included in analyses. Data are presented as mean ± SD. *: p<0.05 vs c-fHP.
Increased inflammation of the kidney in fPE-rats in response to ang II

Ang II infusion did not affect the number of interstitial monocytes/macrophages in NP and fHP-rats. However, three weeks after ang II infusion of fPE-rats the amount of interstitial monocytes/macrophages was significantly higher as compared to sham treated fPE-rats (Figure 4B). No differences were seen after sham or ang II infusion between NP-, fHP-, and fPE-rats in the number of CD206 positive macrophages (data not shown). In all groups, treated for three weeks with ang II, we found a significant increase in the number of positive pixels per area for αSMA as compared to the sham treated rats (data not shown), with no differences between these groups.

Figure 4. Proteinuria and macrophages influx upon chronic angiotensin II infusion.

(A) The mean ± SEM of the percentage increase in proteinuria (mg/24h) after three weeks of 200ng/kg/min angiotensin II (ang II) compared to baseline proteinuria in the never pregnant rats (A-NP; white bar), the formerly healthy pregnant rats (A-fHP; black bar), and in the formerly experimental preeclamptic rats (A-fPE; striped bar). Data were analyzed using One-Way ANOVA followed by LSD post-hoc analysis. *: p<0.05

(B) The median with 25th-75th percentile of the number of CD68-positive macrophages (ED1-macrophages) in the interstitial part of the kidney and in sham operated never pregnant rats (c-NP), formerly healthy pregnant rats (c-fHP) and, formerly preeclamptic rats (c-fPE), and in never pregnant rats (A-NP), formerly healthy pregnant rats (A-fHP), and formerly preeclamptic rats (A-fPE) infused with 200ng/kg/min ang II for three weeks. Data were analyzed using Mann-Whitney U test. *: p<0.05

AT1- and AT2-receptor mRNA expression and protein expression in the aorta and kidney

The increased blood pressure and proteinuria response upon infusion of ang II could be due to a different regulation of the AT1-R and AT2-R upon chronic ang II infusion between the three groups. Therefore, the mRNA expression of the AT1-R and AT2-R in the thoracic aorta and the kidneys of all groups were measured. We observed a trend towards an increased mRNA expression of the AT1-R (Figure 5A) in the thoracic aorta of the A-fPE-rats as compared to c-fPE-rats. This effect of chronic ang II infusion was not observed in ang II treated NP-rats and fHP-rats. No differences were seen in AT2-R mRNA expression in the thoracic aorta between the different groups (Figure 5B). No difference in mRNA expression for the AT1-R and the AT2-R was found between the three groups of rats with sham pumps.
The mRNA expression of the AT1-R and the AT2-R in the kidney of sham treated rats was comparable between the three groups. Moreover, no effect of chronic ang II infusion upon expression of either AT1-R mRNA or AT2-R mRNA was observed. However, after chronic ang II infusion, AT1-R mRNA expression was significantly lower in the fPE-rats as compared to the NP-rats, with no differences in AT2-R mRNA expression (Figure 5C and Figure 5D).

Protein expression of the AT1-R in the kidney of sham treated rats was comparable between the three groups (Figure 5E). After chronic ang II infusion, AT1-R protein expression showed a trend towards a lower expression in the fPE-rats as compared to the NP-rats (Figure 5E). Very low levels of AT2-R protein expression were found in the kidney extracts. However, AT2-R protein expression was significantly increased in sham treated fPE-rats as compared to sham treated fHP-rats (Figure 5F). Also, after chronic ang II infusion, protein AT2-R expression was significantly increased in fPE-rats as compared to fHP-rats (Figure 5F). Unfortunately, due to a lack of aorta material, we were not able to study AT1-R and AT2-R protein expression in the aorta.

DISCUSSION

This is the first translational study that analyses ang II sensitivity in formerly early-onset preeclamptic women without any comorbidity and in healthy rats after experimental preeclampsia. We demonstrated increased blood pressure response to ang II infusion, albeit subtle (borderline significant) in formerly early-onset preeclamptic women as compared with formerly healthy pregnant women. Moreover, after experimental preeclampsia we observed an increased blood pressure, proteinuria and macrophage influx into the kidney after chronic infusion of ang II as compared with formerly healthy pregnant rats. Since we studied women without any comorbidity and rats that were healthy and identical pre-pregnancy, our data suggest that (experimental) preeclampsia itself may have induced the persistent increased ang II sensitivity in the present studies. This increased ang II sensitivity postpartum might be one of the pathophysiological mechanisms leading to the increased risk for cardiovascular and renal diseases in fPE-women.

Blood pressure appeared to increase more in response to ang II in fPE-women compared to fHP-women, however, this was just not significant. Our data appear to be in line with a study of Hladunewich et al., however, they found more pronounced differences. This may be due to the fact that they studied women 6-18 months postpartum rather than 1-10 years postpartum 11. Furthermore, their study group showed increased baseline blood pressure, BMI and age compared with controls. Similarly, differences between our study and the study of Spaanderman et al. 27 and Saxena et al. 10 may be explained by differences in study design, such as a mixture of phenotypes and increased blood pressure in the study group, the ang II concentration and low sodium intake used, and no standardization of menstrual cycle in both studies. Although in the present study, the fPE-women showed a higher BMI, the waist/hip ratio, which is a superior risk factor for cardiovascular disease than BMI 28, was not different between the groups. The white-coat effect observed in our study may be related to the increased risk for cardiovascular disease 29.
Figure 5. mRNA and protein expression.
The mRNA expression of the AT1-R (A, C) and the AT2-R (B, D) in the thoracic aorta (A, B) and the kidney (C, D) and the protein expression of the AT1-R (E) and the AT2-R (F) in the kidney in sham operated never pregnant rats (c-NP), formerly healthy pregnant rats (c-fHP), and formerly preeclamptic rats (c-fPE), and in never pregnant rats (A-NP), formerly healthy pregnant rats (A-fHP), and formerly preeclamptic rats (A-fPE) infused with 200ng/kg/min angiotensin II for three weeks. Representative western blot analysis of AT1-R and AT2-R and the associated β-actin protein expression in the kidney in the 6 groups of rats (G). Data were analyzed using Mann-Whitney U test. *: p<0.05, #: p<0.1
To investigate cause and effect relationships between preeclampsia and postpartum ang II sensitivity in a clean model, we studied blood pressure and kidney response to chronic ang II infusion after experimental preeclampsia, induced by infusion of low dose LPS. Previous studies have shown that LPS-infused pregnant rats develop a preeclampsia like syndrome, as characterized by hypertension and proteinuria. Since the sensitivity to ang II is increased in this experimental preeclampsia model during pregnancy, this appears to be an appropriate model to study the relationship between the increased ang II sensitivity during and after preeclampsia. In accordance with our human data, in fPE-rats we found no baseline hypertension or proteinuria. In fact, fPE-rats even showed a significantly lower SBP before implantation of the minipumps, compared with the NP-rats. This might be related to the decreased vascular response to ang II found in the aorta of these fPE-rats (see below). Still, three weeks after chronic ang II infusion we observed an increased blood pressure and proteinuria response upon ang II in fPE-rats as compared to NP-rats and fHP-rats. Our data are in line with a study in preeclamptic mice (due to overexpression of soluble fms-like tyrosine kinase-1 (sFlt-1)), in which no long-term differences in blood pressure and vascular function were observed, while different expression of plasma proteins involved in cardiovascular function was seen, indicating postpartum effects induced by experimental preeclampsia. In our animal model, preeclampsia-like symptoms are induced by LPS via activation of Toll-like receptor 4. Many other animal models for preeclampsia exist, for instance overexpressing sFlt-1, ATP-infusion, and renin-angiotensin aldosterone system (RAAS) overactivity or genetic adjustments. In these models, the preeclampsia-like symptoms may be induced via other pathways. Therefore, our animal data should be confirmed in other models of preeclampsia.

To test ex-vivo ang II sensitivity in our rat model, we used the easily accessible aorta, and performed aortic ring contraction experiments in response to ang II. The aorta is a conductance vessel rather than a resistance vessel. The aorta is therefore not typically involved in blood pressure regulation. However, the use of aortic rings is a well-established read-out for vascular pharmacological responses, including studying angiotensin sensitivity. Although the total response to ang II (i.e. AUC) did not differ between the groups, logEC50 was significantly lower in the fHP-rats. This suggests, in contrast to our hypothesis, a decreased sensitivity to ang II in the aorta of fPE-rats as compared to fHP-rats. The question arises whether this decreased sensitivity in fPE-rats is related to the slightly decreased blood pressure measured in these rats at baseline. It may be speculated that the decreased ang II sensitivity in the aorta may decrease vascular smooth muscle tone and thus aortic stiffness and therefore central blood pressure and possibly peripheral blood pressure. Further studies in other vascular beds, such as small resistance or renal arteries are needed to confirm that changes in the ang II sensitivity in fPE-rats are linked to blood pressure.

The ang II response via the AT2-R showed a decreased relaxation upon ang II in fPE-rats. This was, however, not accompanied by changes in the mRNA expression of the AT2-R in the aorta. The decreased ex-vivo responsiveness of the AT2-R to ang II in the fPE-rats might contribute to the increased blood pressure response upon ang II. This hypothesis, however, needs to be confirmed in resistance vessels. In contrast to differences in AT2-R responses in fPE-rats, we did not find differences in the AT1-R responses in fPE-rats. This is in contrast to the situation.
during experimental preeclampsia, in which the response of the aorta to the AT1-R was increased 
24. Thus, although we have observed changes in ang II sensitivity during and after preeclampsia in 
the rat, during preeclampsia the AT1-R may be involved, while after preeclampsia the AT2-R may be 
involved. Further postpartum follow-up studies, also in other models for preeclampsia, are needed 
to establish the time course of changes and mechanisms in the AT1-R and AT2-R sensitivity.

We also observed increased proteinuria in response to ang II infusion in fPE-rats without 
differences in serum and urinary creatinine. This rise in urinary protein level was associated with an 
increased influx of interstitial macrophages. Since we found no increase in the number of CD206 
positive macrophages, i.e. M2 macrophages, the increased macrophages are most likely of the 
M1 or inflammatory phenotype. It can be suggested that the increase in interstitial macrophages 
was secondary to proteinuria, since excessive tubular reabsorption of proteins results in tubulo-
interstitial infiltration of monocytes 40. As macrophages themselves may also inflict kidney injury 41, 
this macrophage infiltration may induce a vicious circle of kidney damage leading to proteinuria. 
On the other hand, as infiltration of macrophages in glomeruli and renal interstitium sometimes 
precedes the onset of glomerular injury and interstitial fibrosis leading to proteinuria 42, the infiltrated 
macrophages may also be the cause of proteinuria. Profibrotic changes, as measured by αSMA 
expression, were similarly increased in all groups after ang II infusion. Whether infusion duration or 
higher dosages of ang II would have resulted in more pronounced changes in renal damage in fPE-
rats (i.e. focal glomerular sclerosis) as compared to the other groups, remains to be investigated.

The mechanism of increased ang II sensitivity during and after preeclampsia is unknown. 
The study of Hladunewich et al. suggested that the balance between the AT1-R and AT2-R could be 
involved 11. Indeed, our data suggest differential expression and regulation of the AT1-R and AT2-R 
with ang II infusion in fPE-rats as compared with the other groups of rats. However, the present 
results for AT1-R and AT2-R mRNA and protein expression are not conclusive, since differential 
responses of the aorta and kidney were observed. Other RAAS mechanisms like, heterodimerization 
of the AT1-R with the bradykinin B(2)-receptor 43 or alterations in the vasodilatory ang 1-7 acting on 
the MAS-receptor 44 could be involved and should be studied in more detail. Furthermore, increased 
levels of AT1-AA postpartum could be involved in increased ang II sensitivity 17, 45. Indeed in our 
study, although just not significant, more fPE-women tested positive for having AT1-AA as compared 
to fHP-women, but not correlating with ang II sensitivity. It seems unlikely that this contributes in 
our experimental rat model since we did not find AT1-AA in pregnant LPS-infused rats (unpublished 
results).

Conclusion Our translational study suggests that preeclampsia per se leads to subtle long-term 
increased blood pressure and proteinuria in response to acute and chronic ang II infusion in 
otherwise healthy humans and rats. However, we cannot rule out that underlying pre-pregnancy 
(risk) factors may also play a role. Preeclampsia might merely identify women with an unfavorable 
cardiovascular system or aggregation of cardiovascular risk factors might occur before and during 
the preeclamptic pregnancy.
Acknowledgements

We thank Mrs. L. B. Klein Schaarberg, Mrs. M. Dekker, the animal microsurgeons, and Mr. P.A. Klok for their assistance. We greatly appreciate all the help of the employees during the study days. We acknowledge Spacelabs Healthcare for the supply of the ABPM-device.
REFERENCES


