Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION

American Heart Association®

*Learn and Live*sm

Mice Overexpressing Both Human Angiotensinogen and Human Renin as a Model of Superimposed Preeclampsia on Chronic Hypertension

Stéphanie Falcao, Ekatherina Stoyanova, Guy Cloutier, Roch L. Maurice, Jolanta Gutkowska and Julie L. Lavoie

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/cgi/content/full/54/6/1401

Data Supplement (unedited) at:

http://hyper.ahajournals.org/cgi/content/full/HYPERTENSIONAHA.109.137356/DC1

Subscriptions: Information about subscribing to Hypertension is online at
http://hyper.ahajournals.org/subscriptions/

Permissions: Permissions & Rights Desk, Lippincott Williams & Wilkins, a division of Wolters
Kluwer Health, 351 West Camden Street, Baltimore, MD 21202-2436. Phone: 410-528-4050. Fax:
410-528-8550. E-mail:
journalpermissions@lww.com

Reprints: Information about reprints can be found online at
http://www.lww.com/reprints

Mice Overexpressing Both Human Angiotensinogen and Human Renin as a Model of Superimposed Preeclampsia on Chronic Hypertension

Stéphanie Falcao, Ekatherina Stoyanova, Guy Cloutier, Roch L. Maurice,
Jolanta Gutkowska, Julie L. Lavoie

Abstract—Preeclampsia is the major cause of maternal and fetal mortality/morbidity. Because hypertension is an important risk factor for preeclampsia, we investigated whether hypertensive mice that overexpress human renin and angiotensinogen develop superimposed preeclampsia. Given that the mechanisms underlying this disease are still poorly understood, animal models are of great use for elucidatation. Blood pressure and proteinuria were measured by telemetry and ELISA, respectively. Heart function was evaluated by echocardiography, whereas pathological cardiac hypertrophy–related genes were assessed by real-time PCR. Soluble fms-like tyrosine kinase 1 plasma concentrations were quantitated by ELISA and placental expression by real-time PCR. Transgenic mice develop de novo proteinuria during gestation and marked blood pressure elevation, which are hallmarks of superimposed preeclampsia on chronic hypertension. Abnormal placentation present in these mothers produced a significant decrease in pup and placental weight and was associated with an increased placental expression of soluble fms-like tyrosine kinase 1. We also found heightened circulating levels of this receptor, when adjusted for placental mass, as has been observed in women who suffer from preeclampsia. Cardiac hypertrophy could be observed in the transgenic mice and was exacerbated by gestation. As a result, heart function was significantly decreased, and markers of pathological hypertrophy were increased. Our data, thus, confirm the characterization of a new model of superimposed preeclampsia on chronic hypertension. Because chronically hypertensive women are at risk of developing the pathology, our model reflects a clinical reality and is, thus, an excellent tool to elucidate the molecular mechanisms triggering this disease. (*Hypertension*. 2009;54:1401-1407.)

Key Words: preeclampsia ■ mouse model ■ renin-angiotensin system ■ cardiac hypertrophy ■ hypertension

Preeclampsia (PE) has been studied extensively in the last 2 decades, because it is the most common cause of fetal and maternal mortality and morbidity.[1] However, thus far, apart from delivery, there are no available treatments, because antihypertensive medications are deleterious to the fetus.[2] This human pregnancy-associated syndrome is characterized by the new occurrence of proteinuria and hypertension, in previously normotensive women, or a progression of chronic hypertension to superimposed PE (SPE).[3] Other symptoms that can also be associated with this disease are placental pathology and cardiac hypertrophy.[1,4] Because it is difficult to predict disease onset, studies in humans are challenging and require a tremendous number of subjects to be of any significance. Moreover, few animal models develop PE spontaneously.[5–7] Because it has been suggested that PE may not be a homogenous disease, similar to essential hypertension, many different animal models may be required to characterize the different faces of this pathology that is still so poorly understood.

The renin-angiotensin system (RAS) is postulated to be one of the mechanisms responsible for the development of PE.[8] Indeed, although renin, angiotensinogen, angiotensin II (Ang II), and aldosterone plasma levels are elevated in pregnant women, response to vasoconstrictors is strikingly diminished.[8] In contrast, PE is characterized by a heightened sensitivity to vasoconstrictors when compared with normal pregnancy,[5,9] which, in part, may be explained by an upregulation of the Ang II type 1 receptors.[10] Moreover, Ang II type 1–activating autoantibodies have been detected in the serum of preeclamptic women[10–13] and have been shown to specifically stimulate Ang II type 1 receptors and trigger the same pathways as Ang II. In addition, it is known that all of the RAS components are present in human placenta throughout pregnancy,[14] which makes it a potential player in the modulation of maternal blood pressure. On the basis of these findings, a novel animal model of PE was characterized when transgenic female mice[15] or rats[16] overexpressing human angiotensinogen were mated with males carrying the human

renin gene (female hAogen×male hRen). Because angiotensinogen cleavage by renin is species specific, both female and male mice that are single transgenic are normotensive. In this model, it has been shown that overexpression of the uteroplacental RAS and release of placental human renin into the circulation trigger the PE-like symptoms in these rodents.

On the other hand, transgenic mice or rats overexpressing both human renin and human angiotensinogen (R^+A^+) have been developed in different laboratories and have been established as a model of hypertension.[17–20] Indeed, male mice have elevated blood pressure, ≈150 mm Hg,[17] and show high plasma levels of Ang II with evidence of end-organ damage, such as endothelial dysfunction.[21] Although these phenotypes have not been characterized in females, they should be similar, because transgene expression is equivalent. Therefore, because chronic hypertension is an important risk factor for PE,[22] we hypothesized that hypertensive R^+A^+ double-transgenic female mice would develop SPE, and, therefore, we propose these mice as an animal model of this clinical reality.

Materials and Methods

An expanded Materials and Methods section is available in the online Data Supplement at http://hyper.ahajournals.org.

Animals

R^+A^+ transgenic mice were produced by breeding heterozygous human renin (Ren9 line) mice[23] with heterozygous human angiotensinogen (204/1 line) mice.[24] Both of these single transgenic lines, obtained from Dr Curt D. Sigmund at the University of Iowa, were maintained in our animal facility by backcrossing with C57BL/6 mice (Charles River, St-Constant, Quebec, Canada). The mouse genotype was determined as described previously.[23,24] The animals were kept on a 12-hour light/dark cycle with water and standard laboratory chow (2018; Teklab Premier Laboratory Diets) ad libitum. Female mice in these experiments were 12 to 15 weeks of age and were separated into 4 groups (nonpregnant R^+A^+ and nontransgenic [NT; R^-A^-]; pregnant R^+A^+ and R^-A^-). Their care met the standards set forth by the Canadian Council on Animal Care for the use of experimental animals. All of the procedures were approved by the university animal care committee of the Centre Hospitalier de l'Université de Montréal Research Centre.

Blood Pressure Measurement

Arterial pressure and heart rate were directly measured by telemetry, as done previously.[25,26]

Proteinuria

Urine samples were collected before and on day 18 of gestation, and albumin and creatinine concentrations were quantified as described previously.[25,26]

Tissue Collection and Histology

On day 18 of gestation, blood was collected by heart punction and placed in chilled 1.5-mL tubes containing EDTA (EMD). The pups were weighed, and their tails were cut and kept for genotyping. Kidneys, hearts, and placentas were all collected, weighed, and either flash frozen in liquid nitrogen or fixed until further assessment, as published previously.[25,26]

Placental alterations were characterized by 5 criteria: necrosis, hyalinization, microcalcification, giant cell island loss, and labyrinthine trophoblast structure loss as done previously.[25,26] The latter 2 are, respectively, analogous to human extravillous cytotrophoblasts cells and chorionic villi structure.[5] A score from 0 to 3 was assigned for each criterion: 0 for no change, 1 for mild, 2 for moderate, and
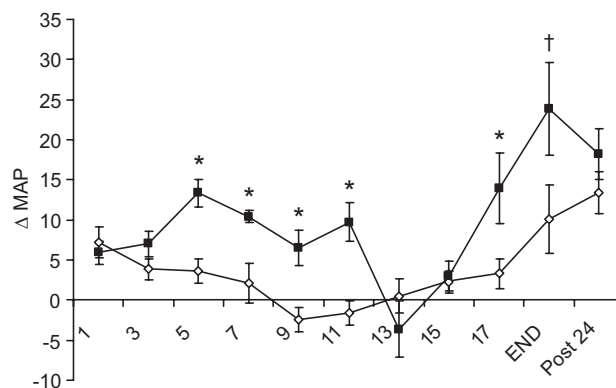
Figure 1. Changes in MAP during pregnancy. MAP variation vs baseline significantly increased in R^+A^+ mice (■, n=6) at days 5, 7, 9, 11, and 17 and at the end of pregnancy in comparison with R^-A^- mice (◇, n=7), as measured by telemetry. Values are expressed as mean±SE. *$P≤0.05$ and †$P≤0.001$, statistically different from R^-A^- mice. END indicates end of gestation; Post 24, 24 hours postpartum.

3 for severe alteration. All of the scores were then summed up for total evaluation of the placental pathology present. The investigator scoring the tissues was blinded to the genotype of the pups and mothers to avoid any bias.

Plasma Ang II Concentration

Plasma Ang II was concentrated using an extraction kit with a C18 SEP-column (Peninsula Laboratories). Reconstituted sample concentrations of Ang II were determined by radioimmunoassay with a commercial kit (Peninsula Laboratories) using the directions and reagents supplied by the manufacturer.

Plasma Soluble fms-Like Tyrosine Kinase 1 Levels

Plasmatic soluble fms-like tyrosine kinase-1 (sFlt-1) concentrations were measured using a commercial ELISA kit (R&D-Quantikine). Plasmas were diluted 1:20 in the manufacturer dilutor before measurements so that values were within the standard curve.

Echocardiography

Transthoracic echocardiographic studies were performed before and at the end of pregnancy. The mice were anesthetized by inhalation of isoflurane 2% to 3% in oxygen and then maintained on 2% isoflurane. Their hearts were investigated as described previously[27] by high-resolution ultrasound biomicroscopy (Vevo660; Visualsonics) equipped with 25- to 55-MHz probes that allow tracings of time-varying M-mode dimensions of the left ventricle (LV). The ejection fraction (EF), LV volumes, LV fractional shortening (FS), and stroke volume, as well as cardiac output, were all calculated on the basis of data acquired.

Western Blot

Protein samples were separated by electrophoresis and transferred on a nitrocellulose membrane. Proteins were detected with antiplacental growth factor 2 (PlGF-2; Abcam) antiserum using enhanced chemiluminescence West Pico kits (Pierce). Total protein was subsequently measured by staining of the membrane with Amido black (Sigma), and the PlGF-2 signal was normalized to total protein content of each sample.

Real-Time PCR

Brain natriuretic peptide (BNP) and nerve growth factor 1-A–binding protein (Nab1) were both amplified in LV, whereas sFlt-1 was amplified in placentas. BNP and Nab1 are both reported to be specific markers of pathological cardiac hypertrophy, because they are markedly increased in this condition, whereas no differences are detected in physiological hypertrophy, such as exercise training.[28,29]
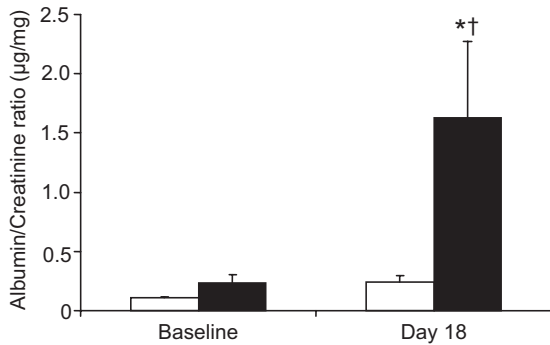
Figure 2. Changes in proteinuria during gestation. Proteinuria was assessed by measuring the albumin:creatinine ratio in urine by ELISA. R^+A^+ mice (black, n=6) show an elevated albumin:creatinine ratio at the end of gestation (day 18) when compared with baseline. Furthermore, this was also significantly different from that observed in R^-A^- mice (white, n=9) at day 18. Values are expressed as mean±SE. *$P \leq 0.05$, statistically different from baseline and †$P \leq 0.001$, statistically different from R^-A^- mice.

Thus, their expression was measured to differentiate physiological heart hypertrophy in pregnancy from pathological cardiac remodeling. Total RNA was extracted from frozen samples using TRIzol (Invitrogen) according to the manufacturer's protocol. Removal of genomic DNA, reverse-transcription reaction, and PCR were all done as described previously.[30] mRNA levels were expressed as values relative to 18s mRNA.

Statistical Analysis

All of the data are expressed as mean±SE. The impact of pregnancy and genotype on most parameters was analyzed by 2-way ANOVA. Repeated-measures ANOVAs were performed to assess the impact on blood pressure and albumin:creatinine ratio, and cardiac parameters measured by echocardiography followed by Tukey post hoc test when an interaction was detected. Placental pathology scores were analyzed by the nonparametric Mann–Whitney U test, and changes in placental and pup weight were assessed by t test. Real-time PCR values, as well as plasma concentrations, were analyzed by 1-way ANOVA. Furthermore, plasma sFlt-1 concentrations were analyzed by 1-way ANOVA using placental mass (total weight of all of the placentas in a litter) as a cofactor.

Results

There were no differences in age (82.6±2.8 versus 97.6±7.4 days in R^-A^- and R^+A^+ mice, respectively) or in body weight both before and at the end of gestation (please see Table S1 in the online Data Supplement at http://hyper.ahajournals.org). As expected, we detected increased levels of Ang II in plasma from R^+A^+ mice collected at the end of gestation (334.7±4.7 versus 141.4±2.6 pg/mL in R^+A^+

mice compared with R^-A^- mice; $P \leq 0.001$). Before pregnancy, it was noted that R^+A^+ mice had significantly higher mean arterial pressure (MAP; 134.2±2.5 mm Hg) when compared with R^-A^- mice (99.7±1.6 mm Hg), as observed previously in males.[17] Concurrently, during gestation, the MAP was always significantly higher in our model as compared with their NT littermates (please see Figure S1 in the online Data Supplement). Of interest, MAP increased significantly compared with baseline in the R^+A^+ at days 5 and 17 and at the end of gestation, and these changes in blood pressure were significantly different from those observed in R^-A^- mice (Figure 1). Indeed, MAP at the end of gestation was 157.4±4.9 compared with 109.8±3.9 mm Hg in NT mice. In addition, MAP in R^+A^+ mice started decreasing by 24 hours postpartum, and the change in MAP was no longer significantly different from R^-A^- females (Figure 1), although MAP had not completely returned to normal (Figure S1). Furthermore, whereas the albumin:creatinine ratios in R^+A^+ mice were not different from the NT mice at baseline, they developed marked proteinuria by the end of gestation compared with R^-A^- mice (Figure 2). To confirm that the proteinuria observed in the pregnant R^+A^+ mice was not simply attributable to disease progression with age in these mice, we also measured proteinuria in nonpregnant double-transgenic mice of comparable age or older. Their albumin:creatinine ratio (0.414±0.120) was not different from the levels observed in all mice before gestation. Furthermore, it was significantly lower than the ratio observed at the end of gestation (D18) in pregnant mice of the same genotype ($P \leq 0.05$). However, the proteinuria developed with gestation in the R^+A^+ mice was not accompanied by renal pathology, because no glomerulosclerosis, endotheliosis, or any other signs of nephropathy could be observed by our pathologist by light microscopy. Conversely, placental abnormalities were very pronounced in samples collected from R^+A^+ mothers in comparison with placentas from NT mice independent of pup genotype (please see Figure S2). This severe placental pathology was characterized by increased necrosis and loss of labyrinthine trophoblast structure in samples collected from R^+A^+ mice (Table 1), which resulted in a reduction of both pup and placental weight in comparison with NT mice (Table 2). We did not, however, observe any differences in litter size (6.9±0.9 versus 6.9±0.8 for R^-A^- and R^+A^+ mothers, respectively).

Table 1.   Characterization of Placental Pathology

Maternal Genotype	N	Placental Pathology Parameters											
		Necrosis		Hyalinization		Microcal		GCIL		LLTS		Total	
		Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%	Mdn	75%
R^-A^-	52	1	1	1	1	0	0	0	1.0	0	1	2	3
R^+A^+	31	1†	2	2*	2	0	0	0	0.8	1*	2	5*	6

The results are expressed as the median (Mdn) and the 75th percentile of the score given in histology. Each parameter was given a score from 0 to 3, whereas total was the sum of the score given to a placenta for each parameter. GCIL indicates giant cell island loss; LLTS, loss of labyrinthine trophoblast structure; Microcal, microcalcification.

*$P \leq 0.001$, data are statistically different from the R^-A^- genotype.

†$P \leq 0.01$, data are statistically different from the R^-A^- genotype.

Table 2.   Pup and Placental Weight

| Maternal Genotype	N	Pup Weight, mg	Placental Weight, mg
R^-A^-	59	806±162	129±27
R^+A^+	54	682±142*	102±18*

Values are expressed as mean±SE.

*$P \leq 0.001$, data are statistically different from R^-A^- genotype.

sFlt-1 mRNA expression was significantly increased in placentas from R^+A^+ mice (0.88±0.22; n=7) compared with NT mice (0.37±0.12; n=8) when expressed as sFlt1:18s ratio ($P<0.05$). Furthermore, NT mice tended to have lower circulating levels of sFlt-1 compared with R^+A^+ mice (52.8±9.2 versus 57.7±2.1 ng/mL). However, given that we observed heightened placental expression in our transgenic mice, we further investigated this issue. Because it is well known that increased circulating sFlt-1 levels during pregnancy originates from the placenta[31] and that mice have a variable number of pups per litter, we evaluated the role of the total placental mass in the levels of circulating sFlt-1. Doing so, we determined that its plasmatic concentrations were significantly higher in R^+A^+ mice when total placental mass was used as a cofactor in 1-ANOVA analysis ($P \leq 0.05$). PlGF-2 protein expression was significantly decreased in placentas from R^+A^+ mothers (0.74±0.10; n=5) compared with those from R^-A^- mice (1.85±0.37, n=5) when expressed as PlGF-2 signal/total protein ($P<0.05$; please see Figure S3 for representative image of Western blot).

Interestingly, at baseline, the heart:body weight ratio was significantly higher in R^+A^+ compared with R^-A^- mice. At the end of gestation, this ratio was also increased in NT mice, and the phenotype was exacerbated in R^+A^+ animals (Figure 3). Statistical analyses were performed on a subgroup of age-matched R^+A^+ mice to confirm that the exacerbated heart hypertrophy observed with gestation was not a normal progression of the disease but rather an effect of pregnancy. Indeed, it demonstrated that the gestational impact on heart hypertrophy was maintained when pregnant R^+A^+ mice (7.54±0.29) were compared with age-matched nonpregnant females (6.42±0.20; $P \leq 0.001$). Heart hypertrophy was further assessed by echocardiography (please see Table S2). Furthermore, the EF and FS were calculated to evaluate LV performance. Although gestation had no effect on EF, this parameter was significantly lower in R^+A^+ mice before and at the end of gestation compared with NT mice (Table 3). At the end of gestation, FS was significantly decreased in R^+A^+ mothers compared with baseline as well as compared with NT animals. However, although we found no significant changes in systolic LV volume ($P=0.09$), a significant difference was noted in diastolic volume compared with pregestation data (Table 3). As expected, cardiac output was increased with pregnancy,[32] and although it tended to be lower in R^+A^+ mice, this difference did not reach statistical significance.

BNP and Nab1 gene expressions were evaluated to confirm that cardiac hypertrophy observed in R^+A^+ mice was indeed pathological. We detected a significant effect of mice genotype on both BNP and Nab1, because their expressions were significantly higher in the LV from R^+A^+ mice both before
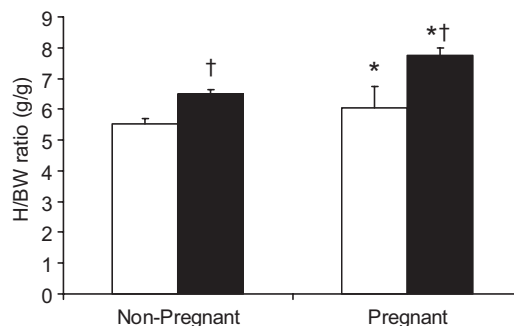
Figure 3. Heart:body weight ratio. The heart:body weight (H/BW) ratio was significantly higher in R^+A^+ mice (black, n=9) compared with R^-A^- mice (white, n=9) before pregnancy, and the phenotype was exacerbated by gestation. Also, the H/BW ratio was significantly increased by pregnancy in R^-A^- mothers. Values are expressed as mean±SE. *$P \leq 0.05$, statistically different from nonpregnant mice and †$P \leq 0.001$, statistically different from R^-A^- mice.

and at the end of gestation compared with NT mice (Figure 4), supporting the pathological phenotype of cardiac hypertrophy.

Discussion

Our results clearly demonstrate that we have characterized, to our knowledge, the first model of SPE. Chronic hypertension being a major risk factor for development of the disease, R^+A^+ mice, thus, represent a clinical reality. In fact, it has been demonstrated that the incidence of PE increases to 15% to 25% in previously hypertensive women and is detected after de novo proteinuria with a rise in arterial pressure.[22,33] In the present study, as in clinical SPE, hypertensive R^+A^+ mice manifested a significant elevation of MAP and developed severe proteinuria during gestation compared with NT animals. The fact that nonpregnant transgenic mice of similar ages or older did not develop proteinuria and that blood pressure decreased postpartum demonstrates that the phenomenon observed with gestation in these mice is not a simple progression of hypertension but is induced by gestation. Furthermore, the placentas were impaired, which resulted in a decreased pup and placental weight, indicating the presence of intrauterine growth restriction that is often associated with PE. They also manifested increased cardiac hypertrophy at the end of gestation accompanied with a decreased heart function, indicating end-organ damage. Hence, these mice truly seem to mimic the disease.

Many epidemiological studies have suggested a relation between alleles of the RAS and PE.[34] For instance, women carrying specific polymorphisms of angiotensin-converting enzyme[35] or angiotensinogen[36] genes had an increased risk of developing PE. Interestingly, these alleles have been shown to contribute to a rise in systemic RAS.[37] Hence, although PE is typically associated with a decreased circulating RAS, our model is in line with these genetic association studies, because R^+A^+ mice have elevated levels of circulating Ang II.[17]

Male R^+A^+ mice have already been well characterized.[21] For instance, it has been shown that endothelial dysfunction is present and that increased oxidative stress in this model also contributed to the vascular dysfunction.[38] We can, thus,

Table 3. Heart Parameters Calculated After Echocardiography

Time Point	Maternal Genotype	N	LV Vol Systole, μL	LV Vol Diastole, μL	LV Mass, mg	EF, %	FS, %	SV, μL	CO, mL/min
Before pregnancy	R⁻A⁻	7	23.0±2.9	56.0±4.4	85.0±7.4	59.4±3.0	31.3±2.1	27.1±2.1	12.7±1.0
	R⁺A⁺	9	29.1±3.5	57.5±4.8	112.3±4.6γ	52.9±3.0‖	30.1±2.7	25.7±2.4	11.6±1.3
End of pregnancy	R⁻A⁻	7	33.9±4.5	71.0±2.3‡	100.3±7.0‡	57.2±4.0	30.3±2.6	33.9±2.4*	15.7±1.4†
	R⁺A⁺	9	32.3±1.3	66.7±1.5‡	140.4±2.0‡§	48.3±2.8‖	22.9±0.8‖‡	32.6±1.9*	14.2±0.7†

Values are expressed as mean±SE. Vol indicates volume; SV, stroke volume; CO, cardiac output.

*$P \leq 0.001$, data are statistically different from the nonpregnant state.

†$P \leq 0.01$, data are statistically different from the nonpregnant state.

‡$P \leq 0.05$, data are statistically different from the nonpregnant state.

γ$P < 0.01$, data are statistically different from the R⁻A⁻ genotype.

§$P \leq 0.001$, data are statistically different from the R⁻A⁻ genotype.

‖$P \leq 0.05$, data are statistically different from the R⁻A⁻ genotype.

hypothesize that endothelial dysfunction may also be present in the female R⁺A⁺ mice and may, therefore, contribute to the development of SPE symptoms in this model.
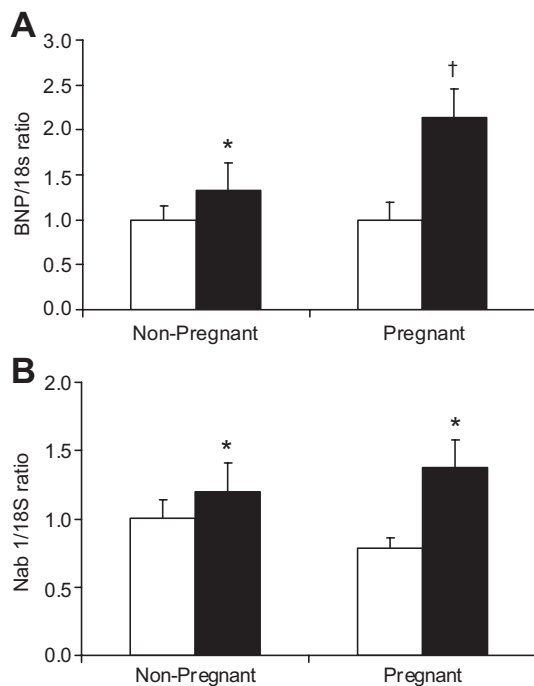
Although mice developed severe proteinuria, we did not observe any signs of renal pathology. We can hypothesize that, because these animals are exposed to increased levels of Ang II from birth, there may be some local adaptation of the renal RAS, which may account for their resistance to developing renal damage. Additional studies will be required to further investigate this issue.

In addition, placental pathology was apparent in samples obtained from R⁺A⁺ mice. More specifically, this was characterized by necrosis and loss of labyrinthine trophoblast

Figure 4. BNP (A) and Nab1 (B) gene expression in the LV. BNP and Nab1 gene expressions are significantly increased in R⁺A⁺ mice (black) compared with NT mice (white). Values are expressed as mean±SE. *$P \leq 0.05$ and †$P \leq 0.01$ are statistically different from R⁻A⁻ mice. For nonpregnant and pregnant Nab1 data, n=6 for NT and n=5 for R⁺A⁺ mice. For BNP, n=6 for nonpregnant R⁺A⁺ and NT mice, n=5 for R⁺A⁺ pregnant mice, and n=4 for NT pregnant mice.

structure. Interestingly, impaired placental perfusion and trophoblast invasion have been suggested to be implicated in the development of PE.[5] We suggest that the release of substances by the poorly perfused placenta may have systemic influences and may contribute to the rise in MAP during gestation in the R⁺A⁺ mice.

Angiogenic imbalance has been suggested by many to be implicated in the development of PE.[39] It is known that sFlt-1 levels increase significantly during pregnancy,[40] but it has been reported that these levels are higher in women suffering from PE.[41] In our model, we found that placental mRNA expression of sFlt-1 is higher in placentas from R⁺A⁺ compared with NT mothers, and the circulating concentrations are increased when analyses are adjusted for total placental mass. In line with these findings, we found a decrease in PlGF-2 protein in placentas from R⁺A⁺ mothers. Put together, these data strongly suggest the implication of an angiogenic imbalance as a possible mechanism involved in the development of SPE in our model.

In our model, the heart:body weight ratio was significantly enhanced compared with NT mice at baseline, as has been observed previously in male R⁺A⁺ mice.[38,42] Control mice also developed cardiac hypertrophy with gestation, because pregnancy is associated with physiological cardiac hypertrophy attributed to volume expansion that characterizes this state and is reversible after delivery.[43] Therefore, we suggest that the cardiac hypertrophy observed in R⁺A⁺ mice is pathological and may result from the increased pressure overload encountered by the heart during gestation, although other mechanisms may be implicated, such as different factors released by the hypoxic placenta.[44] Conversely, increased heart:body weight ratio in NT mice during gestation is a normal physiological response. The results obtained by echocardiography supported this assumption, because the EF was only affected by the genotype, clearly demonstrating that heart function is only decreased in the R⁺A⁺ mice. Furthermore, the significant deterioration in FS at the end of gestation in R⁺A⁺ mothers reinforces our hypothesis that cardiac hypertrophy is aggravated by gestation in our model. In addition, the pathological cardiac hypertrophy markers BNP[29,45] and Nab1[28] were found to be increased only in R⁺A⁺ LV, further confirming our hypothesis.

In conclusion, our results confirm that R^+A^+ mice are a novel model of SPE on chronic hypertension and, to our knowledge, it is the only characterized animal model of its kind. Because many characteristics of the pathology are observed, the model will be an important tool to study the molecular mechanisms underlying this frequently reported disease of pregnancy.

Perspectives

Additional studies will be required to further investigate these animals to determine whether other features of the disease, such as liver dysfunction and low platelets, are also present. Moreover, it will be an interesting model to investigate the impact of SPE on the maternal heart, which is still a poorly understood phenotype. Additional characterization of our model could help target pathological mechanisms implicated in the disease to eventually prevent, treat, and protect the mother and fetus.

Disclosures

None.

References

1. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. *Lancet*. 2005;365:785–799.
2. Roberts JM, Lain KY. Recent Insights into the pathogenesis of pre-eclampsia. *Placenta*. 2002;23:359–372.
3. Brown MA, Lindheimer MD, de SM, Van AA, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy*. 2001;20:IX–XIV.
4. Moutquin JM, Garner PR, Burrows RF, Rey E, Helewa ME, Lange IR, Rabkin SW. Report of the Canadian Hypertension Society Consensus Conference: 2–nonpharmacologic management and prevention of hypertensive disorders in pregnancy. *CMAJ*. 1997;157:907–919.
5. Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? *Clin Genet*. 2003;64:96–103.
6. Kanasaki K, Palmsten K, Sugimoto H, Ahmad S, Hamano Y, Xie L, Parry S, Augustin HG, Gattone VH, Folkman J, Strauss JF, Kalluri R. Deficiency in catechol-O-methyltransferase and 2-methoxyoestradiol is associated with pre-eclampsia. *Nature*. 2008;453:1117–1121.
7. Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. *Hypertension*. 2007;50:1142–1147.
8. Shah DM. Role of the renin-angiotensin system in the pathogenesis of preeclampsia. *Am J Physiol Renal Physiol*. 2005;288:F614–F625.
9. Gant NF, Daley GL, Chand S, Whalley PJ, MacDonald PC. A study of angiotensin II pressor response throughout primigravid pregnancy. *J Clin Invest*. 1973;52:2682–2689.
10. Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jupner A, Baur E, Nissen E, Vetter K, Neichel D, Dudenhausen JW, Haller H, Luft FC. Patients with preeclampsia develop agonistic autoantibodies against the angiotensin AT1 receptor. *J Clin Invest*. 1999;103:945–952.
11. Dechend R, Homuth V, Wallukat G, Kreuzer J, Park JK, Theuer J, Juepner A, Gulba DC, Mackman N, Haller H, Luft FC. AT(1) receptor agonistic antibodies from preeclamptic patients cause vascular cells to express tissue factor. *Circulation*. 2000;101:2382–2387.
12. Rothermel A, Kurz R, Ruffer M, Weigel W, Jahnke HG, Sedello AK, Stepan H, Faber R, Schulze-Forster K, Robitzki AA. Cells on a chip–the use of electric properties for highly sensitive monitoring of blood-derived factors involved in angiotensin II type 1 receptor signalling. *Cell Physiol Biochem*. 2005;16:51–58.
13. Dechend R, Muller DN, Wallukat G, Homuth V, Krause M, Dudenhausen J, Luft FC. Activating auto-antibodies against the AT1 receptor in pre-eclampsia. *Autoimmun Rev*. 2005;4:61–65.
14. Cooper AC, Robinson G, Vinson GP, Cheung WT, Broughton PF. The localization and expression of the renin-angiotensin system in the human placenta throughout pregnancy. *Placenta*. 1999;20:467–474.
15. Takimoto E, Ishida J, Sugiyama F, Horiguchi H, Murakami K, Fukamizu A. Hypertension induced in pregnant mice by placental renin and maternal angiotensinogen. *Science*. 1996;274:995–998.
16. Bohlender J, Ganten D, Luft FC. Rats transgenic for human renin and human angiotensinogen as a model for gestational hypertension. *J Am Soc Nephrol*. 2000;11:2056–2061.
17. Merrill DC, Thompson MW, Carney C, Schlager G, Robillard JE, Sigmund CD. Chronic hypertension and altered baroreflex responses in transgenic mice containing the human renin and human angiotensinogen genes. *J Clin Invest*. 1996;97:1047–1055.
18. Fukamizu A, Sugimura K, Takimoto E, Sugiyama F, Seo M-S, Takahashi S, Hatae T, Kajiwara N, Yagami K, Murakami K. Chimeric renin-angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes. *J Biol Chem*. 1993;268:11617–11621.
19. Sugiyama F. Development of genetically engineered mice with hypertension and hypotension. *Exp Anim*. 1997;46:171–182.
20. Ganten D, Wagner J, Zeh K, Koeller M, Bader M, Michel JB, Paul M, Zimmermann F, Ruf P, Hilgenfeld U, Ganten U, Kaling M, Bachmann S, Mullins JJ, Murakami K. Species-specificity of renin kinetics in transgenic rats harboring the human renin and angiotensinogen genes. *Proc Natl Acad Sci U S A*. 1992;89:7806–7810.
21. Lavoie JL, Bianco RA, Sakai K, Keen HL, Ryan MJ, Sigmund CD. Transgenic mice for studies of the renin-angiotensin system in hypertension. *Acta Physiol Scand*. 2004;181:571–577.
22. Podymow T, August P. Hypertension in pregnancy. *Adv Chronic Kidney Dis*. 2007;14:178–190.
23. Sigmund CD, Jones CA, Kane CM, Wu C, Lang JA, Gross KW. Regulated tissue- and cell-specific expression of the human renin gene in transgenic mice. *Circ Res*. 1992;70:1070–1079.
24. Yang G, Merrill DC, Thompson MW, Robillard JE, Sigmund CD. Functional expression of the human angiotensinogen gene in transgenic mice. *J Biol Chem*. 1994;269:32497–32502.
25. Falcao S, Solomon C, Monat C, Berube J, Gutkowska J, Lavoie JL. Impact of diet and stress on the development of preeclampsia-like symptoms in p57kip2 mice. *Am J Physiol Heart Circ Physiol*. 2009;296:H119–H126.
26. Falcao S, Bisotto S, Gutkowska J, Lavoie JL. Hyperhomocysteinemia is not sufficient to cause preeclampsia in an animal model: the importance of folate intake. *Am J Obstet Gynecol*. 2009;200:198.e1–198.e5.
27. Stoyanova E, Trudel M, Felfly H, Garcia D, Cloutier G. Characterization of circulatory disorders in beta-thalassemic mice by noninvasive ultrasound biomicroscopy. *Physiol Genomics*. 2007;29:84–90.
28. Buitrago M, Lorenz K, Maass AH, Oberdorf-Maass S, Keller U, Schmitteckert EM, Ivashchenko Y, Lohse MJ, Engelhardt S. The transcriptional repressor Nab1 is a specific regulator of pathological cardiac hypertrophy. *Nat Med*. 2005;11:837–844.
29. Iso T, Arai M, Wada A, Kogure K, Suzuki T, Nagai R. Humoral factor(s) produced by pressure overload enhance cardiac hypertrophy and natriuretic peptide expression. *Am J Physiol*. 1997;273:H113–H118.
30. Jankowski M, Wang D, Mukaddam-Daher S, Gutkowska J. Pregnancy alters nitric oxide synthase and natriuretic peptide systems in the rat left ventricle. *J Endocrinol*. 2005;184:209–217.
31. Mutter WP, Karumanchi SA. Molecular mechanisms of preeclampsia. *Microvasc Res*. 2008;75:1–8.
32. Chapman AB, Abraham WT, Zamudio S, Coffin C, Merouani A, Young D, Johnson A, Osorio F, Goldberg C, Moore LG, Dahms T, Schrier RW. Temporal relationships between hormonal and hemodynamic changes in early human pregnancy. *Kidney Int*. 1998;54:2056–2063.

33. Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol*. 2000;183:S1–S22.

34. Mutze S, Rudnik-Schoneborn S, Zerres K, Rath W. Genes and the preeclampsia syndrome. *J Perinat Med*. 2008;36:38–58.

35. Mello G, Parretti E, Gensini F, Sticchi E, Mecacci F, Scarselli G, Genuardi M, Abbate R, Fatini C. Maternal-fetal flow, negative events, and preeclampsia: role of ACE I/D polymorphism. *Hypertension*. 2003; 41:932–937.

36. Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C, Farrington PF, Ogasawara M, Suzumori K, Tomoda S, Berrebi S, Sasaki M, Corvol P, Lifton RP, Lalouel J-M. A molecular variant of angiotensinogen associated with preeclampsia. *Nature Genetics*. 1993;4:59–61.

37. Medica I, Kastrin A, Peterlin B. Genetic polymorphisms in vasoactive genes and preeclampsia: a meta-analysis. *Eur J Obstet Gynecol Reprod Biol*. 2007;131:115–126.

38. Didion SP, Ryan MJ, Baumbach GL, Sigmund CD, Faraci FM. Superoxide contributes to vascular dysfunction in mice that express human renin and angiotensinogen. *Am J Physiol Heart Circ Physiol*. 2002;283:H1569–H1576.

39. Maynard S, Epstein FH, Karumanchi SA. Preeclampsia and angiogenic imbalance. *Annu Rev Med*. 2008;59:61–78.

40. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA. Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med*. 2004;350:672–683.

41. Baumann MU, Bersinger NA, Surbek DV. Serum markers for predicting pre-eclampsia. *Mol Aspects Med*. 2007;28:227–244.

42. Ryan MJ, Didion SP, Mathur S, Faraci FM, Sigmund CD. PPAR(gamma) agonist rosiglitazone improves vascular function and lowers blood pressure in hypertensive transgenic mice. *Hypertension*. 2004;43: 661–666.

43. Eghbali M, Wang Y, Toro L, Stefani E. Heart hypertrophy during pregnancy: a better functioning heart? *Trends Cardiovasc Med*. 2006;16: 285–291.

44. Maynard SE, Venkatesha S, Thadhani R, Karumanchi SA. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. *Pediatr Res*. 2005;57:1R–7R.

45. Uhler MD, McKnight GS. Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. *J Biol Chem*. 1987; 262:15202–15207.

ONLINE SUPPLEMENT

MICE OVEREXPRESSING BOTH HUMAN ANGIOTENSINOGEN AND HUMAN RENIN
AS A MODEL OF SUPERIMPOSED PREECLAMPSIA ON CHRONIC HYPERTENSION

Stéphanie Falcao[1, 2], Ekatherina Stoyanova[2, 3], Guy Cloutier[3, 4], Roch Listz Maurice[3, 4], Jolanta Gutkowska[1, 5], Julie L. Lavoie[1, 5]

[1]Centre de Recherche du Centre hospitalier de l'Université de Montréal (CRCHUM), Montréal, Québec, Canada. [2]Department of Biomedical Sciences, Université de Montréal, Montréal, Québec, Canada. [3]Laboratory of Biorheology and Medical Ultrasonics, CRCHUM, Montréal, Québec, Canada. [4]Department of Radiology, Radio-Oncology and Nuclear Medicine, and Institute of Biomedical Engineering, Université de Montréal, Montréal, Québec, Canada. [5]Department of Medicine, Université de Montréal, Montréal, Québec, Canada.

Short Title: A new mouse model of superimposed preeclampsia

Word count of manuscript: 2 066 words

Number of Figures: 3

Corresponding author: **Dr Julie L. Lavoie**
                                   Centre de recherche, CHUM-Technopôle Angus
                                   2901 Rachel St. East, Suite 310
                                   Montréal, Québec H1W 4A4, Canada
                                   Tel: (514) 890-8000 ext. 23612
                                   Fax: (514) 412-7655
                                   email: julie.lavoie.3@umontreal.ca

Materials and Methods

Animals. Transgenic mice overexpression human renin and angiotensinogen (R^+A^+) were produced by breeding heterozygous human renin (Ren9 line) mice[1] with heterozygous human angiotensinogen (204/1 line) mice[2]. Both these single transgenic lines, obtained from Dr. Curt D. Sigmund at the University of Iowa, were maintained in our animal facility by backcrossing with C57BL/6 mice (Charles River, St-Constant, QC, Canada). The mouse genotype was determined as described previously[1, 2]. The animals were kept on a 12-h light/dark cycle with water and standard laboratory chow (2018; Teklab Premier Laboratory Diets, Madison, WI) *ad libitum*. Mice in these experiments were 12-15 weeks of age, and were separated in 4 groups (Non-pregnant R^+A^+ and non-transgenic (R^-A^-); Pregnant R^+A^+ and R^-A^-). Their care met the standards set forth by the Canadian Council on Animal Care for the use of experimental animals. All procedures were approved by the University Animal Care Committee of the CHUM Research Centre.

Blood pressure measurement. Arterial pressure (AP) and heart rate (HR) were directly measured by telemetry with the TA11PA-C10 probes (Data Sciences International, St. Paul, MN). Probe catheter was inserted into the left carotid artery of female mice anesthetized by inhalation of isoflurane (induction at 4% and maintained at 2%) in oxygen[3, 4]. HR and AP were recorded for 3 consecutive days after 10 days of recovery post-surgery. Male mice were then placed in the cages for timed mating. Gestation was confirmed by the presence of a vaginal plug and was considered as day 1. Starting on this day, AP and HR were assessed every 2 days up to day 19 with measurements collected continuously until 24 h post-partum.

Proteinuria. Urine samples were collected before and on day 18 of gestation by briefly restraining the mice and directly retrieving urine in 1.5-ml tubes as done previously[3, 4]. This method avoids the unnecessary stress of placing the animals in metabolic cages for 24 hours. Indeed, it has been shown that mice of both sex present a significant rise in mean AP (MAP) and HR when placed in metabolic cages[5]. The urine samples were kept frozen at -80°C until albumin and creatinine concentrations were quantitated with Albuwell and Creatinine companion mouse ELISA kits (Exocell, Philadelphia, PA), respectively, according to the manufacturer's protocol. Each sample was thawed and diluted 1:10 before quantitation in duplicate.

Tissue collection and histology. On day 18 of gestation, mice were anesthetized by intra-peritoneal injection of ketamine/xylazine (0.1ml/20g of mouse body weight of a 100mg/ml:20mg/ml solution). Blood was collected by heart punction and placed in chilled 1.5 ml tubes containing EDTA (EMD, Gibbstown, NJ, USA). Plasma was separated by centrifugation and samples were kept frozen at -80°C until assayed. The pups were weighed, and their tails were cut and kept for genotyping. Kidneys, hearts, and placentas were all collected, weighed, and either flash-frozen in liquid nitrogen or placed overnight in 4% paraformaldehyde for fixation. The fixed tissues were washed 24 h after with phosphate buffer and then embedded in paraffin. Sections were obtained by cross-sectionally cutting the fixed tissues in a microtome. To evaluate renal and placental morphology, the sections were stained with hematoxylin phloxine saffron (HPS) and evaluated by light microscopy. Embedding, sectioning and staining were achieved by the histology platform of the Research Institute in Immunology and Cancerology at the Université de Montréal.

Placental alterations were characterized by 5 criteria: necrosis, hyalinization, microcalcification, giant cell island loss and labyrinthine trophoblast structure loss as done previously[3, 4]. The latter two are respectively analogous to human extravillous cytotrophoblasts cells and chorionic villi structure[6]. A score from 0 to 3 was assigned for each criterion: 0 for no change, 1 for mild, 2 for moderate, and 3 for severe alteration. All scores were then summed up for total evaluation of the placental pathology present. The investigator scoring the tissues was blinded to the genotype of the pups and mothers to avoid any bias.

Echocardiography. Transthoracic echocardiographic studies were performed before and at the end of pregnancy. The mice were anesthetized by inhalation of isoflurane 2-3% in oxygen, and then maintained on 2%. Their hearts were investigated as previously described[7] by high-resolution ultrasound biomicroscopy (Vevo660; Visualsonics, Toronto, ON, Canada) equipped with 25-55 MHz probes that allow tracings of time-varying M-mode dimensions of the left ventricle (LV). Positioning of the M-lines was guided by B-mode echocardiography. The parasternal long-axis view served to capture M-mode tracings through the anterior and posterior LV walls at the level of the papillary muscle. The M-mode sampling line was positioned perpendicularly to the ascending aorta at the LV exit to record aortic diameter (AoD). Aortic flow velocity was measured in pulsed-wave Doppler mode, and the envelope of the Doppler tracing was delineated manually to compute the velocity time integral (VTI). LV mass was directly calculated by the apparatus's software. The ejection fraction (EF) was estimated by the formula: $EF = (LVEDV - LVESV) \times 100 / LVEDV$, where LVEDV and LVESV are respectively LV end-diastolic and end-systolic volumes. LV volumes were determined as $7 \times LVD^3 / (2.4 + LVD)$, where left ventricular diameter (LVD) is substituted by LVEDD for LV diastolic volume or LVESD for LV systolic volume. LV fractional shortening (FS) was given by $(LVEDD - LVESD) \times 100 / LVEDD$, whereas stroke volume (SV) was measured as: $SV = (AoD / 2)^2 \times \pi \times VTI$. SV times HR gives estimated cardiac output (CO).

Western Blot. Frozen placentas were lyophilized and subsequently homogenised in lysis buffer. Total protein content was measured in supernatants by standard Bradford assay. Samples containing 50µg of protein were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and separated by electrophoresis. Proteins were transferred on a nitrocellulose membrane (Whatman inc., Piscataway, NJ). Non-specific sites were blocked for one hour at room temperature in 5% non-fat milk in SuperBlock buffer (Thermo Fisher Scientific, Rockford, IL). Membrane was then incubated with the primary monoclonal antibody anti-PlGF-2 (1:100, 1:10 Superblock in 0.2% Tween 20 (Fischer Scientific, Ottawa, ON, Canada) in TBS) (Abcam, Cambridge, MA) overnight at 4˚C. Secondary antibody, anti-rat conjugated with horseradish peroxidase (1:1000, 1:10 Superblock in 0.2% Tween 20 (Fischer Scientific) in TBS) (Santa Cruz Biothecnology, Santa Cruz, CA) was applied for 1h at room temperature. Bands were revealed using the ECL West Pico kit (Pierce, Rockford, IL). Total protein was subsequently measured by staining of the membrane with Amido Black (Sigma, St-Louis, MO). PlGF-2 signal was normalized to total protein for each band.

Real-time PCR. Brain natriuretic peptide (BNP) and NGF1-A-binding protein (Nab1) were both amplified in left ventricles, while fms-like tyrosine kinase-1 (sFlt-1) was amplified in placentas. BNP and Nab1 are both reported to be specific markers of pathological cardiac hypertrophy, as they are markedly increased in this condition, whereas no differences are detected in

physiological hypertrophy such as exercise training[8, 9]. Thus, their expression was measured to differentiate physiological heart hypertrophy in pregnancy and hypertension-derived pathological cardiac remodelling. Total RNA was extracted from frozen samples using Trizol (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. To remove genomic DNA, RNA samples were incubated with 2 U deoxyribonuclease I (DNase I; Invitrogen)/ug RNA for 30 min at 37°C. Single-stranded cDNA was synthesized by reverse-transcriptase reaction with Moloney Murine Leukemia Virus (M-MLV) (Invitrogen). PCR was undertaken in the iCycler IQ Real Time PCR detection System (Bio-Rad Laboratories, Hercules, CA), using SYBR® green chemistry[10]. In brief, 2 µl of diluted cDNA was added to an 18 µl reaction mixture containing 1X iQ SYBR Green Supermix (Bio-Rad Laboratories) and 200 nM forward and reverse primers (Invitrogen). The following primer sets were employed to generate amplicons: Nab1 strand (s): CAGGCCGGGTATGAGAGG; Nab1 anti-strand (as): GCTGTCGGTTCTGCACACT; BNP (s): CTGAAGGTGCTGTCCCAGAT; BNP (as): GTTCTTTTGTGAGGCCTTGG; sFlt-1 (s): AGGTGAGCACTGCGGCA ; sFlt-1 (as): ATGAGTCCTTTAATGTTTGA;18S Universal 18S internal standards (#1718; Ambion, Streetsville, ON, Canada). Each sample was run and analyzed in duplicate. mRNA levels were expressed as values relative to 18S mRNA.

Drugs. The following drugs were purchased for mouse anesthesia: ketamine (Bimeda-MTC, Cambridge, ON, Canada), xylazine (Bayer, Toronto, ON, Canada) and isoflurane (Abbott, St-Laurent, QC, Canada).

Results

LVID was significantly smaller at systole in R^+A^+ mice, both before and at the end of gestation, as their LVPW was significantly thicker when compared to non-transgenic mice (Table S5). The IVS thickness remained similar in all animals (Table S5). LV mass followed the same pattern as the total heart-to-body weight ratio.

References

1. Sigmund CD, Jones CA, Kane CM, Wu C, Lang JA, Gross KW. Regulated tissue- and cell-specific expression of the human renin gene in transgenic mice. *Circ Res*. 1992; 70:1070-1079.

2. Yang G, Merrill DC, Thompson MW, Robillard JE, Sigmund CD. Functional expression of the human angiotensinogen gene in transgenic mice. *J Biol Chem*. 1994; 269:32497-32502.

3. Falcao S, Solomon C, Monat C, Berube J, Gutkowska J, Lavoie JL. Impact of diet and stress on the development of preeclampsia-like symptoms in p57kip2 mice. *Am J Physiol Heart Circ Physiol*. 2009; 296:H119-H126.

4. Falcao S, Bisotto S, Gutkowska J, Lavoie JL. Hyperhomocysteinemia is not sufficient to cause preeclampsia in an animal model: the importance of folate intake. *Am J Obstet Gynecol*. 2009; 200:198.e1-198.e5.

5. Hoppe CC, Moritz KM, Fitzgerald SM, Bertram JF, Evans RG. Transient hypertension and sustained tachycardia in mice housed individually in metabolism cages. *Physiol Res*. 2009; 58:69-75.

6. Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? *Clin Genet*. 2003; 64:96-103.

7. Stoyanova E, Trudel M, Felfly H, Garcia D, Cloutier G. Characterization of circulatory disorders in beta-thalassemic mice by noninvasive ultrasound biomicroscopy. *Physiol Genomics*. 2007; 29:84-90.

8. Buitrago M, Lorenz K, Maass AH, Oberdorf-Maass S, Keller U, Schmitteckert EM, Ivashchenko Y, Lohse MJ, Engelhardt S. The transcriptional repressor Nab1 is a specific regulator of pathological cardiac hypertrophy. *Nat Med*. 2005; 11:837-844.

9. Iso T, Arai M, Wada A, Kogure K, Suzuki T, Nagai R. Humoral factor(s) produced by pressure overload enhance cardiac hypertrophy and natriuretic peptide expression. *Am J Physiol*. 1997; 273:H113-H118.

10. Jankowski M, Wang D, Mukaddam-Daher S, Gutkowska J. Pregnancy alters nitric oxide synthase and natriuretic peptide systems in the rat left ventricle. *J Endocrinol*. 2005; 184:209-217.

Table S1. Maternal body weight

| Maternal genotype	N	Pre-gestational BW (mg)	End of gestation BW (mg)
R^-A^-	10	20.69 ± 1.17	33.59 ± 3.26
R^+A^+	9	22.76 ± 2.11	34.67 ± 4.43

BW, body weight

Table S2. Heart parameters calculated following echocardiography.

Time point	Maternal genotype	N	LV ID systole (mm)	LV ID diastole (mm)	LV PW systole (mm)	LV PW diastole (mm)	IVS systole (mm)	IVS diastole (mm)
Before pregnancy	R^-A^-	7	2.50 ± 0.14	3.63 ± 0.12	1.10 ± 0.09	0.77 ± 0.06	0.89 ± 0.11	1.32 ± 0.14
	R^+A^+	9	2.63 ±0.14†	3.71 ± 0.10	1.26 ± 0.04†	0.91 ± 0.03*	0.98 ± 0.04	1.47 ± 0.09
End of pregnancy	R^-A^-	7	2.80 ± 0.13	4.01 ±0.06‡	1.02 ± 0.06	0.73 ± 0.04	0.93 ± 0.08	1.36 ± 0.11
	R^+A^+	9	2.97 ±0.11†	3.89 ±0.12‡	1.28 ± 0.06†	1.05 ± 0.04*	1.15 ± 0.05	1.56 ± 0.06

Values are expressed as means ± SE. * $p \leq 0.001$ and † $p \leq 0.05$, statistically different from the R-A- genotype, and ‡ $p \leq 0.05$, statistically different from the non-pregnant state. LVID, Left ventricular internal diameter; LVPW, Left ventricular posterior wall thickness; IVS, Interventricular septum.
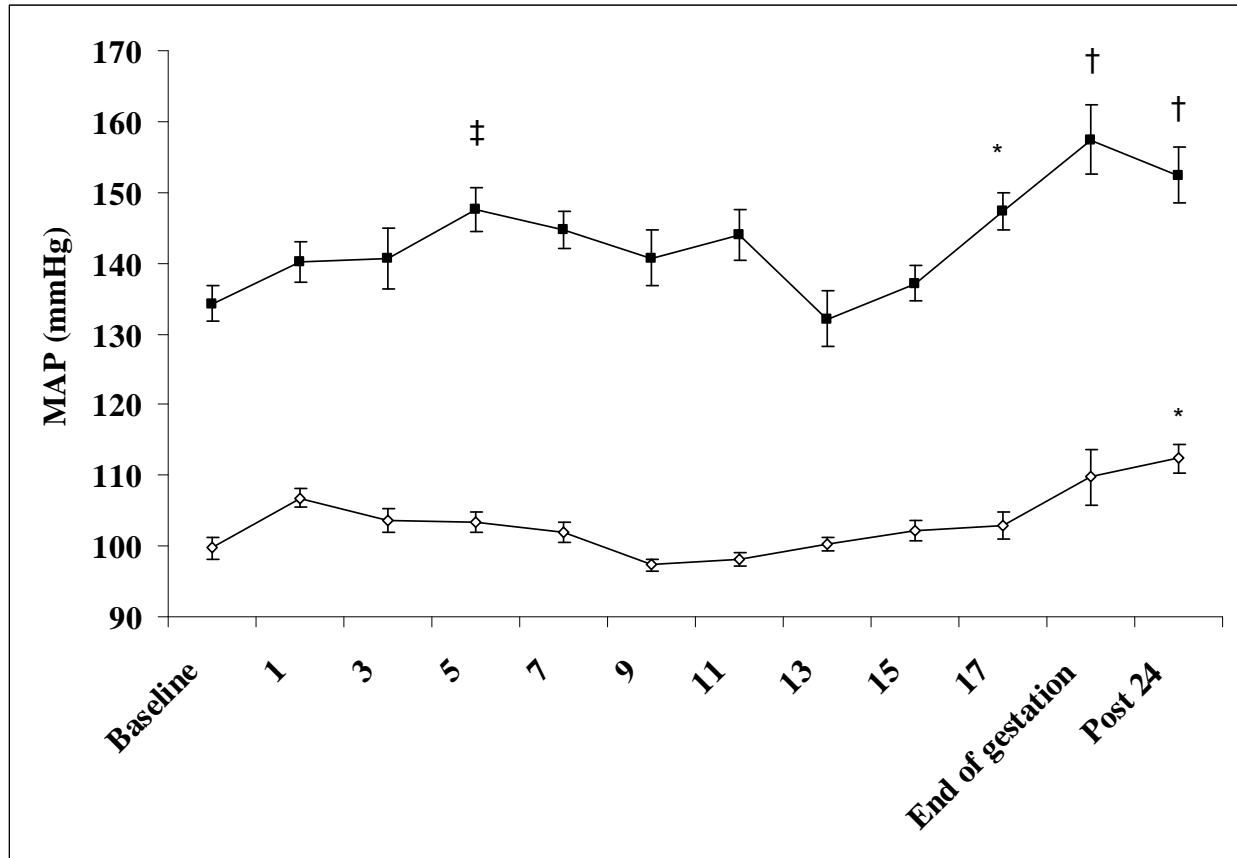
Figure S1. MAP throughout pregnancy. Average MAP during pregnancy significantly increased in R^+A^+ mice (black squares, n = 6) at days 5, 17, at the end of pregnancy and 24PP postpartum in comparison to their baseline as measured by telemetry. In R^-A^- mice (white diamonds, n = 7), it was also significantly increased at 24h postpartum in comparison to their baseline. Values are expressed as means ± SE. * p≤0.05 and † p≤0.001, and ‡ p≤0.01 statistically different from baseline. END, end of gestation; Post 24, 24 h post-partum.
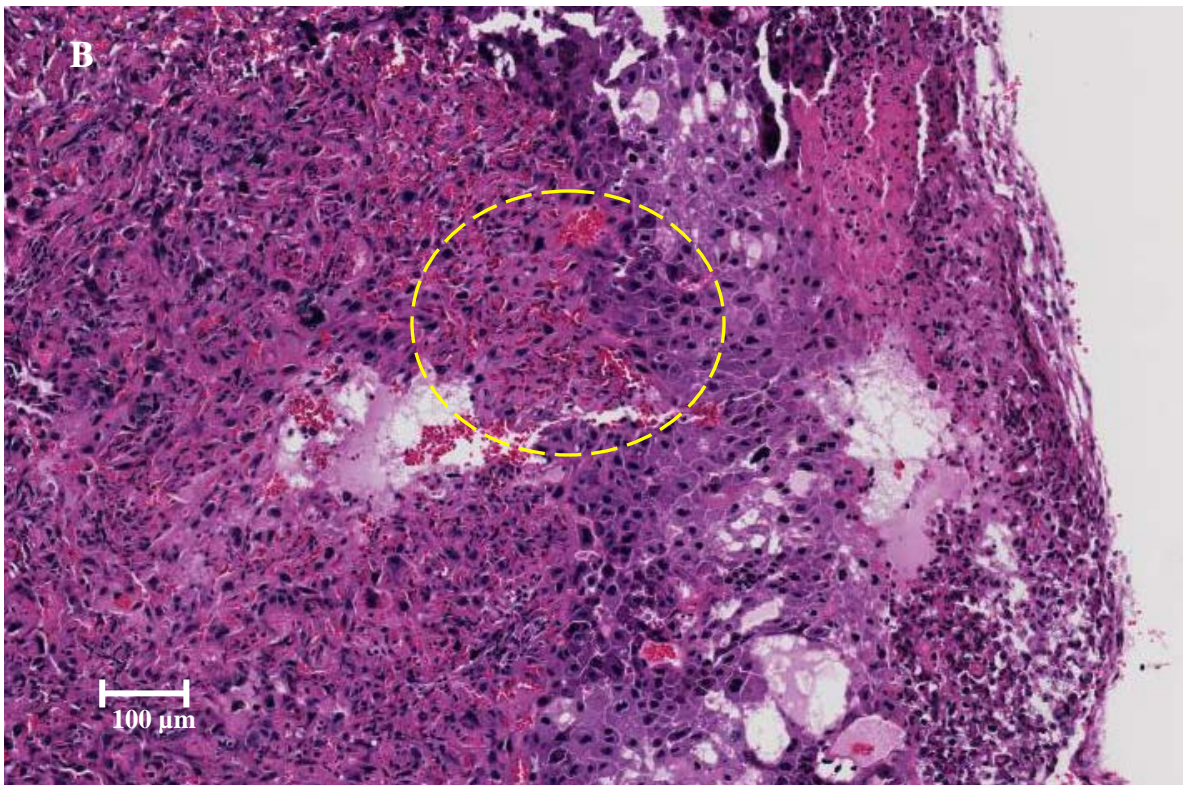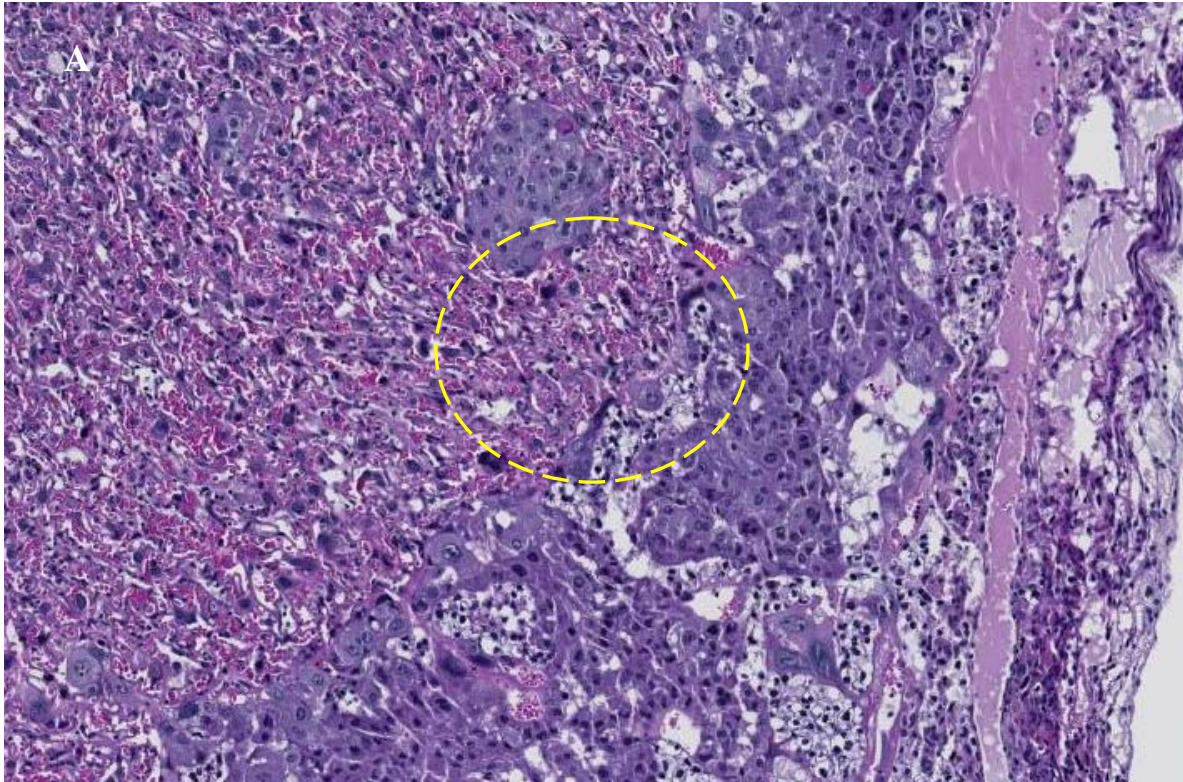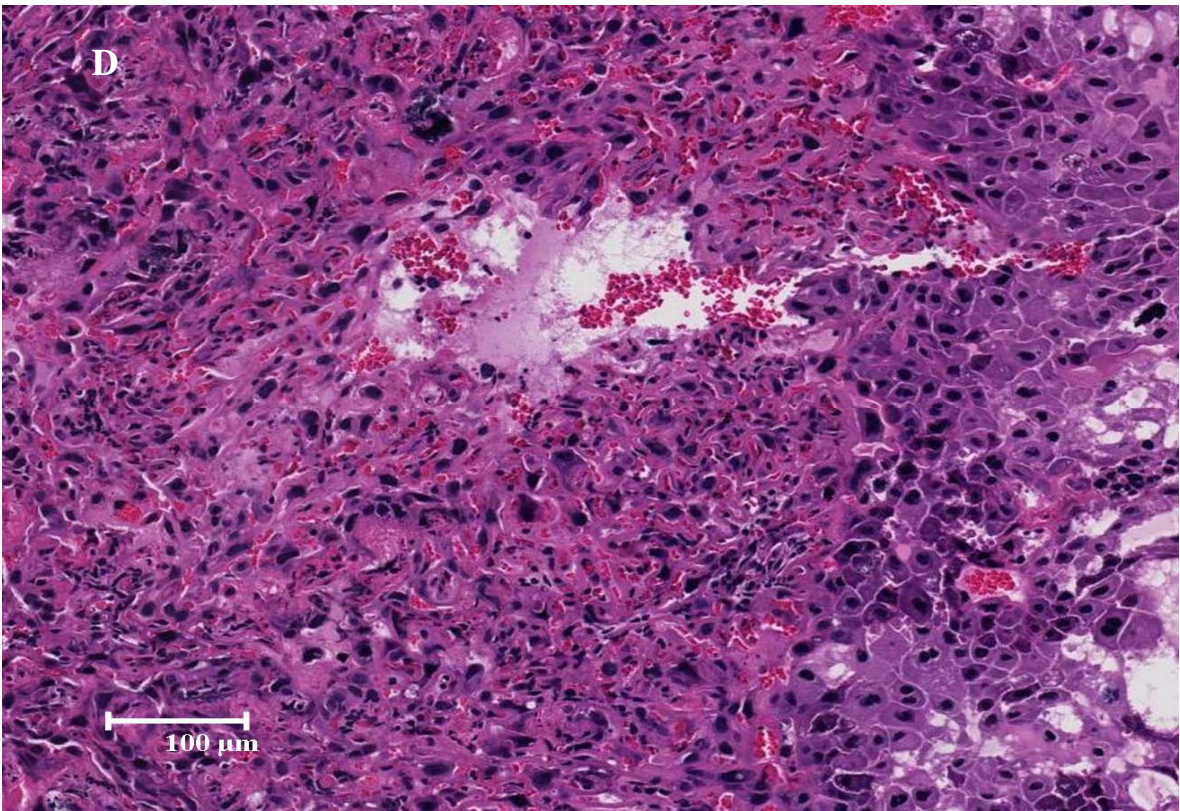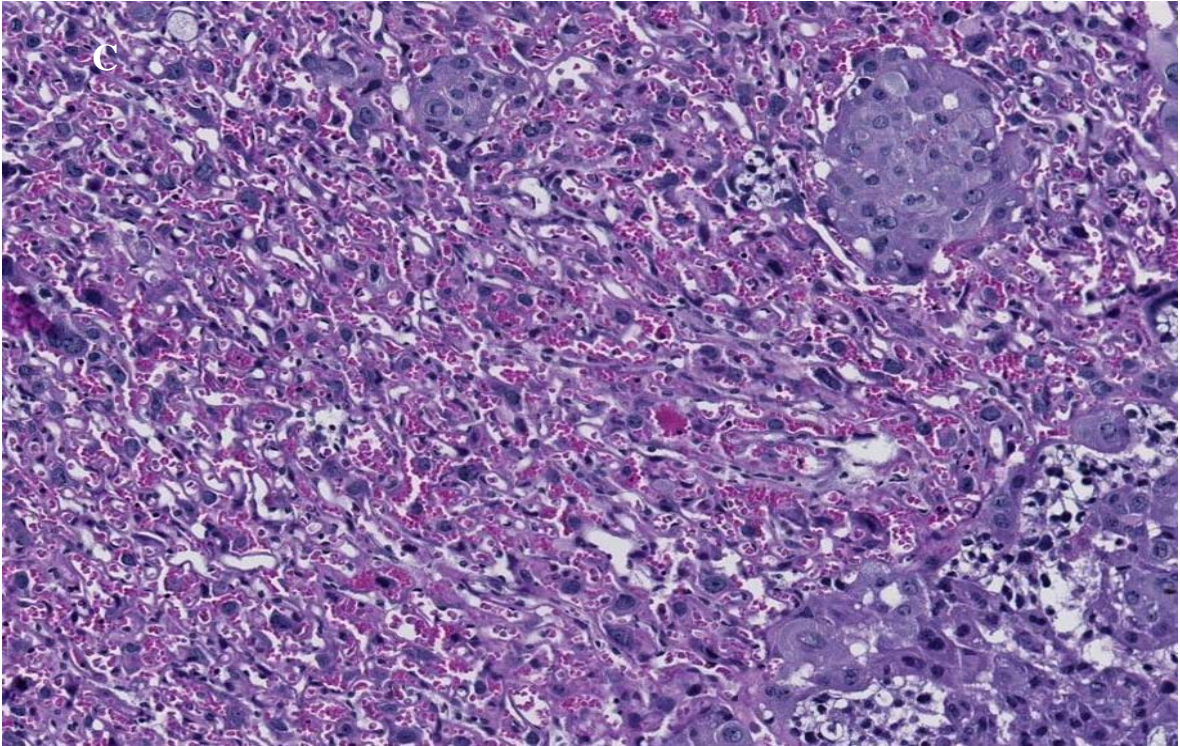
Figure S2. Placental histology. These images demonstrate typical HPS staining in placentas from A) R⁻A⁻ and B) R⁺A⁺ mothers. C) and D) represent a greater magnification of the encircled region of the previous images in the same order. Placental pathology can be clearly observed in the R⁺A⁺ placenta as loss of labyrinthine trophoblast structure on this panel. This is characterized by a much more compact appearance of the labyrinth layer as compared to R⁻A⁻ where cells are well defined and dispersed as is delineated by a yellow circle on both panels. HPS, hematoxylin phloxine saffron.
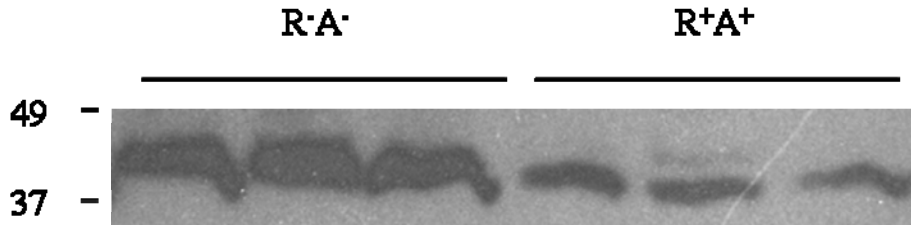
Figure S3. PlGF-2 Western Blot. This image is a representative Western Blot of PlGF-2 on placentas from R⁻A⁻ and R⁺A⁺ mothers. Signal was observed at 38 kDa.