Preeclampsia

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 elucidation. 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 women10–13 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 mice15 or rats16 overexpressing human angiotensinogen were mated with males carrying the human
renin gene (female hAogen×male hRen). Because angioten-
sinogen 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 utero-
placental 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.21–23 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) mice23 with heterozygous human angioten-
sinogen (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 libi-
tum. Female mice in these experiments were 12 to 15 weeks of age
and were separated into 4 groups (nonpregnant R′A′ mice [R′A′];
pregnant R′A′ mice [R′A′]; pregnant R′A′ and R′A′ mice, as measured
by telemetry. Values
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 concen-
trations 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

Transhormonal 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 previously27
by high-resolution ultrasound microscopy (Vevo660; Visualson-
ics) 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 (PIGF-2; Abcam) antiserum using enhanced chemi-
luminescence West Pico kits (Pierce). Total protein was subse-
quently measured by staining of the membrane with Amido black
(Sigma), and the PIGF-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.26,29
Table 1. Characterization of Placental Pathology

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>Necrosis</th>
<th>Hyalinitization</th>
<th>Microcal</th>
<th>GCIL</th>
<th>LLTS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Mdn</td>
<td>75%</td>
<td>Mdn</td>
<td>75%</td>
<td>Mdn</td>
</tr>
<tr>
<td>R^-A^-</td>
<td>52</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>R^+A^+</td>
<td>31</td>
<td>1†</td>
<td>2</td>
<td>2*</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

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<0.001, data are statistically different from the R^-A^- genotype.
†P<0.01, data are statistically different from the R^-A^- genotype.
Table 2. Pup and Placental Weight

<table>
<thead>
<tr>
<th>Maternal Genotype</th>
<th>N</th>
<th>Pup Weight, mg</th>
<th>Placental Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>R(^<em>) A(^</em>)</td>
<td>59</td>
<td>806±162</td>
<td>129±27</td>
</tr>
<tr>
<td>R(^<em>) A(^</em>)</td>
<td>54</td>
<td>682±142*</td>
<td>102±18*</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE. *P<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 placentental expression in our transgenic mice, we further investigated this issue. Because it is well known that increased circulating sFlt-1 levels during pregnancy originate from the placenta\(^31\) and that mice have a variable number of pups per litter, we evaluated the role of the total placentical mass in the levels of circulating sFlt1. Doing so, we determined that its plasmatic concentrations were significantly higher in R\(^*\) A\(^*\) mice when total placentical mass was used as a cofactor in 1-ANOVA analysis (P<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≤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 both 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 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,
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 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 that the circulating concentrations are increased when analyses are adjusted for total placental mass. In line with these findings, we found a decrease in PIGF-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.

### Table 3. Heart Parameters Calculated After Echocardiography

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

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

*P<0.001, data are statistically different from the nonpregnant state.
†P<0.01, data are statistically different from the nonpregnant state.
‡P<0.05, data are statistically different from the nonpregnant state.
§P=0.001, data are statistically different from the R\(^+\)A\(^+\) genotype.
∥P=0.01, data are statistically different from the R\(^+\)A\(^+\) genotype.

![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).](image)

**A**

- **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<0.05 and †P<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.
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.

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**Disclosures**

None.

**References**

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

Animals. Transgenic mice overexpression human renin and angiotensinogen (R+A+) were produced by breeding heterozygous human renin (Ren9 line) mice1 with heterozygous human angiotensinogen (204/1 line) mice2. 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 previously1, 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 oxygen3, 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 previously3, 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 cages5. 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 intraperitoneal injection of ketamine/xylazine (0.1ml/20g of mouse body weight of a 100mg/ml:20mg/ml solution). Blood was collected by heart puncture 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. The latter two are respectively analogous to human extravillous cytotrophoblasts cells and chorionic villi structure. 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 by high-resolution ultrasound microscopy (Veo660; 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: $EF = \frac{(LVEDV – LVESV) \times 100}{LVEDV}$, 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-PIGF-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 Biootechnology, 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). PIGF-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. 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) 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. 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): ATGAGTCCTTTAATGTTTG; 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


**Table S1. Maternal body weight**

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>N</th>
<th>Pre-gestational BW (mg)</th>
<th>End of gestation BW (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R'A^-</td>
<td>10</td>
<td>20.69 ± 1.17</td>
<td>33.59 ± 3.26</td>
</tr>
<tr>
<td>R'^A'^+</td>
<td>9</td>
<td>22.76 ± 2.11</td>
<td>34.67 ± 4.43</td>
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</tbody>
</table>

BW, body weight
Table S2. Heart parameters calculated following echocardiography.

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

Values are expressed as means ± SE. * p ≤ 0.001 and † p ≤ 0.05, statistically different from the R⁻A⁻ genotype, and ‡ p ≤ 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.