Novel Role of the Renin–Angiotensin System in Preeclampsia Superimposed on Chronic Hypertension and the Effects of Exercise in a Mouse Model

Dominique S. Genest, Stéphanie Falcao, Catherine Michel, Sonia Kajla, Mark F. Germano, Andrée-Anne Lacasse, Cathy Vaillancourt, Jolanta Gutkowska, Julie L. Lavoie

Abstract—Gestational hypertensive disorders, such as preeclampsia, affect 6% to 8% of all pregnancies in North America, and they are the leading cause of maternal mortality in industrialized countries, accounting for 16% of deaths. Women with hypertension have an increased risk (15% to 25%) of developing preeclampsia. Our aim was to investigate the mechanisms implicated in preeclampsia superimposed on chronic hypertension and in the protective effects of exercise in a mouse model. Female mice overexpressing human angiotensinogen and human renin were used as a model of preeclampsia superimposed on chronic hypertension. In the trained group, mothers were placed in cages with access to a wheel before mating, and they remained within these throughout gestation. Blood pressure was measured by telemetry. We found that angiotensin II type I receptor was increased, whereas the Mas receptor was decreased in the placenta and the aorta of pregnant sedentary transgenic mice. This would produce a decrease in angiotensin-(1–7) effects in favor of angiotensin II. Supporting the functional contribution of this modulation, we found that the prevention of most pathological features in trained transgenic mice was associated with a normalization of placental angiotensin II type 1 and Mas receptors and an increase in aortic Mas receptor. We also found reduced circulating and placental soluble Fms-like tyrosine kinase-1 in trained transgenic mice compared with sedentary mice. This study demonstrates that modulation of the renin–angiotensin system is a key mechanism in the development of preeclampsia superimposed on chronic hypertension, which can be altered by exercise training to prevent disease features in an animal model. (*Hypertension. 2013;62:1055-1061.*)

Key Words: angiogenic factor • angiotensin-(1–7) • exercise • preeclampsia • renin–angiotensin system

Gestational hypertensive disorders, such as preeclampsia, affect 6% to 8% of all pregnancies in the United States and Canada, and they have been identified as the leading cause of maternal mortality in industrialized countries, accounting for 16% of deaths. Chronic hypertension is an important risk factor for this pathology because 15% to 25% of the women with hypertension develop preeclampsia superimposed on chronic hypertension (PESCH) during their pregnancy. PESCH is thought to be comparable in pregnancy outcomes with that of severe preeclampsia. However, few studies have investigated the mechanisms implicated in this specific type of preeclampsia. For instance, there are no data on what may trigger the sudden increase in blood pressure during pregnancy, which was observed only in some women with hypertension. As such, given the poorly understood cause of this disease, the only treatment available is the premature delivery of the fetus.

We have recently characterized a new animal model of PESCH. These mice overexpress both human angiotensinogen and renin (R’A’), and as such, these mice are hypertensive at baseline. During their pregnancy, these mice spontaneously develop PESCH-like features as their blood pressure increases, and they develop proteinuria. To our knowledge, it is the only model of its kind. Furthermore, it is particularly relevant because many studies are now pointing to the involvement of the renin–angiotensin system (RAS) in the development of preeclampsia. Indeed, preeclampsia is characterized by heightened sensitivity to vasoconstrictors when compared with normal pregnancy partly because of an upregulation of the angiotensin II (Ang II) type 1 receptors (AT1R). Interestingly, autoantibodies against the AT1R have been identified in the circulation of women destined to develop preeclampsia, which, along with Ang II, can, for instance, stimulate the secretion of soluble Fms-like tyrosine kinase-1 (sFlt-1).
Conversely, 1 axis of the RAS, which has been poorly investigated in the context of preeclampsia, is the Ang-(1–7) axis in which the main reaction cascade starts with the angiotensin-converting enzyme 2 (ACE2) that converts Ang II into Ang-(1–7). Ang-(1–7) has been shown to counteract the effects of Ang II, for instance, by its vasodilatory and antitrophic effects through the stimulation of the Mas receptor (MasR). Circulating Ang-(1–7) is reduced in human preeclampsia compared with normal pregnancy. In parallel, Brosnihan’s group found that although there was no decrease in Ang-(1–7) in chorionic villi from human preeclampsia pregnancies, a significant decrease in MasR and an increase in angiotensinogen, Ang II, and AT1R could be observed. They proposed that the increased Ang II effect in the chorionic villi could produce a decrease in fetal blood flow, which would contribute to a reduction in fetal oxygen and nutrients as well as to the development of intrauterine growth restriction. However, to date, there are no data about the modulation of the Ang-(1–7) axis in women with PESCH.

The beneficial effect of exercise training (ExT) in the prevention and treatment of cardiovascular diseases has been well documented in men and nonpregnant women. Physical activity has also been suggested to be beneficial during normal pregnancy by improving maternal cardiovascular and metabolic adaptations as well as placental and fetal development. However, for preeclampsia, drawing conclusions on the effect of exercise on pregnancy outcomes is made difficult by the limited number of studies that are largely observational and have small sample sizes. Although guidelines from the American College of Obstetricians and Gynecologists (ACOG) recommend that pregnant women participate in moderate intensity activity has also been suggested to be beneficial during normal pregnancy by improving maternal cardiovascular and metabolic adaptations as well as placental and fetal development.

However, for preeclampsia, drawing conclusions on the effect of exercise on pregnancy outcomes is made difficult by the limited number of studies that are largely observational and have small sample sizes. Although guidelines from the American College of Obstetricians and Gynecologists (ACOG) recommend that pregnant women participate in moderate intensity exercise on most, if not all, days of the week during their pregnancy, this does not apply to women at risk of complications, given the lack of data, although they may benefit from regular physical activity. Conversely, we have recently demonstrated in a mouse model of preeclampsia that ExT, both before and during pregnancy, can markedly reduce preeclampsia-like features. Hence, more research is warranted to eventually modify the present ACOG guidelines. Therefore, the aim of this study was to investigate the mechanisms implicated in the development of PESCH and those involved in the protective effects of ExT on the disease in our unique mouse model.

Materials and Methods

Materials and Methods section.

Animals

R+A+ transgenic mice were produced by breeding heterozygous human renin mice (Ren9 line) with heterozygous human angiotensinogen mice (204/1 line). Both single transgenic lines were a generous gift from Dr Curt D. Sigmund of the University of Iowa. Female mice in these experiments were separated into 4 groups (sedentary R+A+ and nontransgenic; R−A−; trained 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 Animal Care Committee of the CHUM Research Center.

Exercise Training

Mice were placed in cages with free access to a running wheel for 1 month before mating, and they remained in these cages throughout gestation, as done previously in a different mouse model of preeclampsia in which we have shown significant improvement in maternal outcomes with this type of ExT regimen.

Arterial Pressure Measurement

Arterial pressure and heart rate were quantified in the carotid artery by telemetry using TAl1PA-C10 probes (Data Sciences International, St. Paul, MN), as done previously.

Proteinuria

Urine samples were collected on day 0 (before mating) and on day 18 (before euthanasia), and albumin and creatinine were assayed, as done previously.

Echocardiography

Transthoracic echocardiographic studies were performed before mating and at the end of pregnancy by high-resolution ultrasound microscopy (Veo660; Visualsonics, Toronto, ON, Canada) equipped with a 25- to 55-MHz probe, as previously described.

Tissue Collection and Histology

On day 18 of gestation, mice were anesthetized, and blood was collected by intrathoracic cardiac puncture. The pups were weighed, and their tails were cut and kept for genotyping. Heart and placentas were all collected, weighed, and either snap-frozen in liquid nitrogen or fixed for future experiments, as done previously.

Immunohistochemistry

Immunohistochemical analysis was assessed from 3 different placentas issued from 3 different mothers/group, as done previously. Paraffin-embedded placentas were sectioned at 4 μm and deparaffinized to assess cytokeratin (Abcam, Cambridge, MA) and Histone H3 (Abcam) to evaluate trophoblasts at all stages of gestation and mitosis, respectively.

Statistical Analysis

All values are expressed as mean±SE. A P value of ≤0.05 was considered significant. Differences in tissue weights and ratios, number of pups, circulating glucose and sFlt-1 levels, as well as data obtained by real-time polymerase chain reaction, Western blot, and immunohistochemistry were computed by 2-way ANOVA. Two-way repeated-measures ANOVA was used to analyze echocardiography parameters, blood pressure, and albumin/creatinine ratio. These analyses were all followed by Tukey post hoc test if an interaction was detected. Placental alterations were analyzed using a nonparametric Mann–Whitney rank-sum test.

Results

As shown previously, R+A+ mice were hypertensive at baseline (136.6±3.1 and 98.9±1.9 mmHg in R+A+ and nontransgenic, respectively; P<0.001) and before and throughout gestation. Sedentary R+A+ mice experienced a further increase in blood pressure at the end of pregnancy (Figure 1A). Similarly, proteinuria, assessed by the albumin/creatinine ratio, was significantly increased in R+A+ compared with their nontransgenic littermates (Figure 1B).

RAS components were studied in the mouse aorta (Figure 2) to identify potential mechanisms implicated in blood pressure regulation. In sedentary R+A+ mice, we found that aortic AT1R protein was significantly increased by 85%, whereas the MasR and ACE2 were diminished by 24% and 32%, respectively. In contrast, the aortic MasR was significantly increased by 50% in trained R+A+ mice, and this was associated with a lack of gestational increase in blood pressure, although ExT did not decrease blood pressure before pregnancy. Moreover, ExT significantly lowered the levels of albumin/creatinine ratio, although these
values were not completely normalized (Figure 1B). We observed no change in aortic AT1R and ACE2 protein with ExT.

As a result of PESCH, placental alterations could be detected in sedentary R+A+ mice, which produced a decrease in placental weight and intrauterine growth restriction, compared with their nontransgenic littermates (Table). In addition, R+A+ mice had smaller litters compared with nontransgenic controls. Placental alterations were characterized by a significant increase in hyalinization, giant cell island loss, and loss of labyrinthine structure. These alterations were corroborated by our immunohistochemistry results demonstrating a significant increase in placental cytokeratin and histone H3 staining in placentas from sedentary transgenic mothers (Figures S1 and S2 in the online-only Data Supplement). In addition, R+A+ mice had smaller litters compared with nontransgenic controls. Placental alterations were characterized by a significant increase in hyalinization, giant cell island loss, and loss of labyrinthine structure. These alterations were corroborated by our immunohistochemistry results demonstrating a significant increase in placental cytokeratin and histone H3 staining in placentas from sedentary transgenic mothers (Figures S1 and S2 in the online-only Data Supplement). Again, similar to what we observed in the aorta, modulation of the RAS was implicated in this phenotype because placental AT1R protein was significantly increased by 23%, whereas that of the MasR and ACE2 were diminished by 16% and 30%, respectively, in the sedentary R+A+ mice compared with nontransgenic littermates (Figure 3). Importantly, the normalization of the placental alterations with ExT (Table S1; Figures S1 and S2) was paralleled by those of both AT1R and MasR expressions, whereas ACE2 was unaffected by training. Incidentally, ExT also reduced AT1R by 28% in nontransgenic controls. These improvements had functional effects because they translated into a normalization of total fetal and placental weight as well as litter size with ExT in R+A+ mice (Table). Interestingly, we also observed an increase in fetal:placental weight ratio (Table) with ExT, independent of genotype, suggesting a modified placental efficiency even in nontransgenic animals.

Placental vascular endothelial growth factor (VEGF) protein was significantly increased in the sedentary R+A+ mice compared with their sedentary controls, whereas a significant decrease was observed with ExT (Figure 4A). A similar pattern was observed for placental mRNA and circulating sFlt-1, which were found to be increased in the sedentary
transgenic group compared with their nontransgenic controls, and normalized with ExT in R’A+ (Figure 4B and 4C). As such, the decreased placental sFlt-1 transcription in trained R’A+ mice is likely responsible for the reduced maternal circulating sFlt-1 levels observed in this study.

As reported previously, cardiac hypertrophy, characterized by elevated ventricular mass, was observed in R’A+ at the end of gestation, but it was unaffected by training (Table S2). Interestingly, the right atrium had a significantly increased mass in trained mice, independently of genotype, whereas the right ventricle was also significantly enlarged by ExT but only in nontransgenic mice. NGFI-A-binding protein 1, a marker of pathological cardiac hypertrophy, was found to be increased in the transgenic mice, as reported previously, whereas ExT caused a significant reduction, although these values were not completely normalized (Figure S3A). The brain natriuretic peptide, a marker of left ventricular dysfunction, was significantly reduced, although these values were not completely normalized (Figure S3B). Echocardiographic analysis demonstrated similar trends wherein ejection fraction and fractional shortening were significantly decreased in R’A+ and tended to be increased with ExT (Tables S3 and S4).

Circulating glucose was also examined because diabetes mellitus is a known risk factor for preeclampsia. Although no effect of genotype could be observed on circulating glucose levels, exercise did cause a significant reduction of 8% to 11% (Figure S4), as has been previously published by others.26

Similar exercise patterns were observed in both R’A+ and R’A- trained mice before and during pregnancy (Figure S5). The distance travelled daily diminished drastically at day 1 of gestation and continued to steadily decrease as pregnancy progressed. Furthermore, there was no effect of ExT or genotype on length of gestation, baseline body weight, and gestational weight gain (Table S5).

**Discussion**

Our study demonstrates a functional implication of the RAS and angiogenic balance in PESCH. Indeed, in the aorta, we found that AT1R protein was significantly increased, whereas the MasR and ACE2 were significantly decreased. Hence, these modulations may contribute to promoting endothelial dysfunction because it would increase the sensitivity to Ang II, as has been reported in women with preeclampsia.7 Moreover, it would decrease both the production and sensitivity to Ang-(1–7), thus countering the normal regulation of blood pressure during pregnancy11 because its effects oppose those of Ang II.11 Hence, together, the changes in the vascular RAS could strongly contribute to the increase in blood pressure in this model. Moreover, it may induce the development of placental alterations if similar modifications are present in uterine blood vessels. Importantly, we observed an increase in the MasR in the aorta with ExT, which could contribute to minimize the Ang II–associated vasoconstriction, which is often exaggerated in preeclampsia, and could promote the vasodilatory properties of Ang-(1–7). This is in line with reports that ExT stimulates the cardiac27,28 and aortic Ang-(1–7) axis by increasing ACE2 and the MasR, which has been suggested to contribute to both the development of ExT-associated physiological cardiac hypertrophy and the prevention of ventricular dysfunction in a heart failure mouse model.27

Changes similar to that observed in the aorta were also detected in the placenta. Indeed, our sedentary R’A+ mice had a significant increase in AT1R, whereas the MasR and ACE2 were significantly decreased. Ang II in the placenta, via the AT1R, is known to induce the production and secretion of sFlt-1, along with mediation of oxidative stress, inflammation, and vasoconstriction.10,30,31 In line with this, we observed a significant increase in placental sFlt-1 mRNA in our model, with a concomitant elevation in circulating levels of this antiangiogenic factor similarly to what has been reported in women with preeclampsia.32 Given that high levels of sFlt-1 are known to inhibit trophoblastic invasion, this may directly contribute to the development of placental alterations observed in our model. Moreover, decreased placental ACE2 has been suggested to be implicated in the development of intrauterine growth restriction, for instance, with maternal protein restriction33 and in a rat model of preeclampsia.34 Indeed, it is proposed that the resulting reduction in placental Ang-(1–7) decreases local vasodilation and thus impedes placental–fetal blood flow. Hence, placental dysregulation of the RAS may contribute to the development of placental alterations.

In support of this hypothesis, the ExT normalization of placental development was accompanied by a significant reduction in AT1R and elevation in MasR in the placenta. This may thus be responsible for the decrease in both placental sFlt-1 mRNA and circulating sFlt-1 observed with ExT. Similarly, others have found AT1R reductions with ExT in other tissues, such as the paraventricular nucleus and rostral ventrolateral medulla, in spontaneously hypertensive rats.35 Moreover, the increase in MasR may enhance Ang-(1–7)–mediated vasodilation and improve placental perfusion. As such, the ExT effect on the RAS may not
only contribute to preventing the development of placental alterations associated with preeclampsia but also be beneficial for other conditions that are at risk of intrauterine growth restriction, for instance, because of uteroplacental insufficiencies.

In our study, ExT may have improved endothelial function during pregnancy not only by altering RAS components but also by diminishing circulating levels of glucose and promoting angiogenic balance. For instance, ACE2 was unaffected by ExT. Values are expressed as mean±SE. \( *P<0.05 \) and \( †P<0.01 \), statistically different from R−A−; \( ‡P<0.05 \) and \( §P<0.005 \), statistically different from sedentary mice. Sedentary R−A−, n=3 to 5; trained R−A−, n=6 to 8; sedentary R+A+, n=6 to 7; and trained R+A+, n=5 to 6.

Interestingly, our sedentary transgenic mice also had a significant increase in placental VEGF protein expression, which was reduced with ExT. Normal pregnancy is characterized by an increase in angiogenic factors, such as VEGF, at the start of pregnancy, which usually decrease as pregnancy progresses and placental development nears completion. The presence of high VEGF levels at the end of gestation in our model may result from the inadequate placental development. As such, in response to hypoxia, increased VEGF levels may be present to stimulate placental vascularization. Alternatively, this increase may occur to counterbalance the increase in sFlt-1 associated with our PESCH model. These results are thus in

### Figure 3

Modulation of placental angiotensin II type 1 receptors (AT1R), Mas receptor (MasR), and angiotensin-converting enzyme 2 (ACE2) protein by a preeclampsia superimposed on chronic hypertension and exercise training (ExT). A significant increase in placental AT1R (A) was observed in sedentary R+A+ mice, along with a significant reduction in MasR (B) and ACE2 (C). Both MasR and AT1 were normalized with ExT, and AT1R was even decreased in nontransgenic mice. Conversely, ACE2 was unaffected by ExT. Values are expressed as mean±SE. \( *P<0.05 \) and \( †P<0.01 \), statistically different from R−A−; \( ‡P<0.05 \) and \( §P<0.005 \), statistically different from sedentary mice. Sedentary R−A−, n=3 to 5; trained R−A−, n=6 to 8; sedentary R+A+, n=6 to 7; and trained R+A+, n=5 to 6.

### Figure 4

Effect of exercise training (ExT) and preeclampsia superimposed on chronic hypertension on angiogenic balance. Compared with their nontransgenic littermates, sedentary R+A+ mice presented with significant increase in placental vascular endothelial growth factor (VEGF) protein (A) and soluble Fms-like tyrosine kinase-1 (sFlt-1) mRNA (B), which was significantly decreased with ExT. Comparably, a significant increase in circulating sFlt-1 (C) was observed among sedentary R+A+ mice, which was absent in trained transgenic mice. Sedentary R−A−, n=5 to 6; trained R−A−, n=6 to 9; sedentary R+A+, n=5 to 11; and trained R+A+, n=6 to 9. Values are expressed as mean±SE. \( *P<0.05 \) and \( †P<0.01 \), significantly different than R−A− mice; \( ‡P<0.05 \) and \( §P<0.001 \), significantly different from sedentary mice.
line with the hypothesis that preeclampsia is associated with an angiogenic shift that is initiated by the placenta. We propose that ExT restores angiogenic balance; therefore, the healthy placenta no longer releases angiogenic mediators that would favor endothelial dysfunction into the circulation.

The ExT molecular adaptations reported in our study are certainly involved in the normalization of placental alterations in R+A+ mice. The placentas of trained transgenic mice were characterized by a normalization of placental immunostaining to histone H3 and cytokeratin, which are markers of mitosis and trophoblastic cells in all stages of gestation, respectively, and were found to be increased in sedentary transgenic mice. These increased placental markers in conjunction with the increase in labyrinthine structure loss observed by histology support the premise that trophoblasts in R+A+ placentas are highly proliferative and fail to differentiate. This is in line with studies that suggest that preeclampsia is produced not only by an inadequate trophoblastic invasion but also by a highly proliferative and underdeveloped placenta. As such, our data suggest that our PESCH model truly represents a clinical reality. In addition, the cytokeratin and histone H3 results support the hypothesis that ExT promotes healthy placental development by increasing placental trophoblastic volume and enhancing the exchange of nutrients and oxygen toward the growing fetus. As such, we observed an increase in the ratio of fetal to placental weight with ExT, suggesting an improved placental efficiency. Moreover, fetal weight and litter size were completely normalized in trained R+A+ females, suggesting that these placental modifications had a functional effect on the fetal development. Hence, it is clear that ExT both before and during gestation can be beneficial to fetal outcome.

As previously reported, sedentary R+A+ mice develop pathological cardiac hypertrophy compared with the nontransgenic mice. Although ExT did not have any effect on heart weight, modifications in cardiac pathological markers were observed. This suggests that there may be modifications in the type of hypertrophy present in the trained transgenic mice. Indeed, NGFI-A–binding protein 1 and brain natriuretic peptide were both significantly decreased with ExT in transgenic dams, although NGFI-A–binding protein 1 was not completely normalized. Fractional shortening and ejection fraction were significantly compromised in the sedentary R+A+ mice, whereas ExT tended to increase these parameters.

Cages with free access to an exercise wheel were used to investigate the effect of training in PESCH prevention in our study. Of note, the degree of training observed in our mice corresponds to what is reported in the literature, and it did not vary according to genotype. Three weeks of free wheel training is associated with many cardiovascular benefits in nonpregnant rodents, including an increase in \( V_{O_2\text{max}} \). Because similar improvements are observed in nonpregnant women after a moderate aerobic ExT regimen, we suspect that the beneficial effects of this type of ExT on pregnancy outcome observed in our mouse model may also translate to pregnant women.

**Perspective**

This study has advanced our understanding of the role of the local RAS in the vasculature and in the placenta in many hallmark features of PESCH. The fact that the observed PESCH-associated modulations are prevented by ExT strongly supports a functional role for these components. Further studies will be required to determine whether parallel observations can be made in women.

Moreover, to our knowledge, this is the first study that has investigated the potential beneficial effect of ExT on PESCH features, and it is in line with the numerous retrospective studies, which have demonstrated a protective effect on preeclampsia in women. This preventive and therapeutic approach, if proven effective, could minimize the prevalence of future cardiovascular diseases, deficits associated with preterm births, and perinatal mortality and morbidity. Indeed, in contrast to pharmacological treatments inhibiting the RAS, which are teratogenic, ExT may efficiently modulate this system to obtain the required benefits without the adverse fetal outcomes. However, large-scale randomized studies are needed to confirm these effects in women and eventually lead to modifications of the ACOG guidelines, which presently do not support the prescription of ExT to women at risk of gestational complications.

**Acknowledgments**

We thank Dr Louis Gabboury for his assessment of pathological alterations present in placental histological slides as well as Morgan Michalet for his help in the data analysis.

**Sources of Funding**

This work was supported by grants from the Canadian Institutes of Health Research, Fonds de recherche du Québec - Santé, the Université de Montréal, and Pfizer, Canada.

**Disclosures**

None.

**References**

Novelty and Significance

What Is New?

- We have found a novel modulation of the renin–angiotensin system in preeclampsia superimposed on chronic hypertension in an animal model. There is a modification of the balance between the good (angiotensin-[1–7]) and the bad (angiotensin II) axis of the renin–angiotensin system favoring angiotensin-II.
- Exercise training prevented most features of preeclampsia superimposed on chronic hypertension in an animal model.

What Is Relevant?

- Women with hypertension are at higher risks (15%–25%) of developing preeclampsia.
- Preeclampsia is a gestational disease, which is characterized by the development of hypertension (or increase in blood pressure in the case of women with hypertension) and proteinuria after 20 weeks of gestation.

Summary

Developing treatments targeting the good axis (angiotensin-[1–7]) of the renin–angiotensin system may be an interesting avenue because there are presently no treatments available apart from the premature delivery of the fetus. Exercise training may be a potential treatment for women with hypertension to prevent the development of preeclampsia.
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Hypertension. 2013;62:1055-1061; originally published online October 7, 2013; doi: 10.1161/HYPERTENSIONAHA.113.01983
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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NOVEL ROLE OF THE RENIN-ANGIOTENSIN SYSTEM IN PREECLAMPSIA SUPERIMPOSED ON CHRONIC HYPERTENSION AND THE EFFECTS OF EXERCISE IN A MOUSE MODEL.
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Short title: Renin-angiotensin system in a form of preeclampsia

Word count: 4,172 words

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Expanded material and methods

**Animals.** R+ A+ transgenic mice were produced by breeding heterozygous human renin mice (Ren9 line)(1) with heterozygous human angiotensinogen mice (204/1 line)(2). Both single transgenic lines were originally obtained from Dr. Curt D. Sigmund of the University of Iowa, and were maintained in our animal facility by backcrossing with C57BL/6 mice (strain code 027, Charles River, St-Constant, QC, Canada) for over 20 generations. The genotypes were determined by performing a polymerase chain reaction on genomic DNA obtained from tail biopsies using primers specific to hREN and hANG (see Table S6 for sequence). Each reaction contained 1μl 10X buffer; 0.2 μl 10mM dNTP, 0.1 μl of each primer, 6.4 μl of water, 0.5 μl taq polymerase (Feldan, Québec, QC, Canada) and 2 μl of genomic DNA. The PCR products were subsequently analyzed by southern blot using a 1% agarose gel. The animals were kept on a 12h light/dark cycle with water and standard laboratory chow (2018; Teklab Premier Laboratory Diets, Madison, WI) ad libitum. Female mice in these experiments were 8-10 weeks of age, and were separated into 4 groups (Sedentary R+A+ and non-transgenic (R-A-); Trained R+A+ and R-A-). R+A+ mice were bred with non-transgenic (NT) males. Control mice corresponded to NT littermates. The care of all mice met the standards set forth by the Canadian Council on Animal Care for the use of experimental animals. All procedures were approved by the Animal Care Committee of the CHUM Research Centre.

**Exercise training.** Mice were placed in cages with free access to a running wheel, for one month and remained in these cages throughout gestation. In fact, studies have shown significant improvements in aerobic capacity with only 3 weeks of exercise training (ExT) with a running wheel(3;4). In addition, we have shown significant improvement in maternal outcomes in a different mouse model with this type of ExT regimen(5). The use of voluntary exercise was chosen to prevent the stress that is usually associated with treadmill running(6). ExT was measured, as each cage was connected to a computer and, the number of revolutions were recorded and used to confirm training status (Compte-tour5, Aquila, Boucherville, Qc, Canada).

**Arterial pressure measurement.** Arterial pressure (AP) and heart rate (HR) were quantified in the carotid artery by telemetry using TA11PA-C10 probes (Data Sciences International, St. Paul, MN) as done previously(5;7-9). The probe’s catheter was inserted into the left carotid artery of female mice anesthetized by inhalation of isoflurane 2-3% in oxygen, and maintained on 2% isoflurane. After 7-10 days of recovery post-surgery, AP and HR were recorded for 3 consecutive days to represent baseline values. The mice were then put in cages with access to an exercise wheel for 1 month. Sedentary animals were placed in standard cages. After this period, male mice were introduced into the cages for timed-mating of trained animals, while the reverse was performed for sedentary females. Gestation was confirmed by the presence of a vaginal plug, which was recorded as day 1 of pregnancy. AP and HR measurements were recorded every 2 days, beginning on day 1 up to day 19. Since mice normally give birth between day 19 and 21 of gestation, the day prior to delivery was considered as “end of gestation” to permit an adequate comparison.

**Proteinuria.** Urine samples were collected on day 0 (prior to mating) and on day 18 (prior to sacrifice) as done previously(5;7-9). To do so, the mice were momentarily restrained and their urine was directly collected in a 1.5mL tube. Collecting urine via this method is associated with
minimal amount of stress, which is far less than that associated with placing mice in metabolic cages for 24h(10). Once collected, the urine samples were maintained at -80°C until assayed. Albuwell and Creatinine companion ELISA kits were used to determine urine albumin and creatinine clearance, respectively (Exocell, Philadelphia, PA, United States). Urine samples were diluted 1:10, and each sample was done in duplicate. Proteinuria was assessed by calculating the albumin/creatinine ratio (ACR).

Echocardiography. Transthoracic echocardiographic studies were performed prior to training and mating, and at the end of pregnancy. The mice were anesthetized by inhalation of isoflurane 2-3% in oxygen, and maintained on 2% isoflurane and ultrasound transmission gel (Ecogel 200, EcoMed Inc., Mississauga, ON) was placed over the cardiothoracic region to provide an acoustic coupling medium between the probe and the animal. Their heart were investigated by high-resolution ultrasound biomicroscopy (Vevo660; Visualsonics, Toronto, ON, Canada) equipped with a 25-55 MHz probe. 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 left ventricular (LV) walls at the level of the papillary muscle. The ejection fraction (EF) was estimated by the following formula: EF = (LVEDV – LVESV) × 100 / LVEDV, where LVEDV and LVESV are respectively LV end-diastolic and end-systolic volumes. LV fractional shortening (FS) was given by (LVEDD – LVESD) × 100 / LVEDD. Lastly, LV volumes during diastole and systole were determined as 7 × LVD³ / (2.4 + LVD), where left ventricular diameter (LVD) is substituted by LVEDD for LV diastolic volume or LVESD for LV systolic volume, respectively.

Tissue collection and histology. On day 18 of gestation, mice were anesthetized by isoflurane 2-3% in oxygen, and then maintained on 2% isoflurane. Blood was collected by intrathoracic cardiac puncture and placed in a chilled 1.5 ml tube containing 15ul of 500mM EDTA (pH: 8.0) (EMD, Gibbstown, NJ, USA). Plasma was separated by centrifugation and samples were snap frozen in liquid nitrogen, and stored at -80°C until assayed. The pups were weighed, and their tails were cut and kept for genotyping. Kidneys, heart, and placentas were all collected, weighed, and either snap-frozen in liquid nitrogen or placed overnight in 4% paraformaldehyde for fixation. 24h after the tissues had been fixed, they were washed with phosphate buffer and subsequently embedded in paraffin. Sections were obtained by cross-sectionally cutting the fixed tissue using a microtome. To evaluate placental morphology, the sections were stained with hematoxylin phloxine saffron (HPS) and evaluated by light microscopy. Embedding, sectioning and staining were performed 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. The latter two are analogous to human extravillous cytotrophoblasts cells and chorionic villi structure, respectively(11). 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 alterations. To avoid any bias, the investigator scoring the tissues was blinded to the genotype of both mother and pups.

Real-time PCR. 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)/μg 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(12). 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 genes were investigated: 40S ribosomal protein S16 (S16), sFlt-1, NGFI-A-binding protein 1 (Nab1) and brain natriuretic peptide (BNP). Primer sequences are described in Table S6. Each placental and aortic sample was run and analyzed in duplicate. mRNA levels are expressed as values relative to s16 mRNA.

Immunohistochemistry. Immunochemical analysis was assessed on 3 different placentas from 3 different litters. Paraffin-embedded tissues were sectioned at 4 μm and deparaffinised in citrisolv (Fisher Scientific, Ottawa, ON, Canada). Antigen retrieval was performed by boiling the sections in sodium citrate buffer (10 nM, pH 6.0) for 3min. Immunostaining was then carried out with Catalyzed Signal Amplification System (Dako, Carpinteria, CA), according to the manufacturer’s instructions. The following modification were used: Peroxidase activity was blocked for 15min at room temperature with peroxidase block solution. Samples were rinsed in 3 baths for 3min in TBS-T (0.05 M tris-HCl, 0.3 M NaCl, 0.1% tween 20, pH 7.6). The sections were then incubated with endogenous avidin and biotin blocking solutions (Thermo Scientific, Ottawa, ON, Canada) for 15min at room temperature. Non-specific antigen binding was blocked by incubation in protein block solution for 30min at room temperature. The sections were incubated overnight at 4ºC with specific immunohistochemical primary antibodies diluted in TBS with 1% bovine serum albumin at the following concentrations: anti-pan-cytokeratin (1:800; ab9377, Abcam, Cambridge, MA) and histone H3 (1:1600; ab5176-100, Abcam) to evaluate the presence of trophoblast and mitosis, respectively(13;14). Tissue sections were incubated with secondary antibody conjugated to biotin (1:5000, donkey anti-rabbit; AP182B, Chemicon international, Millipore, Billerica, MA) solution for 1h at room temperature. Primary antibodies were omitted in the negative control. Samples were incubated with streptavidin-biotin complex for 15min and subsequently, with amplification reagent for 15min at room temperature. The sections were then incubated with a streptavidin-peroxidase for 15 min. Lastly, staining with substrate-chromogen solution for 4min and counterstaining with Mayer’s hematoxylin (Sigma-Aldrich, Oakville, ON, Canada) blue in 0.3% ammonia water were performed. Sections were viewed and photographed with a Leitz Diaplan microscope equipped with a Nikon CoolPix 990 camera (Nikon Instruments, Melville, NY).

Western Blot. Frozen placentas and aortas were lyophilized and subsequently homogenised in lysis buffer (50mM HEPES pH 7.5, 137mM NaCl, 1mM MgCl₂, 1mM CaCl₂, 2mM Na₃VO₄, 10mM Na pyrophosphate, 10mM NaF, 2mM EDTA, 1% NP-40, 10% glycerol, 34mg/L PMSF along with a protease inhibitor cocktail (Roche, Mississauga, ON, Canada)). Total protein content was measured in supernatants by standard Bradford assay. Samples containing 50μg of protein were loaded on 10% or 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel, depending on the molecular weight of the protein studied, and separated by electrophoresis. Proteins were transferred on a nitrocellulose membrane (Amersham, Baie d’Urfé, QC). Placental and aortic non-specific sites were blocked overnight at 4°C in SuperBlock buffer (Thermo Fisher Scientific, Rockford, IL) or in Bløk™-Chemiluminescent blocker (Millipore, Temecula, MA),
respectively. Membranes were then incubated with the primary antibody in 10% Superblock and 0.1% Tween 20 (Fisher Scientific, Ottawa, ON, Canada) in tris buffered solution (TBS) (Abcam, Cambridge, MA) overnight at 4°C. The following antibodies and concentrations were used: vascular endothelial growth factor (VEGF) (1:1000, ab46154, Abcam, Cambridge, MA); angiotensin converting enzyme 2 (ACE2) (1:500, SC-20998, Santa Cruz Biotechnology, Santa Cruz, CA); Angiotensin-(1-7)-Mas receptor (MasR) (1:2000, AAR-013, Alamone Labs, Jerusalem, Israel); AT1R (1:2000, SC-578, Santa Cruz Biotechnology, Santa Cruz, CA); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000, SC-20357, Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were subsequently washed with 0.1% TBS-T for 30min and incubated for 1h at room temperature with their respective horseradish peroxidase conjugated secondary antibody (1:3000, 1:10 Superblock in 0.1% Tween 20 in TBS) independent of primary antibody used. The GAPDH antibody was already linked to horseradish peroxidise, and thus, a secondary antibody was not use for this experiment. Bands were revealed using the ECL West Pico kit (Pierce, Rockford, IL). Each protein signal was normalized to its respective GAPDH band.

**Plasma sFlt-1 levels:** Circulating sFlt-1 concentrations were measured using a commercial ELISA kit (R&D-Quantikine, Minneapolis, MN). Plasma samples were diluted 1:20 using the manufacturer’s dilutor prior to the experiment and each sample was measured in duplicate.

**Plasma glucose levels:** Circulating glucose levels were determined using a commercially available enzymatic kit (Autokit Glucose, WAKO Diagnostics, Richmond, VA), following the manufacturer’s instructions. Each sample was measured in duplicate.

**Statistical analysis.** All values are expressed as means ± SE. A p-value of ≤ 0.05 was considered significant. Differences in tissue weights and ratios, number of pups, circulating glucose and sFlt-1 levels, as well as data obtained by real-time PCR, Western blot and immunohistochemistry were computed by 2-way ANOVA. 2-way repeated measures ANOVA was used to analyze echocardiography parameters, blood pressure and albumin/creatinine ratio. These analyses were all followed by Tukey’s post-hoc test if an interaction was detected. Placental alterations were analyzed using a non-parametric Mann-Whitney Rank Sum Test.
References


### Table S1. Characterization of placental pathology.

<table>
<thead>
<tr>
<th>Training Status</th>
<th>Mother’s genotype</th>
<th>N</th>
<th>Necrosis</th>
<th>Hyalinization</th>
<th>Microcal.</th>
<th>GCIL</th>
<th>LLTS</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>R^-A</td>
<td>22</td>
<td>0.0</td>
<td>1.0</td>
<td>0.5</td>
<td>1.0</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>R^+A</td>
<td>21</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0*</td>
<td>2.0</td>
<td>0.0</td>
<td>4.0*</td>
</tr>
<tr>
<td>Trained</td>
<td>R^+A</td>
<td>25</td>
<td>1.0</td>
<td>1.0</td>
<td>0.0†</td>
<td>1.0</td>
<td>0.0</td>
<td>3.0†</td>
</tr>
</tbody>
</table>

Results are expressed as the median (Mdn) and the 75th percentile of the score given in histology. * p ≤ 0.05 significantly from non-transgenic mice; † p ≤ 0.05 significantly different from sedentary mice. GCIL, Giant cell island loss; LLTS, Loss of labyrinthine trophoblast structure; Mdn, Median; Microcal., Microcalcification.
<table>
<thead>
<tr>
<th>Training Status</th>
<th>Mother’s genotype</th>
<th>N</th>
<th>Heart/tibia ratio</th>
<th>LV/tibia ratio</th>
<th>RV/tibia ratio</th>
<th>LA/tibia ratio</th>
<th>RA/tibia ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>R-/A-</td>
<td>5</td>
<td>7.13 ± 0.32</td>
<td>5.27 ± 0.29</td>
<td>1.21 ± 0.07</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.02</td>
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<tr>
<td></td>
<td>R+/A+</td>
<td>5</td>
<td>9.54 ± 0.23†</td>
<td>7.35 ± 0.28†</td>
<td>1.47 ± 0.04*</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>Trained</td>
<td>R-/A-</td>
<td>7</td>
<td>7.73 ± 0.18</td>
<td>5.17 ± 0.34</td>
<td>1.47 ± 0.07‡</td>
<td>0.18 ± 0.01</td>
<td>0.21 ± 0.01‡</td>
</tr>
<tr>
<td></td>
<td>R+/A+</td>
<td>6</td>
<td>9.86 ± 0.36†</td>
<td>7.18 ± 0.26†</td>
<td>1.41 ± 0.04</td>
<td>0.22 ± 0.03</td>
<td>0.24 ± 0.02‡</td>
</tr>
</tbody>
</table>

Table S2. Effect of ExT and PESCH on ratio of the whole heart and its compartments to tibia length at the end of pregnancy. Transgenic mice were found to have a statistically larger hearts, left ventricles, right ventricles and right atria, when corrected to left tibia length in comparison with their non-transgenic littermates without any effect of ExT. Right ventricles were also increased among the trained non-transgenic animals. Values are expressed as mean ± SE. * p ≤ 0.05, † p ≤ 0.001, significantly different from R-/A- mice; ‡ p ≤ 0.05 significantly different than sedentary mice. N, number; LV, left ventricle; RV, right ventricle; LA, left atrium; RA, right atrium.
**Table S3: Cardiac parameters calculated following echocardiography.**

Left ventricular diastolic volume was increased at the end of pregnancy in all groups among the sedentary R\(^+\)A\(^+\) both before and at the end of pregnancy. There was a non-significant decrease in fractional shortening and ejection fraction, accompanied by an increase. Similarly, a non-significant increase in left ventricular systolic volume was observed among sedentary transgenic dams, in comparison with their sedentary and non-transgenic counterparts. Values are expressed as means ± SE. * p≤0.05, statistically different from the R\(^-\)A\(^-\) mice; † p≤0.05, statistically different from the non-pregnant state. N, number; LV, left ventricular; FS, Fractional shortening; EF, Ejection fraction.
Table S4: Cardiac parameters measured following echocardiography.

A significant increase in diastolic LVID was observed at the end of pregnancy, independent of genotype and training. Additionally, systolic IVS was significantly increased at the end of pregnancy among nontransgenic animals. The above values are expressed as mean ± SE. * p≤0.05, statistically different from the R’A- mice; † p≤0.05, statistically different from the non-pregnant state. N, number; LVID, left ventricular internal diameter; LVPW, left ventricular posterior wall thickness; IVS, interventricular septum.
<table>
<thead>
<tr>
<th>Mother’s genotype</th>
<th>N</th>
<th>Baseline weight (grams)</th>
<th>Weight gain (grams)</th>
<th>Length of pregnancy (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R′A−</td>
<td>11</td>
<td>21.67 ± 0.23</td>
<td>13.9 ± 0.68</td>
<td>19.0 ± 0.0</td>
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<tr>
<td>R′A+</td>
<td>10</td>
<td>22.54 ± 0.42</td>
<td>13.95 ± 0.84</td>
<td>19.1 ± 0.01</td>
</tr>
<tr>
<td>Trained</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R′A−</td>
<td>10</td>
<td>21.14 ± 0.59</td>
<td>12.61 ± 0.80</td>
<td>19.0 ± 0.0</td>
</tr>
<tr>
<td>R′A+</td>
<td>11</td>
<td>21.32 ± 0.43</td>
<td>14.44 ± 0.52</td>
<td>19.2 ± 0.2</td>
</tr>
</tbody>
</table>

**Table S5: Maternal characteristics.**

No statistical significance could be detected on baseline body weight, weight gain and length of pregnancy. Values are expressed as mean ± SE. N, number; BW, body weight; LV, left ventricle; LK, left kidney.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
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<td>s16</td>
<td>5’-ATCTCAAAGGCCCTGGTCGC-3’</td>
<td>5’-ACAAAGGTAACCCCGATCC-3’</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>5’- AGGTGAGCAGTGCAGGCA-3’</td>
<td>5’-ATGAGTCCTTTAATGTTTGA-3’</td>
</tr>
<tr>
<td>Nab1</td>
<td>5’-CTGGCCAGGGTTTCTC-3’</td>
<td>5’-TGGCACAGATTCCTGGAAAGTC-3’</td>
</tr>
<tr>
<td>BNP</td>
<td>5’-AATTTCAAGGGTACACATATATCTC-3’</td>
<td>5’-GGTCTTCTCTACAACAAACTTCAG-3’</td>
</tr>
<tr>
<td>hREN</td>
<td>5’-TGACACTGGTTCGTCATAATG-3’</td>
<td>5’-ATA CGC GAG GGT GAG TTC TG-3’</td>
</tr>
<tr>
<td>hANG</td>
<td>5’-TGGTGCTAGTCGCTGCAAAACTTGACACCG-3’</td>
<td>5’-CAGGGAGCAGCCAGTCTCCCATCCCTGTCAC-3’</td>
</tr>
</tbody>
</table>

Table S6: Primer sequences used for real-time PCR
Supplemental Figures

Figure S1: Effects of exercise training and SPE on placental cytokeratin immunostaining.

Cytokeratin is a marker of trophoblast proliferation at all stages of gestation. Histological analysis revealed a significant increase in cytokeratin positive trophoblastic cells among sedentary transgenic mice (Image C, n=3), compared to their nontransgenic counterparts (Image A, n=3). Interestingly, training led to a significant reduction in trophoblast positive staining among R⁻A⁺ mice (Image D, n=3). A small increase in cytokeratin was observed among trained R⁻A⁻ (Image B, n=3), compared to their sedentary counterparts (Image A, n=3). These results suggest that both phenotype and training may affect trophoblast function, and, consequently, fetal development. Additionally, these results are in line with studies demonstrating an increase in placental villous area and perfusion with exercise training. Magnification 200 X.
Figure S2: Effects of exercise training and SPE on placental histone H3 immunostaining.

Histone H3-phospho-immunostaining is a marker of mitosis, which allows distinguishing proliferating from endoreplicating cells (a phenomenon specific to trophoblastic giant cell). A weak increase in histone H3 staining was observed with training among the R'A' mice (Image B, n=3), compared to sedentary R'A' mothers (Image A, n=3). Sedentary transgenic dams has significantly more histone H3 immunostaining (Image C, n=3), which disappeared with training. In sedentary R'+A' mice, the staining was primarily localized in peripheral trophoblasts, demonstrating an increase in number of cells undergoing endoreplication. Magnification 200 X.
Figure S3. Left ventricular gene expression of Nab1 and BNP.
Nab1 (A) and BNP (B) gene expression were significantly decreased with ExT in both R'A⁻ and R⁺A⁺ mice compared to their sedentary counterparts. Nab1 expression however was also significantly greater among R⁺A⁻ mice, compared to non-transgenic mice. Values are expressed as means ± SE. * p<0.05 statistically different from R'A⁻; † p<0.05 and ‡ p<0.005 statistically different from sedentary mice; Sedentary R'A⁻ n=10-11; trained R'A⁻ n=8-9; sedentary R⁺A⁺ n=14; trained R⁺A⁺ n=10.
Figure S4: Changes in circulating glucose levels with ExT and SPE-like phenotype.

Circulating glucose levels were consistently lower among trained animals compared with their sedentary counterparts, irrespective of genotype (n=10, per group). Values are expressed as means ± SE. * p<0.05 statistically significant compared to sedentary animals.
Figure S5: Distance travelled prior to and throughout gestation.

Exercise training patterns were similar regardless of genotype (R⁻A⁻ (n=10) and R⁺A⁺ (n=11)). Training was greater prior to pregnancy in both groups, and decreased progressively as pregnancy advanced, reaching a nadir prior to sacrifice. W, average distance travelled weekly; N, distance travelled during nighttime; END, end of pregnancy.