Evidence Implicating Peroxisome Proliferator-Activated Receptor-γ in the Pathogenesis of Preeclampsia

Fergus P. McCarthy, Sascha Drewlo, Fred A. English, John Kingdom, Edward J. Johns, Louise C. Kenny, Sarah K. Walsh

Abstract—Preeclampsia, a major cause of maternal and perinatal mortality and morbidity, is thought to be attributed, in part, to impaired trophoblast invasion. Peroxisome proliferator-activated receptors are ligand-activated transcription factors expressed in trophoblasts, which regulate the expression of a number of genes involved in cell differentiation and proliferation. We investigated the effect of the administration of a peroxisome proliferator-activated receptor-γ antagonist during uncomplicated pregnancy in rats. Using an intraperitoneal miniosmotic pump, healthy pregnant rats were administered either vehicle or the peroxisome proliferator-activated receptor-γ-specific antagonist, T0070907 (1 mg/kg per day from gestational days 11–15). Rats treated with T0070907 developed key features of preeclampsia, including elevated mean arterial blood pressure, proteinuria, endothelial dysfunction, reduced pup weight, and increased platelet aggregation. T0070907-treated rats had reduced plasma vascular endothelial growth factor and increased plasma soluble fms-like tyrosine kinase 1. Furthermore, increases in total placental soluble fms-like tyrosine kinase 1 mRNA and fms-like tyrosine kinase 1 protein were also demonstrated, suggesting the placenta as the main contributor to the increased circulating levels of soluble fms-like tyrosine kinase 1. The labyrinthine trophoblast in the placentas of T0070907-treated rats were less differentiated, had increased cellular proliferation, and were strongly immunopositive for CD-31 staining, indicating adaptive angiogenesis. The present study suggests that peroxisome proliferator-activated receptor-γ may play a pivotal role in the progression of a healthy pregnancy and may critically regulate the risk of preeclampsia. These findings have important implications regarding the underlying etiology of preeclampsia and potential therapeutic targets. (Hypertension. 2011;58:882-887.) • Online Data Supplement

Key Words: peroxisome proliferator activated receptor-γ • T0070907 • preeclampsia • vascular dysfunction

Preeclampsia is a major cause of maternal and perinatal mortality and morbidity worldwide causing ≈15% of all direct maternal deaths and mediating a 5-fold increase in perinatal mortality.1 Although the underlying etiology of preeclampsia is poorly understood, this condition is characterized by a relatively hypoperfused placenta, which stimulates the maternal response manifesting as hypertension, vascular dysfunction, and a pro-oxidant state.2 Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of a number of genes involved in cell differentiation and proliferation.3 PPAR-γ plays a predominant role in normal vascular function4,5 and in the differentiation of labyrinthine trophoblast lineages,6 which, along with the fetal endothelium, form the vascular exchange interface with maternal blood.7 Homozygous PPAR-γ−deficient embryos die because of placental dysfunction,8 and PPAR-γ null placentas develop a malformed labyrinth zone,9 suggesting a critical role for PPAR-γ in the progression of normal pregnancy. More recently, a role for PPAR-γ in the pathogenesis of preeclampsia has been suggested, because administration of a PPAR-γ agonist to a rodent model of preeclampsia alleviated several of the hallmarks of this condition.10 In addition, recent data have demonstrated that serum concentrations of endogenous agonists of PPAR-γ are significantly reduced in women with severe early onset preeclampsia compared with healthy pregnant women.11 In contrast, Holdsworth-Carson et al12 demonstrated that placentas from women with preeclampsia did not exhibit any changes in either PPAR-γ activity or placental expression of PPAR-γ mRNA and PPAR-γ protein, thus whether aberrant PPAR-γ activity is involved in the development of pregnancies complicated by hypertension is not known. Furthermore, because of the limitations of conducting studies in pregnant women, and the embryonic lethality of homozygous PPAR-γ receptor gene knockout in rodents,9 the functional role of PPAR-γ in normal pregnancy remains unclear. We hypothesized that the antagonism of PPAR-γ would adversely affect the progression of normal rodent
pregnancy and lead to the development of several of the characteristic features of preeclampsia.

Materials and Methods

Animals

Sprague-Dawley timed pregnant rats were supplied and maintained by the University College Cork Biological Services Unit. Animals were maintained at a temperature of 21 ± 2°C, with a 12-hour light/dark cycle and free access to food and tap water. All of the procedures were performed in accordance with national guidelines and the European Community Directive 86/609/EC and approved by the University College Cork Local Animal Experimentation Ethics Committee (2009/04).

PPAR-γ Antagonist In Vivo Experimental Protocol

PPAR-γ antagonism during pregnancy was investigated in a series of experiments via the administration of either T0070907 (1 mg/kg per day; Cayman Chemical), a PPAR-γ antagonist, or vehicle (40% dimethylsulphoxide solution) over a 5-day period (gestational days [GDs] 11–15) using an intraperitoneal miniosmotic pump (ALZET 2ML1). Under anesthesia with isoflurane (2% to 5% inhalation), on GD11, a laparotomy incision was made, pup number noted, pump inserted, and the animal allowed to recover. On GD18, animals were anesthetized with isoflurane, a chronic indwelling catheter (0.58 mm ID × 0.99 mm OD) inserted into the carotid artery, which was exteriorized at the back of the neck and animals allowed to recover before conscious mean arterial blood pressure measurement on GD19. After completion of mean arterial blood pressure measurements, animals were anesthetized with isoflurane, blood collected via the abdominal aorta into precooled heparinized vacutainers, and the mesenteric arterial arcade excised and placed in ice-cold physiological salt solution. All of the pups and placentas were removed, weighed, and litter size noted. The concentration of T0070907 used in this study was chosen on the basis of a previously published study.14,15

Isometric Myography

After culling of the rat, third-order mesenteric arteries were dissected out within 1 hour and mounted onto a 4-channel wire myograph (model 610 mol/L, Danish Myo Technology, Aarhus, Denmark) containing oxygenated (95% O₂ and 5% CO₂) physiological salt solution at 37°C. Vessels were normalized to achieve a transmural pressure of 100 mm Hg using the Danish Myo Technology Normalization software. Isometric tension was recorded and displayed using Powerlab and Chart Software (both AD Instruments). The viability of the smooth muscle was tested via the addition of a 123-mmol/L KCl solution. After 2 physiological salt solution washes, cumulative concentration responses were carried out with the thromboxane mimetic, U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F₂α, 10⁻⁹ to 3 × 10⁻⁵ mol/L), and either bradykinin (10⁻⁹ to 10⁻⁵ mol/L) or sodium nitroprusside (10⁻⁹ to 10⁻⁵ mol/L). U46619 induced a concentration-dependent increase in active wall tension in all of the groups, but the maximum contraction to the vasoconstrictor did not differ significantly between groups.

Platelet Aggregation Studies

Ex vivo platelet aggregation studies were performed in response to either adenosine diphosphate (ADP) or collagen (for extended method please see the online Data Supplement at http://hyper.ahajournals.org).

Urine Analysis

On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight for analysis of microalbumin:creatinine ratios (for extended method please see the online Data Supplement).

Plasma Induced Alterations in Vascular Function

Previously, we have demonstrated that treatment of mesenteric vessels from normal pregnant rats with plasma from the reduced uterine perfusion pressure (RUPP) rat (a rodent model of preeclampsia) induces endothelial dysfunction.16 To investigate whether plasma from PPAR-γ antagonist-treated rats behaved similarly to RUPP plasma, in terms of adversely affecting vascular function in mesenteric vessels from normal pregnant rats, we performed a separate series of experiments in which vessels were incubated overnight in plasma and their vascular function assessed. For plasma preparation, whole blood was collected as described previously and centrifuged at 2400g for 10 minutes at 4°C, the plasma removed and stored in 250-µL aliquots at −80°C. Pregnant rats (GD19) were euthanized via CO₂ asphyxiation and third-order mesenteric arteries dissected out and incubated overnight at 4°C in 3% plasma from rats treated with either vehicle or T0070907. This plasma concentration was chosen because an equivalent concentration of RUPP plasma was shown previously to induce endothelial dysfunction in mesenteric vessels.16 After overnight incubation, vessels were mounted onto a wire myograph and vascular function assessed as described previously.

Measurement of Angiogenic Mediators via ELISAs

Blood was collected as described above. Circulating soluble fms-like tyrosine kinase (sFlt) 1, vascular endothelial growth factor (VEGF), heme oxygenase 1 (HO-1), and interleukin 6 levels were measured in plasma samples using commercial ELISA kits available from R&D Systems (sFlt1, VEGF, and interleukin 6; Quantikine) and Enzo Life Sciences (HO-1) according to the manufacturer’s directions.

Histological Analysis

Kidneys and placentas were removed from rats and fixed in 4% formal buffered saline for 48 hours. Samples were processed (Tissue Tek VIP 5JR, Syntec Scientific Ltd, Dublin, Ireland), embedded in paraffin wax, and 4-µm serial sections cut, which were subsequently stained with hematoxylin and eosin.

Immunohistochemistry for sFlt-1, HO-1, CD-31, and Ki-67

Paraffin-embedded placental sections (4 µm) were incubated with the following primary antibodies used at the following dilutions: sFlt-1, 1:400 (Abcam ab2350); HO-1, 1:100 (Abcam ab13248); Ki-67, 1:100 (LabVision RM-9106-S), and CD-31 (Abcam ab28364). Rate of proliferation was assessed by blindly counting the percentage of Ki-67 immunopositive cells in randomly generated images from the placental labyrinths of all placentas (for extended method please see the online Data Supplement).

Quantitative RT-PCR

Total RNA was extracted from snap-frozen placental tissue and PCR performed with specific primers for sFlt-1 (forward primer 5'-ACG TCA CAT TGC CAA AAC-3'; reverse primer 5'-CAA CAC ACG ACA GTT TCA GG-3'), HO-1 (forward primer 5'-ACG TCA CAT TGC CAA AC-3'; reverse primer 5'-CTG AAA GTT CCT CAT GAA CTC-3'), and PPAR-γ (forward primer 5'-GTG TCA CAA TGC CAT CAG GT-3 '; reverse primer 5'-GTG CAG CTG GAT ATC AC-3'). Levels of mRNA expression were calculated using the mathematical formula for ΔΔcycle threshold and gene expression normalized to the geometric means of 2 housekeeping genes (insulin-like growth factor receptor 1 and platelet-derived growth factor A). Analysis was carried out using CFX Manager Software and data expressed as fold changes relative to controls (for extended methods please see the online Data Supplement).

Statistical Analysis

All of the calculations were performed on data from GD19 animals. Concentration-response curves for all vascular data were generated using GraphPad Prism. Concentration responses between groups...
were compared via repeated-measures ANOVA and Bonferroni post hoc test. Both EC$_{50}$ values (expressed as $-\log[\text{mol/L}] \pm \text{SEM}$) and R$_{\text{max}}$ values (maximal relaxation as a percentage of induced tone) were compared using a 2 tailed t test. For all of the other data, either a 2 tailed t test or Mann-Whitney test was used to determine significance. For all of the experimental groups, data were expressed as the mean $\pm$ SEM, and significance was determined as $p<0.05$.

**Solutions and Chemicals**

All of the chemicals were purchased from Sigma-Aldrich (Dorset, United Kingdom) unless otherwise stated.

**Results**

After administration of T0070907, healthy pregnant rats developed elevated mean arterial blood pressure compared with the control group (116±3 vs 106±3 mm Hg; $p<0.05$; n=8–9; Figure 1). PPAR-γ antagonist-treated rats demonstrated reduced pup weight (29.9±0.2 vs 39.9±0.4 g; $p=0.057$; n=7–9; Figure S1A, available in the online Data Supplement) and increased placental weights (0.54±0.02 vs 0.45±0.01 g; $p<0.001$; n=8; Figure S1B) compared with the control group. No differences were observed in pup number between the control and treatment groups (14±1 versus 11±2 g; $p$ value not significant; n=8–10; Figure S1C).

Mesenteric arteries from T0070907-treated rats displayed impaired relaxation in response to bradykinin (R$_{\text{max}}$: 56±4% versus 74±3%; $p<0.01$; log EC$_{50}$: 8.32±0.08 vs $-8.42\pm0.07$ mol/L; $p<0.001$; Figure 2) compared with controls. No differences were observed between groups in response to sodium nitroprusside (Figure S2), suggesting that the vascular dysfunction observed was because of adverse changes in the endothelium rather than the smooth muscle, a mechanism central to the pathogenesis of preeclampsia.

T0070907-treated rats had elevated microalbumin:creatinine ratios (24±4 versus 14±4 mg/mmol of creatinine; $p<0.05$; n=7–10; Figure 3) indicating proteinuria. However, histological examination of kidneys from these animals did not reveal any major structural abnormalities consistent with preeclampsia. PPAR-γ antagonist administration resulted in increased ADP-induced platelet aggregation compared with control rats (28.4±5.0 versus 16.3±2.8 area under the curve; $p<0.05$; n=8–9; Figure 4). In contrast, no difference was detected between groups in collagen-induced platelet aggregation (4.0±1.6 versus 3.8±2.5 area under the curve; $p=0.94$; n=8–9; Figure S3).

Pregnant rats administered T0070907 had reduced plasma VEGF (193.6±21.2 versus 399.5±33.2 pg/mL; $p<0.001$; n=8–9; Figure 5) and increased plasma sFlt-1 (3485±306 versus 1851±782 pg/mL; $p<0.05$; n=7–10; Figure 6). Increases in total placental sFlt-1 mRNA (quantitative RT-PCR; $p<0.05$; n=7–10; Figure S4) and Flt-1 protein (immunohistochemistry; Figure S5A and S5B) were also demonstrated, suggesting the placenta as one of the main contributors to the increased circulating levels of sFlt-1. T0070907-treated rats had significantly elevated plasma HO-1 levels compared with vehicle-treated rats (172±35 versus 96±3 pg/mL; $p<0.01$; n=6–8; Figure 7). Furthermore, placental HO-1 mRNA (quantitative RT-PCR;
Plasma levels of the proangiogenic cytokine, interleukin 6, did not differ significantly between both control and PPAR-γ antagonist-treated rats (13.0 ± 0.17 pg/mL; P = 0.91; n = 8–10; Figure S10). Placental PPAR-γ mRNA was reduced by ~30% in T0070907-treated rats compared with vehicle-treated rats (0.68 ± 0.01 versus 1.00 ± 0.14; P < 0.05; n = 7; Figure 8).

Histological examination of placentas demonstrated that control rat placentas had a very thin trophospongium layer consistent with a mature well-differentiated placenta (Figure S9A). In contrast, the labyrinthine trophoblast in the placentas of PPAR-γ antagonist-treated rats retained a prominent trophospongium (less differentiated trophoblast; Figure S9B), normally seen at GD15, indicating restricted placental development. A significant increase in Ki-67–positive cells was detected in the labyrinths of placentas from PPAR-γ antagonist-treated rats (13.0 ± 0.7% versus 7.0 ± 0.2% total cells; P < 0.05; n = 6–7; Figure S10), indicating increased cellular proliferation compared with controls (Figure S11A and S11B). Minimal staining for CD-31 (an indicator of vasculature) was observed in placental tissue from control rats (Figure S12A). In contrast, enhanced CD-31 immunoreactivity was observed in placentas from T0070907-treated rats (Figure S12B), indicating adaptive angiogenesis.

Overnight incubation of mesenteric vessels from healthy pregnant rats with plasma obtained from PPAR-γ antagonist-treated rats resulted in impaired vasorelaxation to bradykinin compared with those incubated with plasma from vehicle-treated rats (Rmax 50 ± 8% versus 88 ± 4%; P < 0.01; Figure 9). In further support of a role for the endothelium in mediating this vascular dysfunction, concentration-response curves to the endothelial-independent vasodilator, sodium nitroprusside, did not differ between groups (Figure S13).

**Discussion**

Accumulating evidence supports a role for PPAR-γ in both placental development and embryogenesis, because studies involving the ablation of the PPAR-γ gene in mice demonstrated a disruption in both the terminal differentiation of the labyrinthine trophoblast and placental vascularization. In the present study, administration of a PPAR-γ antagonist to pregnant rats resulted in the development of elevated blood pressure, endothelial dysfunction, proteinuria, and an imbalance of angiogenic proteins, all classic hallmarks of preeclampsia. These manifestations may have occurred, at least in part, in response to alterations in trophoblast differentiation, because the placentas of T0070907-treated rats were characterized by immature trophospongium development and an increased rate of cell proliferation.

In preeclampsia there is extensive platelet activation and increased adhesion to vascular endothelial cells. Activated platelets act as a major source of reactive oxygen species and may contribute considerably to the oxidative stress associated with preeclampsia. PPAR-γ antagonist administration resulted in increased ADP-induced platelet aggregation compared with control rats, which may have occurred as a result of T0070907-mediated inhibition of eicosanoids, which are

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**Figure 5.** Plasma levels of vascular endothelial growth factor (VEGF) compared with control rats treated (193.6 ± 21.2 vs 399.5 ± 33.2 pg/mL; P < 0.001; n = 6–8).

**Figure 6.** Plasma levels of fms-like tyrosine kinase 1 (sFlt) were significantly elevated in T0070907-treated rats compared with control rats (3485 ± 306 vs 1851 ± 782 pg/mL; P < 0.05; n = 7–10).

**Figure 7.** Plasma levels of heme oxygenase (HO) 1 were significantly elevated in T0070907-treated rats compared with control rats (172 ± 35 vs 96 ± 3 pg/mL; P < 0.01; n = 6–8).

**Figure 8.** T0070907-treated rats had significantly reduced peroxisome proliferator-activated receptor (PPAR)-γ mRNA compared with vehicle-treated rats. Data are expressed as fold difference. *P < 0.05 vs vehicle.
both endogenous ligands of PPAR-$\gamma$ and potent inhibitors of platelet function.

Preeclampsia is characterized by an imbalance of proangiogenic and antiangiogenic factors. PPAR-$\gamma$ antagonists displayed significantly reduced plasma levels of VEGF and significantly elevated levels of sFlt-1 compared with vehicle-treated rats, which would promote an angiogenic state. SFlt-1 is believed to be a significant contributor to endothelial damage in preeclampsia through interference with VEGF signaling. Exogenous administration of sFlt-1 to pregnant rats has shown previously to result in increased arterial blood pressure, proteinuria, glomerular endotheliosis, and associated decreases in plasma-free VEGF and placental growth factor (PLGF) concentrations. These effects also occurred in nonpregnant rats, suggesting that sFlt-1 has a direct effect on the maternal endothelium, and it is likely the increased sFlt-1 levels observed after T0070907 administration contributed significantly to the elevated blood pressure and endothelial dysfunction described. Similarly, overnight incubation of mesenteric vessels from healthy pregnant rats with plasma obtained from PPAR-$\gamma$ antagonist-treated rats also resulted in impaired vasorelaxation to bradykinin compared with those incubated with plasma from vehicle-treated rats. $\text{P}<0.05$ vs vehicle.

Figure 9. Overnight incubation of mesenteric vessels from healthy pregnant rats with plasma obtained from peroxisome proliferator-activated receptor (PPAR)-$\gamma$ antagonist-treated rats resulted in impaired vasorelaxation to bradykinin (BK) compared with those incubated with plasma from vehicle-treated rats.

Although no other studies have replicated our experimental protocol, data from first-trimester PPAR-$\gamma$ studies are not entirely consistent with our findings. In general, these studies have investigated the use of activation of PPAR-$\gamma$ in the first trimester using rosiglitazone, a PPAR-$\gamma$ agonist. The use of rosiglitazone in these studies has been shown to inhibit the trophoblastic invasion process and, in particular, to inhibit extravillous cytotrophoblast invasion and impair early human trophoblast migration. In contrast, PPAR-$\gamma$ antagonists were shown to promote extravillous cytotrophoblast cell invasion. However, results from other studies are more consistent with our findings. Parast et al demonstrated defects in both proliferation and differentiation in PPAR-$\gamma$ null trophoblast mouse stem cells. Barak et al demonstrated that the labyrinthine trophoblasts in PPAR-$\gamma$ null mouse placentas failed to properly differentiate. Our data have also demonstrated an impairment in placental differentiation in PPAR-$\gamma$ antagonist-treated rats. These conflicting results are possibly a result of differing time points for pharmacological and genetic manipulation, differing antagonists, and species variation. Further complementary work is required using additional PPAR-$\gamma$ antagonists and alternative doses and times of PPAR-$\gamma$ inhibition to establish whether dose- and time-dependent effects occur and to help clarify the exact role that PPAR-$\gamma$ plays in the progression of normal pregnancy in each species at all of the time points.

PPAR-$\gamma$ antagonist-treated rats were characterized by restricted placental development, as evidenced by the presence of a prominent trophospongium (less differentiated trophoblast), which is normally present at GD15, although control rat placentas had a very thin trophospongium layer consistent with a mature well-differentiated placenta. A significant increase in Ki-67-positive cells was also detected in the labyrinths of placentas from T0070907-treated rats, indicating increased cellular proliferation. These findings are analogous to data that showed defects in differentiation in PPAR-$\gamma$ null trophoblast stem cells. CD-31 immunoreactivity was enhanced in placentas from T0070907-treated rats indicating adaptive angiogenesis, which may be secondary to the upregulation of placental HO-1, increasing the production of carbon monoxide, a vasodilatory smooth muscle relaxing agent with potent proangiogenic properties.

Perspectives
The development of a preeclamptic-like phenotype, after administration of a PPAR-$\gamma$ antagonist, provides strong experimental support for our working hypothesis that PPAR-$\gamma$ critically regulates the risk of preeclampsia and, similarly, that an aberration in PPAR-$\gamma$ function adversely affects the progression of healthy pregnancy. These data
demonstrate the pivotal role for PPAR-γ in regulating the development of a healthy pregnancy in rodents.

Acknowledgments
We acknowledge Dora Baczyk for her input on immunohistochemistry and quantitative RT-PCR.

Sources of Funding
This work was funded by a PhD fellowship grant awarded to F.P.M. by Molecular Medicine Ireland.

Disclosures
None.

References
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Hypertension. 2011;58:882-887; originally published online September 19, 2011; doi: 10.1161/HYPERTENSIONAHA.111.179440

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:
http://hyper.ahajournals.org/content/58/5/882

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EVIDENCE IMPLICATING PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA IN THE PATHOGENESIS OF PREECLAMPSIA

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Extended method for Urine analysis
On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight. All samples were stored immediately following collection at -80°C. Microalbumin creatinine ratios (MCR) were then calculated following measurement of albumin using an Immuno- turbidimetric test for the quantitative determination of albumin in an OLYMPUS analysers® and creatinine using a kinetic colour test (Jaffé method).

Extended method for Platelet aggregation studies
Ex vivo platelet aggregation studies were performed to characterize the effects of PPAR-γ antagonism in vivo on platelet function. Whole blood was collected via the abdominal aorta into a heparinised vacutainer at room temperature. 0.5 ml of whole blood was placed in a cuvette with 0.5 ml of saline (0.9% NaCl) at 37°C and stirred with a magnetic stir bar. Platelet aggregation in response to either adenosine diphosphate (ADP; 5μM) or collagen (2μg/ml) was then measured using whole blood impedance aggregometry (Chrono-log Aggregometer). Platelet aggregation (expressed as area under the curve (AUC)) was measured over a period of 8 min and data calculated using Aggrolink® software (Chrono-log Corporation, PA, USA).

Extended method for immunohistochemical staining for sFlt-1, HO-1, CD-31 and Ki-67
Sections were cleared in xylene and rehydrated through a series of graded alcohols and then incubated in 0.3% H2O2 in methanol for 30 minutes. Antigen retrieval was accomplished with high heat 10mM sodium citrate buffer (pH 6) treatment for 45 min. Following phosphate buffered saline (PBS) washes, slides were incubated at room temperature (RT) with blocking solution (DAKO, Mississauga, ON) for 1 hour. Sections were incubated with primary antibodies overnight at 4°C and subsequently with biotinylated secondary antibodies (1:300) for 1 hour at RT. Slides were incubated with streptavidin-horseradish peroxidase (1hour, RT) followed by 3,3′-diaminobenzidine detection (Vector, Burlington, ON), then briefly counterstained with Harris’s Hematoxylin. Primary antibodies were used at the following dilutions: sFlt-1(1:400; Abcam ab2350); HO-1 (1:100; Abcam ab13248); Ki-67 (1:100; LabVision RM-9106-S) and CD-31 (1:100; Abcam ab28364). Rate of proliferation was assessed by blindly counting the percentage of Ki-67 immuno-positive cells in randomly generated images from the placental labyrinths of all placentas using newCAST™ software from Visiopharm. A pilot study of 100% analysis of two control placental labyrinths demonstrated that 15 random images per placenta (1% of each labyrinth) accurately reflected Ki-67 positive cell numbers. Representative images are provided for all immunohistochemistry.

Extended method for qRT-PCR
Total RNA was extracted from snap frozen placental tissue using the RNeasy® Plus Minikit (Qiagen). DNase free RNA (1μg) was reverse transcribed (iScript cDNA synthesis kit; Biorad) followed by quantification using a CFX384™ RT system (Biorad). PCR was performed with specific primers for rat sFlt-1 (forward primer 5’- ACG TCA CAG ATG TGC CAA AC- 3’; reverse primer 5’- CAA CAC AGG ACA GTT TCA GG- 3’), HO-1 (forward primer 5’- ACG TCA CAG ATG TGC CAA AC-3’; reverse primer 5’ CTG AAA GTT CCT CAT GAA CTC-3’) and PPAR-γ (forward primer 5’- GTC TCA CAA TGC CAT CAG GT- 3’; reverse primer 5’- GTT CAG CTG GTC GAT ATC AC- 3’). Samples were analyzed in triplicates in 12µl volumes containing 7.5ng of template cDNA and 6µl SYBR Green PCR Master Mix (Biorad). Conditions for the PCR were as follows: 95°C for 30sec; followed by 40 cycles of 95°C for 15sec; 60°C for 30sec (40cycles). The following
housekeeping genes were used for normalization of gene expression, insulin like growth factor receptor 1 (IGF-1R) and platelet derived growth factor A (PDGFA). Housekeeping genes and target genes were tested for efficacy prior to sample testing. Levels of mRNA expression were calculated using the mathematical formula for \( \Delta \Delta \) cycle threshold. Gene expression was normalized to the geometric means of both housekeeping genes. Analysis was calculated using CFX Manager Software™ and expressed as fold changes relative to controls.

**Expanded discussion**
Significant decreases in pup weight and significant increases in placental weight. Growth restriction is a common feature of a preeclamptic phenotype. The exact cause of this growth restriction remains unclear, although it is thought to be due to a combination of maternal endothelial dysfunction and hypertension affecting maternal fetal blood supply and nutrition. The increased placental weights are an interesting finding. We may speculate that this increase is due to a combination of increased vascularity (as demonstrated by increased CD-31 staining, Figure S12) increased proliferation (as demonstrated by increased Ki-67 staining; Figure S11) and possibly impaired differentiation (as demonstrated in Figure S9A & S9B). Our findings are consistent with other animal models of hypertension but no consistent pattern of pup and placental weights has emerged in other animal models of preeclampsia. It is also feasible that the administration of a PPAR-\( \gamma \) antagonist at this stage of gestation may alter the levels of metabolic hormones, such as insulin, IGF-1 and leptin which may also explain the reduced pup weight and increased placental weights.

**Supplementary references**
Figure S1. Effect of T0070907 administration on pup weight (A), placental weight (B) and pup number (C). Pups born to PPAR-γ antagonist (T0070907) treated rats had reduced pup weights (2.9±0.2 vs. 3.9±0.4g; P=0.057; n=7-9) and increased placental weights (0.54±0.02 vs. 0.45±0.01g; P<0.001; n=8) compared with controls. No significant differences were observed in pup number between the control and treatment groups (14±1 vs. 11±2; P=0.09; n=8-10). Data is expressed as mean ± s.e.m.
Figure S2. Effects of administration of T0070907 on vascular relaxation in response to sodium nitroprusside. No differences were observed in third order mesenteric arteries between groups in response to sodium nitroprusside (SNP; \( R_{\text{max}} \) 95 ± 1 versus 93 ± 2%; not significant; Log EC\(_{50}\) -7.89±0.04 vs. -8.07±0.09mol/L; not significant; n=8). Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± s.e.m.
Figure S3. Effect of T0070907 administration on collagen (2µg/ml) induced platelet aggregation. No differences were detected between groups in relation to collagen induced platelet aggregation (4.0±1.6 vs. 3.8±2.5 area under curve (AUC); P=0.94; n=8-9). Data expressed as mean area under the curve ± s.e.m.
Figure S4. Effect of the PPAR-γ antagonist, T0070907, administration on placental expression of sFlt-1 mRNA. Total placental sFlt-1 was 50% increased in T0070907 treated rat placentas compared with controls (P<0.05; n=7). Data expressed as fold difference.
Figure S5. Effect of T0070907 on placental Flt-1 protein expression. Control placental tissue (A) had minimal staining for Flt-1 protein (as indicated by dark red/brown staining) compared with the intense staining for Flt-1 observed in the placentas of T0070907 treated rats (B). This may suggest that the placenta is one of the main contributors to the increased circulating levels of sFlt-1 documented in PPAR-\(\gamma\) antagonist treated rats. Image representative of n=6. Enhanced image at x100 magnification.
Figure S6. Effects of T0070907 administration on expression of HO-1 mRNA. Placental HO-1 mRNA (measured via qRT-PCR) was elevated by more than 50% in T0070907 treated rats compared with controls (P<0.05). Data expressed as fold difference.
Figure S7. Effect of T0070907 on placental HO-1 protein expression. Positive staining for HO-1 (as indicated by brown staining) was virtually undetectable in control placental tissue (A) compared with intense staining for HO-1 in placentas from T0070907 treated rats (B). Image representative of n=6. Enhanced image at x100 magnification.
Figure S8. Effect of T0070907 administration on circulating IL-6. IL-6 levels did not differ significantly between both control and PPAR-γ antagonist treated rats (96±4 vs. 96±2pg/ml; P=0.91; n=8). Data expressed as mean ± s.e.m.
Figure S9A. H&E staining of placentas from control and T0070907 treated rats. Histological examination of placentas using hematoxylin and eosin (H&E) staining, demonstrated that placentas from control rats (A) had a very thin trophospongium layer consistent with a mature well differentiated placenta. In contrast, the labyrinthine trophoblast in the placentas of PPAR-γ antagonist treated rats (B) retained a prominent trophospongium (less differentiated trophoblast), normally seen at GD15, indicating restricted placental development. Image representative of n=6, Enhanced image at x100 magnification. Morphological different layers: 4 - labyrinth; 3 - trophospongium; 2 - giant-cell layer; 1 - stroma.
Figure S10. Percentage of Ki-67 positive cells in placental tissue. A significant increase in Ki-67 positive cells was detected in the labyrinths of placentas from PPAR-γ antagonist treated rats (13±0.7 vs. 7±0.2% total cells; P<0.05; n=6) indicating increased cellular proliferation compared with controls. Data expressed as percentage total cells ± s.e.m.
Figure S11. Representative photomicrographs of Ki-67 positive cells in placental tissue. Ki-67 positive cells are indicated by intense black localised staining appearing as black dots in placental tissue from control (A) and PPAR-γ antagonist treated (B) rats. Image representative of n=6. Enhanced image at x100 magnification
Figure S12. Effect of T0070907 administration of placental vascularity. Minimal staining for CD-31 (an indicator of vascularity and indicated by red staining) was observed in placental tissue from control rats (A). In comparison, enhanced CD-31 immunoreactivity was observed in placentas from PPAR-γ antagonist treated rats (B), indicating adaptive angiogenesis. Image representative of n=6. Enhanced image at x100 magnification.
Figure S13. Effect of plasma from T0070907 treated rats on vascular responses to sodium nitroprusside (SNP). In further support of a role for the endothelium in mediating this vascular dysfunction, no significant differences were observed in concentration response curves to the endothelial-independent vasodilator, SNP ($R_{\text{max}}$ 95±2 vs. 93±3%; not significant; n=8). Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± s.e.m.