Peroxisome Proliferator-Activated Receptor-γ as a Potential Therapeutic Target in the Treatment of Preeclampsia

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

Abstract—Preeclampsia is a multisystemic disorder of pregnancy characterized by hypertension, proteinuria, and maternal endothelial dysfunction. It is a major cause of maternal and perinatal morbidity and mortality and is thought to be attributable, in part, to inadequate trophoblast invasion. Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a ligand-activated transcription factor expressed in trophoblasts, and the vasculature of which activation has been shown to improve endothelium-dependent vasodilatation in hypertensive conditions. We investigated the effects of the administration of a PPAR-γ agonist using the reduced uterine perfusion pressure (RUPP) rat model of preeclampsia. The selective PPAR-γ agonist, rosiglitazone, was administered to pregnant rats that had undergone RUPP surgery. To investigate whether any observed beneficial effects of PPAR-γ activation were mediated by the antioxidant enzyme, heme oxygenase 1, rosiglitazone was administered in combination with the heme oxygenase 1 inhibitor tin-protoporphyrin IX. RUPP rats were characterized by hypertension, endothelial dysfunction, and elevated microalbumin:creatinine ratios. Rosiglitazone administration ameliorated hypertension, improved vascular function, and reduced the elevated microalbumin:creatinine ratio in RUPP rats. With the exception of microalbumin:creatinine ratio, these beneficial effects were abrogated in the presence of the heme oxygenase 1 inhibitor. Administration of a PPAR-γ agonist prevented the development of several of the pathophysiological characteristics associated with the RUPP model of preeclampsia, via a heme oxygenase 1–dependent pathway. The findings from this study provide further insight into the underlying etiology of preeclampsia and a potential therapeutic target for the treatment of preeclampsia. (Hypertension. 2011;58:00-00.) ● Online Data Supplement

Key Words: peroxisome proliferator-activated receptor-γ ▪ preeclampsia ▪ reduced uterine perfusion pressure ▪ hypertension ▪ heme oxygenase 1 ▪ vascular dysfunction

Preeclampsia is a major cause of maternal and perinatal morbidity and mortality 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, it is characterized by a relatively hypoperfused placenta, which stimulates the maternal response manifesting as hypertension, vascular dysfunction, alterations in platelet aggregation, and a pro-oxidant state.2 There is currently no effective pharmacological intervention available for the treatment of preeclampsia, and, consequently, pregnancies are often delivered preterm for maternal benefit, imposing the challenges of iatrogenic pre-maturity on the fetus.3

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.4 PPAR-γ plays a predominant role in normal vascular function5 and in the differentiation of labyrinthine trophoblast lineages6, which, along with the fetal endothelium, form the vascular exchange interface with maternal blood.7 A reduction in the placental expression of PPAR-γ activators has been demonstrated in some women who develop severe preeclampsia,8 and significantly higher PPAR-γ DNA binding activity has been demonstrated in placentas from women with both intrauterine growth restriction and preeclampsia.9 To investigate the role of PPAR-γ as a potential therapeutic target for preeclampsia, we administered the PPAR-γ agonist, rosiglitazone, to rats that had undergone reduced uterine perfusion pressure (RUPP) surgery. As PPAR-γ agonists have been shown to mediate some of their beneficial effects via upregulation of heme oxygenase 1 (HO-1), an antioxidant enzyme that negatively regulates soluble fms-like tyrosine kinase-1 (sFlt-1) expression,10 we examined whether this enzyme played a role in any observed beneficial effects of rosiglitazone in the RUPP rat.
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 with 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).

RUPP Procedure
Using pregnant Sprague Dawley rats (200 to 250 g), uterine perfusion pressure was surgically reduced as described previously and further described in the online Data Supplement at http://hyper.ahajournals.org.

PPAR-γ Agonist In Vivo Experimental Protocol
Five experimental groups were used to investigate the effect of administration of a PPAR-γ agonist (rosiglitazone) in the RUPP rat, and all of the pharmacological interventions were administered on GD16-18. In the first experimental group, normal pregnant (NP; n=12) rats were administered vehicle (10% dimethyl sulfoxide) in drinking water. In the second experimental group, pregnant rats subjected to the RUPP procedure (RUPP; n=14) were administered vehicle (10% dimethyl sulfoxide) in drinking water. In the third treatment group (RUPP and Rosig; n=11), RUPP rats received rosiglitazone (5 mg/kg per day; Cayman Chemical) in drinking water. To investigate whether any observed beneficial effects of rosiglitazone treatment were mediated through an HO-1–dependent pathway, tin-protoporphyrin IX (SnPP; 50 μmol/kg per day; a HO-1 inhibitor; Sigma Aldrich) was administered in combination with rosiglitazone (RUPP and Rosig and SnPP; n=9) to the fourth experimental group. A fifth experimental group was used in which RUPP rats received SnPP alone (RUPP and SnPP; 50 μmol/kg per day; n=8) to examine the effects of HO-1 antagonism alone in RUPP rats. Both SnPP and rosiglitazone were administered orally via drinking water. Pharmacological agents were administered in drinking water after the completion of a pilot study to investigate average daily water intake. After this pilot study, vehicle, rosiglitazone, and/or SnPP were administered in a predefined amount of water, which was replaced daily after consumption of the previous day’s water with or without pharmacological agent/vehicle. Fluid intake was continually monitored over the study period, and fluid intake did not vary after the addition of pharmacological agents to the drinking water. The concentrations of rosiglitazone11,12 and SnPP15,16 used in the present study were chosen on the basis of previously published data.

Concentration-response curves for all of the vascular data were generated using GraphPad Prism. Concentration responses between groups were compared via a repeated-measures ANOVA and a Bonferroni post hoc test. Both EC50 values (expressed as −log [in moles per liter]±SEM) and maximal relaxation as a percentage of induced tone (Rmax values) were compared using a 1-way ANOVA and Dunnett post hoc test. For all of the other data, a 1-way ANOVA and Dunnett post hoc test were used to determine significance. For all of the experimental groups, data were expressed as the mean±SEM, and significance was determined as P<0.05.

Statistical Analysis
All of the calculations were performed on data from GD19 animals. Concentration-response curves for all of the vascular data were generated using GraphPad Prism. Concentration responses between groups were compared via a repeated-measures ANOVA and a Bonferroni post hoc test. Both EC50 values (expressed as −log [in moles per liter]±SEM) and maximal relaxation as a percentage of induced tone (Rmax values) were compared using a 1-way ANOVA and Dunnett post hoc test. For all of the other data, a 1-way ANOVA and Dunnett post hoc test were used to determine significance. For all of the experimental groups, data were expressed as the mean±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
RUPP rats displayed elevated mean arterial blood pressure compared with NP rats (127±5 versus 91±2 mm Hg; P<0.001; n=7 to 10; Figure 1). Treatment of RUPP rats with the PPAR-γ agonist rosiglitazone significantly reduced RUPP-induced hypertension (103±3 versus 127±5 mm Hg;
P<0.001; n=7 to 10; Figure 1). Coadministration of rosiglitazone with the HO-1 inhibitor SnPP abrogated the previously observed mean arterial blood pressure-lowering effects of this PPAR-γ agonist (124±4 versus 103±3 mm Hg; P<0.01; n=7 to 8; Figure 1). Treatment of RUPP rats with SnPP alone did not alter mean arterial blood pressure compared with control RUPP rats (129±3 versus 127±5 mm Hg; P=0.70; n=6 to 7; Figure 1), nor were there any differences observed in any outcomes between NP rats and sham-operated rats (data not shown).

All of the groups subjected to the RUPP procedure had reduced maternal weights (238±6 versus 287±5 g; P<0.001; n=12; Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org) and reduced pup numbers (5±1 versus 14±0.3; P<0.001; n=8 to 12; P<0.001; Figure S2). Both pup (Figure S3) and placental (Figure S4) weights were also reduced in RUPP rats compared with NP rats. Perhaps unsurprisingly, because of the surgical nature of the RUPP model, treatment with rosiglitazone did not alter pup number, pup weight, or placental weight.

Mesenteric arteries from RUPP rats displayed impaired vasoconstriction in response to bradykinin when compared with NP rats (R_{max}: 48±3% versus 72±3%; P<0.001; log EC_{50}: −7.675±0.03 versus −7.79±0.03 mol/L; P<0.05; n=8 to 10; Figure 2). However, no differences were observed in the vascular responses of vessels to the endothelial independent vasodilator, sodium nitroprusside, between RUPP and NP rats, suggesting that this vascular dysfunction was endothelial dependent (Figure S5). In vivo administration of rosiglitazone ameliorated this endothelial dysfunction when compared with control RUPP rats (R_{max}: 65±3% versus 48±3%; P<0.01; n=8 to 10; Figure 2). Coadministration of SnPP with rosiglitazone abrogated the vasculoprotective effect observed when rosiglitazone was administered alone (R_{max}: 48±3% versus 65±3%; P<0.01; n=8 to 10; Figure 2). The vasorelaxant responses of mesenteric vessels (in response to bradykinin) from both control RUPP rats and those treated with SnPP alone did not differ significantly from each other (R_{max}: 48±3% versus 47±4%; P value not significant; n=8 to 10; data not shown).

RUPP rats displayed significantly elevated microalbumin:creatinine ratios when compared with NP rats (63±12 versus 19±3 mg/mmol of creatinine; P<0.001; n=11 to 15; Figure 3). Furthermore, administration of rosiglitazone significantly reduced MCR compared with control RUPP rats (24±6 versus 63±12 mg/mmol creatinine; P<0.01; n=9 to 11; Figure 3). Coadministration of SnPP with rosiglitazone did not reverse the beneficial effects observed with rosiglitazone alone (18±3 versus 16±3 mg/mmol of creatinine; P=0.54; n=9; Figure 3). In addition, administration of SnPP alone had no significant effect on MCR when compared with the RUPP control group (35±11 versus 63±12 mg/mmol of creatinine; P=0.10; n=8 to 11; Figure 3). Similarly, RUPP rats displayed elevated protein:creatinine ratios compared with NP rats (58±10 versus 17±2 mg/mmol of creatinine; P<0.001; n=11 to 15; Figure S6). Administration of rosiglitazone significantly reduced protein:creatinine ratios compared with the control RUPP group (16±3 versus 58±10 mg/mmol of creatinine; P<0.01; n=9 to 11; Figure S6). Coadministration of SnPP with rosiglitazone did not reverse the beneficial effects observed with rosiglitazone alone (30±7 versus 24±6 mg/mmol of creatinine; P=0.60; n=9; Figure S6), and in the experimental group administrated SnPP alone, protein:creatinine ratios did not differ significantly from the vehicle-treated RUPP group (58±10 versus 27±7 mg/mmol of creatinine; P=0.07; n=6 to 11; Figure S6).

No significant differences were observed in ADP-induced platelet aggregation in whole blood from RUPP compared with NP rats (57±14 versus 43±9 area under the curve; P=0.41; n=7 to 9; Figure 4). However, ADP-induced platelet aggregation was completely abolished in blood from RUPP rats administered rosiglitazone when compared with control RUPP rats (0.7±0.7 versus 57.1±14.4 area under the
Rosig vs RUPP and Rosig and SnPP.

### Effects of Rosiglitazone Administration

**Figure 4.** Effects of rosiglitazone administration in the reduced uterine perfusion pressure (RUPP) rat on adenosine diphosphate (ADP)-induced platelet aggregation. No significant increases were observed in ADP-induced platelet aggregation in RUPP rats compared with control rats. Treatment of RUPP rats with rosiglitazone completely abolished ADP-induced platelet aggregation compared with RUPP rats. Coadministration of tin-protoporphyrin IX (SnPP) with rosiglitazone reversed this PPAR-γ agonist–mediated reduction in platelet aggregation. Data are expressed as mean area under the curve (AUC) ± SEM. *P < 0.001 RUPP vs RUPP and Rosig, †P < 0.01 RUPP and Rosig vs RUPP and Rosig and SnPP.

**Figure 5.** Effects of rosiglitazone administration in the reduced uterine perfusion pressure (RUPP) rat on plasma heme oxygenase-1 (HO-1) levels. RUPP rats had significantly lower circulating plasma HO-1 levels compared with normal pregnant (NP) rats. Administration of rosiglitazone resulted in a significant increase in plasma HO-1 levels compared with control RUPP rats. Coadministration of tin-protoporphyrin IX (SnPP) with rosiglitazone significantly reduced plasma HO-1 levels compared with rosiglitazone administration alone. Administration of SnPP alone did not significantly reduce plasma HO-1 levels compared with vehicle-treated RUPP rats. Data are expressed as mean picograms per millilitre ± SEM. *P < 0.05 NP vs RUPP, †P < 0.01 RUPP vs RUPP and Rosig, and #P < 0.05 RUPP and Rosig versus RUPP and Rosig and SnPP.

- **Figure 4** shows the effects of rosiglitazone administration in the reduced uterine perfusion pressure (RUPP) rat on ADP-induced platelet aggregation. Treatment with rosiglitazone resulted in a significant increase in platelet aggregation compared with RUPP rats. Coadministration of tin-protoporphyrin IX (SnPP) with rosiglitazone reversed this effect.
- **Figure 5** demonstrates the effects of rosiglitazone administration on plasma heme oxygenase-1 (HO-1) levels. RUPP rats had significantly lower circulating plasma HO-1 levels compared with NP rats. Administration of rosiglitazone resulted in an increase in plasma HO-1 levels compared with control RUPP rats.

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In models of diabetes mellitus and cardiovascular disease, activation of PPAR-γ has been shown to restore vascular structure and correct endothelial dysfunction, resulting in a reduction in the elevated blood pressure associated with...
hypertensive conditions. In the present study, administration of the PPAR-γ agonist rosiglitazone to RUPP rats ameliorated both hypertension and endothelial dysfunction via a HO-1–dependent pathway. Our data demonstrating a protective role for PPAR-γ in endothelial function is supported by findings from a study by Kleinhenz et al., which demonstrated that endothelial cell-specific PPAR-γ knockout in mice results in both hypertension and impaired vasorelaxation. The present study is one of the first to demonstrate a vasculoprotective role for PPAR-γ in an animal model of preeclampsia and, in addition, proposes a role for HO-1 (antioxidant enzyme) in mediating this protective effect. Previous studies have shown that HO-1 is upregulated by PPAR-γ agonists and that HO-1 induction can prevent tumor necrosis factor-α–induced endothelial dysfunction in vitro; thus, it may be possible that rosiglitazone protects the vasculature via an HO-1–dependent anti-inflammatory mechanism. The coadministration of SnPP with rosiglitazone ameliorated several of the effects observed after administration of rosiglitazone alone to the RUPP rat by inhibiting HO-1 expression under these experimental conditions. The exact mechanism as to how SnPP acts on HO-1 expression and activity is unclear and somewhat dependent on the experimental settings. SnPP treatment has been shown to inhibit enzyme activity, resulting in an induction of protein expression in rat liver allografts. Similar to our data, SnPP treatment has been shown to reduce HO-1 enzyme activity and expression in rat basophil leukemia cells. Although our data demonstrate elevated plasma HO-1 levels in response to rosiglitazone treatment, the exact source of this HO-1 is yet unknown, and the differential expression and regulation of HO-1 requires further investigation.

In addition to the improvement in vascular function and blood pressure, administration of rosiglitazone also reduced ADP–induced platelet aggregation in whole blood from RUPP rats, an effect that was abrogated in the presence of the HO-1 inhibitor, SnPP. Studies have demonstrated that platelet activation/aggregation contributes to endothelial dysfunction via both proinflammatory and pro-oxidant pathways and that antplatelet agents mediate vasculoprotective effects. Furthermore, platelets express PPAR-γ, of which activation by 15-deoxy-D12,14-prostaglandin J2 (an endogenous PPAR-γ ligand) has been shown to inhibit the proinflammatory effects of these cells, including aggregation and the release of both thromboxane A2 and adenosine triphosphate. Thus, it may be possible that part of the vasculoprotective effect of PPAR-γ activation is mediated by its antiplatelet effect; however, this involves further investigation using in vivo studies measuring both inflammatory mediators and possibly reactive oxygen species after PPAR-γ agonist administration.

In the present study, RUPP rats were characterized by increased microalbumin excretion from the kidneys, an additional marker of endothelial dysfunction and a hallmark of preeclampsia. Administration of rosiglitazone reduced microalbumin excretion, and this was unaffected after coadministration with SnPP, which may suggest that pathways other than those dependent on HO-1 mediate the associated renal vascular dysfunction. Recent data from a number of clinical studies have demonstrated a beneficial effect of rosiglitazone on urinary albumin excretion, which has been proposed as being mediated via both an improvement in glycemic control and independent of changes in fasting plasma glucose. Furthermore, a number of experimental studies have demonstrated that the blood pressure–lowering and endothelial-stabilizing effects of rosiglitazone can occur independent of its metabolic effects. On this basis we chose not to investigate the effects of rosiglitazone on glucose regulation; however, we cannot rule out the latter as a possible mechanism for the improvement in vascular function.

Our data are consistent with previous studies that have demonstrated that RUPP rats are characterized by an imbalance of angiogenic factors, in terms of increased sFlt-1 and decreased VEGF plasma levels, and a reduction in placental expression of HO-1. However, although PPAR-γ agonists have been shown previously to restore proangiogenic factors, upregulate HO-1, and increase the production of VEGF in vitro, and in vivo, rosiglitazone failed to alter angiogenic factors in the RUPP rat in the present study. A recent study, involving the induction of HO-1 in RUPP rats via the administration of cobalt (III) protoporphyrin IX chloride, demonstrated that, as a result of cobalt (III) protoporphyrin IX chloride treatment, placental expression of HO-1 was increased, and there was a significant shift in the angiovascular balance ratio (sFlt-1:VEGF). In the present study, neither placental expression or plasma levels of sFlt-1 was reduced nor were plasma levels of VEGF increased after rosiglitazone treatment, which may have occurred as a consequence of a failure of this PPAR-γ agonist to alter placental HO-1 expression (which was associated with beneficial outcomes in both by George et al). Thus, to see a beneficial effect of rosiglitazone on the sFlt-1:VEGF balance (as a consequence of alterations in placental HO-1 expression) it may be necessary to implement treatment at the point of surgical intervention (ie, RUPP surgery on GD14) as per cobalt (III) protoporphyrin IX chloride treatment. However, although rosiglitazone has been shown to upregulate HO-1 expression in both vascular smooth muscle and endothelial cells, which failed to do so in lung fibroblasts, which may suggest a cell-specific effect that does not extend to the most abundant placental cell type, the trophoblast. Thus, in the present study, the beneficial effects of rosiglitazone, in terms of both vascular function and blood pressure, may simply be mediated by direct modulation of endothelial HO-1 activity, because these effects were abrogated by the HO-1 activity inhibitor SnPP.

Although the present study demonstrates the numerous beneficial effects of rosiglitazone administration in the RUPP model of preeclampsia, the timing of pharmacological intervention is roughly equivalent to the second and third trimester of human pregnancies (when placental development is complete), and, thus, the effects of PPAR-γ agonism on placental development have not been examined in this study. Previous research has highlighted the integral role that PPAR-γ plays in early placental development. Activation of the ligand-activated nuclear receptor PPAR-γ has been shown to inhibit the trophoblastic invasion process. Furthermore, rosiglitazone has been shown to inhibit extravillous cytotrophoblast (EVCT) invasion, whereas PPAR-γ antagonists were shown...
to promote EVCT invasion.40 Activation of PPAR-γ by human cytomegalovirus also impaired early human trophoblast migration and invasiveness in the EVCT.41 In recent studies, both Nadra et al42 and Schaff et al43 demonstrated adverse placental development after rosiglitazone administration to healthy pregnant mice, including disorganization of placental layers, altered placental microvasculature, decreased placental expression of angiogenic factors, and thinning of the spongiotrophoblast layer and dilated labyrinthine blood spaces. In both studies, rosiglitazone was administered during the early stages of gestation and at considerably higher concentrations (30 to 100 mg/kg per day) then that used in the present study (5 mg/kg per day). It may, therefore, be possible that this PPAR-γ agonist is only protective as follows: (1) when it is administered in lower doses; (2) when it is administered in disease states, which may be characterized by differential expression/activity of PPAR-γ; and/or (3) when it is administered during the latter stages of gestation, after placental development. Thus, although rosiglitazone has demonstrated a protective effect in the RUPP model of preeclampsia, caution should be exercised in terms of extrapolating these findings in terms of the clinical situation until further investigation of the placental effects of rosiglitazone has been completed.

Perspectives

The underlying etiology of preeclampsia is poorly understood, and as yet there is no effective pharmacological intervention available for this condition. The present study demonstrates that administration of a PPAR-γ agonist to an animal model of preeclampsia can ameliorate a number of the pathophysiological hallmarks associated with this condition and, thus, proposes PPAR-γ as a potential therapeutic target for further investigation.

Acknowledgments

We acknowledge Dora Baczyk for her input on histology, immuno-histochemistry, 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.

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Hypertension. published online June 20, 2011;

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PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA AS A POTENTIAL THERAPEUTIC TARGET IN THE TREATMENT OF PREECLAMPSIA

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Expanded materials and methods

Extended methods for RUPP procedure
Using pregnant Sprague Dawley rats (200-250g) uterine perfusion pressure was surgically reduced as described previously.1,2 In brief, animals were anaesthetized with isoflurane (2-5% inhalation) on gestational day (GD) 14, a midline laparotomy incision made and the abdominal aorta isolated. A silver clip (0.203 mm ID) was placed around the aorta (above the iliac bifurcation) to reduce uterine perfusion pressure by approximately 40%.3 To prevent a redistribution of blood flow via the ovarian arteries, silver clips (0.10 mm ID) were placed on both the right and left uterine arcades between the ovarian branch and the first branch leading to the uterus and first pup.4 The abdomen was then closed and the animal allowed to recover. On GD18, a chronic indwelling catheter was inserted into the carotid artery and on GD19 mean arterial blood pressure (MABP) was recorded in conscious animals. Any rat in which the RUPP procedure resulted in total reabsorption of fetuses was excluded from the study.

Extended methods for Isometric Myography
Following culling of the rat, third order mesenteric arteries were dissected out within one hour and mounted onto a four channel wire myograph (Model 610M, Danish Myo Technology (DMT), Aarhus, Denmark) containing oxygenated (95% O2 & 5% CO2) physiological saline solution (PSS) at 37°C. Vessels were normalized to achieve a transmural pressure of 100mmHg using the DMT Normalization software. Isometric tension was recorded and displayed using a Powerlab and Chart Software (both AD Instruments). The viability of the smooth muscle was tested via the addition of a 123mmol/L KCl solution. Following two PSS washes, cumulative concentration responses were carried out with the thromboxane mimetic, U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α; 10^-9 – 3x10^-7 mol/L) and either bradykinin (BK; 10^-9 – 10^-5 mol/L) or sodium nitroprusside (SNP; 10^-9 – 10^-5 mol/L). In previous work by this group, endothelial dysfunction was detected in RUPP vessels at a concentration as low as 1nM (10^-9) when using bradykinin. This contrasted significantly with ACh where endothelial dysfunction was only evident at 100nM (10^-7). This may suggest that bradykinin is a more physiologically relevant vasodilator under these conditions and as a result bradykinin was the endothelium dependent vasodilator used.

Extended methods for Platelet aggregation studies
Ex vivo platelet aggregation studies were performed to characterize the effects of PPAR-γ agonism 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 adenosine diphosphate (ADP; 5μM) was then measured using 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 methods for Urine analysis
On GD18, each rat was singularly housed in a metabolic cage and urine collected overnight. Microalbumin creatinine ratios (MCR) were 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 method5) for the quantitative determination of creatinine in urine. Protein creatinine ratios were calculated following measurement of protein by adding benzethonium chloride to urine samples followed by turbidimetric quantification at 525 nm using an OLYMPUS analysers®. To
validate the use of rat urine in the OLYMPUS analysers®, 102 rat urine samples were collected on GD11 from NP rats and MCRs were calculated to establish a baseline level.

**Extended methods for measurement of angiogenic mediators via enzyme linked immunosorbent assays**

For plasma preparation, whole blood was collected into precooled heparinised vacutainers under anaesthetic via abdominal aortic puncture. Blood was then centrifuged at 2400g for 10 min at 4°C, the plasma removed and stored in 250μL aliquots at -80°C. Circulating sFlt-1, vascular endothelial growth factor (VEGF) and HO-1 levels were measured in plasma samples from all experimental groups using commercial ELISA kits available from R&D Systems (QuantiKine; s-Flt-1 and VEGF) and Enzo Life Sciences (HO-1) according to the manufacturer’s directions.

**Histological analysis**

Placentas were removed from rats, weighed 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 4μm serial sections cut which were subsequently stained with hematoxylin and eosin (H&E).

**Extended methods for Immunohistochemistry for sFlt-1, CD-31 and Ki-67**

For immunohistochemical staining, sections on polysine slides were cleared in xylene, rehydrated through a series of graded alcohols and 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. Following PBS washes, slides were incubated with streptavidin-horseradish peroxidase (1hour, RT) followed by DAB detection (Vector, Burlington, ON). Sections were briefly counterstained with Harris’s Hematoxylin and dehydrated by sequential immersion of slides in ascending ethanol series. Following xylene clearing, slides were cover-slipped with Cytoseal mounting media (Cole-Parmer, Vernon Hills, IL). Primary antibodies were used at the following dilutions: sFlt-1 (1:400; Abcam ab2350); Ki-67 (1:100; LabVision RM-9106-S) and CD-31 (1:100; Abcam ab28364; used as an indicator of vascularity). 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.

**Extended methods 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’). 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 \( \Delta \Delta \text{Ct} \) 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.

Supplemental References


Figure S1. Effect of rosiglitazone administration on maternal body weight in the RUPP rat. RUPP rats weighed significantly less than normal pregnant rats (238 ± 6 versus 287 ± 5 grams; P<0.001; n=12). Treatment of RUPP rats with rosiglitazone did not significantly alter maternal weight compared to control RUPP rats (228 ± 4 versus 238 ± 6 grams; P=0.21; n=11-12). Similarly, co-administration of SnPP with rosiglitazone did not significantly alter maternal weight compared to RUPP rats treated with rosiglitazone alone (238 ± 6 versus 227 ± 4 grams; P=0.77; n=9-11). SnPP administered alone to RUPP rats did not significantly alter maternal weight compared to vehicle treated RUPP rats (255 ± 10 versus 238 ± 6 grams; P=0.16; n=8-12). Data expressed as mean maternal weight (grams) ± s.e.m. *P<0.001 Normal pregnant versus RUPP.
Figure S2. Effect of rosiglitazone administration on pup number in the RUPP rat.
As the RUPP procedure results in the reabsorption of pups, the normal pregnant group had significantly more pups compared to RUPP, RUPP & Rosig, RUPP & Rosig & SnPP and RUPP & SnPP (14 ± 0 versus 5 ±1 & 6 ± 1 & 4 ± 1 & 5 ± 1 respectively; P<0.001; n=8-12). Data expressed as mean pup number ± s.e.m. *P<0.001 Normal pregnant versus RUPP, RUPP & Rosig, RUPP & Rosig & SnPP and RUPP & SnPP.
Of the remaining viable pups, RUPP pups were smaller in weight compared with the normal pregnant control group (2.2 ± 0.2 versus 2.6 ± 0.1 grams; P<0.05; n=8-12). Administration of rosiglitazone did not significantly alter pup weight compared to control RUPP pups (2.2 ± 0.2 versus 2.3 ± 0.2 grams; P=0.74; n=10-11). Similarly, no differences were observed following co-administration of rosiglitazone with SnPP compared to rosiglitazone alone (2.6 ± 0.1 versus 2.3 ± 0.1 grams; P=0.06; n=9-11). The administration of SnPP alone to RUPP rats had no significant effect on pup weight compared to pups of vehicle treated RUPP pups (2.2 ± 0.2 versus 2.6 ± 0.2 grams; P=0.09; n=6-10). Data expressed as mean pup weight (grams) ± s.e.m. *P<0.05 Normal pregnant versus RUPP
Figure S4. Effect of rosiglitazone administration on placental weight in the RUPP rat. Placental weight was significantly reduced in control RUPP rats compared with the normal pregnant group (0.44 ± 0.04 versus 0.54 ± 0.04 grams; P<0.05; n=10-12). Administration of rosiglitazone did not significantly alter placental weight compared to the control RUPP group (0.44 ± 0.03 versus 0.43 ± 0.01 grams; P=0.84; n=10-11). No significant differences were observed following co-administration of rosiglitazone with SnPP compared to rosiglitazone alone (0.46 ± 0.01 versus 0.43 ± 0.01 grams; P=0.06; n=9-11). The administration of SnPP alone to RUPP rats had no significant effect on placental weights compared to placental weights from vehicle treated RUPP rats (0.47 ± 0.01 versus 0.43 ± 0.01 grams; P=0.09; n=8-10). Data expressed as mean weight (grams) ± s.e.m. *P<0.05 Normal pregnant versus RUPP.
Figure S5. Effect of rosiglitazone administration in the RUPP rat on vascular response to SNP. No differences were observed in the vascular responses of vessels to SNP in any of the groups. Relaxation is calculated as a percentage of the maximum contraction and expressed as mean ± s.e.m.
Similarly, RUPP rats displayed elevated protein creatinine ratios compared with NP rats. Administration of rosiglitazone significantly reduced protein creatinine ratios compared to the control RUPP group. Data expressed as mean mg/mmol creatinine ± s.e.m. *P<0.001 Normal pregnant versus RUPP, †P<0.01 RUPP versus RUPP & Rosig.
Figure S7. Effect of rosiglitazone administration in the RUPP rat on plasma sFlt-1 levels. RUPP rats had higher plasma levels of sFlt-1 (4133 ± 555 versus 1466 ± 650pg/ml; P<0.01; n=7). Treatment with rosiglitazone did not significantly reduce either plasma levels (3633 ± 15 versus 4133 ± 555pg/ml; P=0.25; n=7-9). Co-administration of SnPP with rosiglitazone did not significantly alter plasma sFlt-1 level compared to rosiglitazone treatment alone (3490 ± 132 versus 3541 ± 250pg/ml; P=0.85; n=8). The administration of SnPP alone to RUPP rats did not significantly alter plasma sFlt-1 levels compared to vehicle treated RUPP rats (3049 ± 663 versus 4133 ± 555pg/ml; P=0.23; n=7). Data expressed as mean pg/ml ± s.e.m. *P<0.01 Normal pregnant versus RUPP.
Figure S8. Effect of Rosiglitazone administration on placental sFlt-1 mRNA. sFlt-1 mRNA was 75% elevated in RUPP rats compared with normal pregnant rats (P<0.001; n=7-8). Administration of rosiglitazone did not significantly alter sFlt-1 mRNA expression (P=0.10; n=8-10). Data expressed as fold difference. *P<0.001 normal pregnant versus RUPP.
Figure S9a. sFlt-1 immunohistochemical staining of normal pregnant rat placenta. Image representative of n=6. Enhanced image at x100 magnification.
Figure S9b. sFlt-1 immunohistochemical staining of RUPP placenta. Image representative of n=6. Enhanced image at x100 magnification. Immunohistochemical staining of placenta, demonstrated increased sFlt-1 immunoreactivity (as indicated by intense brown staining) in placenta from RUPP rats (above) compared with those from normal pregnant rats (Figure S9a.).
Figure S9c. sFlt-1 immunohistochemical staining of placentas from rats treated with rosiglitazone. Image representative of n=6. Enhanced image at x100 magnification. However, no significant differences were observed in the intensity of immunohistochemical staining for sFlt-1 in the placentas of rosiglitazone treated rats (above) compared with control RUPP rats (Figure S9b.).
Figure S10. Effect of rosiglitazone administration in the RUPP rat on circulating VEGF levels. RUPP rats had significantly reduced plasma levels of VEGF compared to normal pregnant rats (281 ± 9 versus 368 ± 24 pg/ml; P<0.01; n=9). Administration of rosiglitazone did not significantly alter VEGF level compared to control RUPP rats (280 ± 11 versus 281 ± 9 pg/ml; P=0.94; n=8-9). Co-administration of SnPP with rosiglitazone, also did not alter VEGF level when compared to rosiglitazone treatment alone (293± 26 versus 280 ± 11 pg/ml; P=0.65). However, SnPP administered alone to RUPP rats significantly reduced VEGF level when compared to vehicle treated RUPP rats (219 ± 9 versus 281 ± 9 pg/ml; P<0.001; n=6). Data expressed as mean pg/ml ± s.e.m. *P<0.01 Normal pregnant versus RUPP, †P<0.001 RUPP versus RUPP & SnPP.
Figure S11a. H&E staining of normal pregnant rat placenta.
Following H&E staining, no significant histological differences were observed between placentas from normal pregnant rats (Figure S11a.) and those from RUPP rats (Figure S11b.). Similarly, rosiglitazone treatment did not result in any significant histological changes when compared to placentas from control RUPP rats (Figure S11c.). Image representative of n=6. Enhanced image at x100 magnification. Morphological different layers: 4 - labyrinth; 3 - trophospongium; 2 - giant-cell layer; 1 - stroma.
Figure S11b. H&E staining of RUPP rat placenta. Image representative of n=6. Enhanced image at x100 magnification. Morphological different layers: 4 - labyrinth; 3 - trophospongium; 2 - giant-cell layer; 1 - stroma.
Figure S11c. H&E staining of placentas of rosiglitazone treated RUPP rats. Image representative of n=6. Enhanced image at x100 magnification. Morphological different layers: 4 - labyrinth; 3 - trophospongium; 2 - giant-cell layer; 1 - stroma.
Figure S12a. CD-31 immunohistochemical staining of normal pregnant rat placentas. Image representative of n=6. Enhanced image at x100 magnification. Positive staining for CD-31 was indicated by localised brown staining.
Figure S12b. CD-31 immunohistochemical staining of RUPP rat placentas. Image representative of n=6. Enhanced image at x100 magnification. No significant differences were observed in terms of positive staining for CD-31 (as indicated by brown staining) in placentas from RUPP rats (above) when compared with those from normal pregnant rats (Figure S12a.).
Figure S12c. CD-31 immunohistochemical staining of placentas from rosiglitazone treated rats. Image representative of n=6. Enhanced image at x100 magnification. Rosiglitazone treatment did not significantly alter the intensity of positive staining for CD-31 in RUPP placentas (above) when compared to control RUPP rats (Figure S12b.)
Figure S13a. Percentage of Ki-67 positive cells in normal pregnant, RUPP and RUPP & Rosiglitazone treated rat placentas. No differences were observed in the percentage of Ki-67 positive cells recorded in placentas from normal pregnant rats compared with RUPP rats (14.5 ± 0.9 versus 16.3 ± 0.4%; P= 0.10; n=6). In addition, percentage Ki-67 cells in placental samples did not differ between control RUPP rats and those treated with rosiglitazone (16.3 ± 0.4 versus 16.4 ± 0.6; P= 0.80; n=6). Data expressed as percentage total cells ± s.e.m.
Figure S13b. Ki-67 positive cells in normal pregnant rat placental tissue. Ki-67 positive cells are indicated by intense black localised staining appearing as black dots. Image representative of n=6. Enhanced image at x100 magnification.
Figure S13c. Ki-67 positive cells in RUPP rat placental tissue. Ki-67 positive cells are indicated by intense black localised staining appearing as black dots Image representative of n=6. Enhanced image at x100 magnification.
Figure S13d. Ki-67 positive cells in rosiglitazone treated rat placental tissue. Ki-67 positive cells are indicated by intense black localised staining appearing as black dots Image representative of n=6. Enhanced image at x100 magnification.