Peroxisome Proliferator-Activated Receptor gamma Agonist Rosiglitazone Reverses the Adverse Effects of Diet-Induced Obesity on Oocyte Quality

Cadence E. Minge; Brenton D. Bennett; Robert J. Norman; Rebecca L. Robker

School of Paediatrics and Reproductive Health, Discipline of Obstetrics & Gynaecology, The University of Adelaide, Australia

This work was supported by the National Health and Medical Research Council of Australia.

Abbreviated title: Rosiglitazone reverses DIO oocyte damage

Key words: Obesity, ovary, oocyte, PPARG

Correspondence and reprint request:
Dr R Robker
Discipline of Obstetrics and Gynaecology
The University of Adelaide
Adelaide, SA 5005
AUSTRALIA
Phone: 61 8 8303 8159
Fax: 61 8 8303 4099
Email: rebecca.robker@adelaide.edu.au

Disclosure statement: The authors have nothing to disclose.
ABSTRACT

Obesity and its physiological consequences are increasingly prevalent among women of reproductive age and are associated with infertility. To investigate, female mice were fed a high fat diet until the onset of insulin resistance followed by assessments of ovarian gene expression, ovulation, fertilization and oocyte developmental competence. We report defects to ovarian function associated with diet-induced obesity (DIO) that result in poor oocyte quality, subsequently reduced blastocyst survival rates, and abnormal embryonic cellular differentiation. To identify critical cellular mediators of ovarian responses to obesity-induced insulin resistance, DIO females were treated for 4 days prior to mating with an insulin-sensitizing pharmaceutical: glucose and lipid-lowering AMP Kinase activator, AICAR, 30mg/kg/day; sodium salicylate, IκK inhibitor that reverses insulin resistance, 50mg/kg/day; or PPARG agonist rosiglitazone, 10mg/kg/day. AICAR or sodium salicylate treatment did not have significant effects on the reproductive parameters examined. However, embryonic development to the blastocyst stage was significantly improved when DIO mice were treated with rosiglitazone, effectively repairing development rates. Rosiglitazone also normalized DIO-associated abnormal blastomere allocation to the inner cell mass. Such improvements to oocyte quality were coupled with weight loss, improved glucose metabolism and changes in ovarian mRNA expression of PPRE-regulated genes, Cd36, Scarb1 and Fabp4 cholesterol transporters. These studies demonstrate that peri-conception treatment with select insulin-sensitizing pharmaceuticals can directly influence ovarian functions, and ultimately exert positive effects on oocyte developmental competence. Improved blastocyst quality in obese females treated with rosiglitazone prior to mating indicates that PPARG is a key target for metabolic regulation of ovarian function and oocyte quality.
INTRODUCTION

A growing body of evidence shows that female fertility is significantly compromised under conditions of overweight and obesity (1-3). Overweight women experience longer times to conception than women with moderate body weights (4) indicating that reproductive function is impaired even at the earliest, pre-implantation stages. However, due to the difficulty of examining pre-implantation processes in humans, the exact nature of the lesions is unclear. While a disproportionate percentage of women undergoing assisted reproduction technologies are overweight or obese, there is some conflicting evidence regarding oocyte quality of obese women undergoing in vitro fertilization (1, 5-8) due to relatively small numbers of highly heterogeneous patients and major differences in technical and analytical methodologies. The present study has utilized a mouse model to determine more precisely whether defects in oocyte developmental quality and/or early embryo development occur in response to obesity.

Our studies used a well-characterized model of obesity in which a high-fat diet in C57BL/6 mice causes excessive weight gain and hyperinsulinemia; metabolic parameters implicated in poor reproductive outcomes (9-12). It is evident that insulin resistance arising from obesity is key in the development of female reproductive dysfunction. Hyperinsulinemia can interfere directly with ovarian cell function or be indirectly associated with other hormonal conditions detrimental to optimal fertility (13-16).

For our studies we treated mice with specific insulin-sensitizing and plasma glucose-reducing pharmaceuticals in order to reverse the effects of obesity/hyperinsulinemia and identify the signalling pathways responsible for disruption of pre-implantation events.

AICAR (5-aminoimidazole 4-carboxamide-riboside) is an adenosine analogue that acts through stimulation of AMP kinase (AMPK) activity (17). In this effect, AICAR is similar in mechanism to metformin, although it is recognised as more specific in action (18, 19). AMPK itself phosphorylates and inactivates a number of key biosynthetic enzymes (20-23) consequently inhibiting glycogen synthesis, fatty acid synthesis and isoprenoid/sterol synthesis. AICAR administration to rats increases the activity of the insulin receptor signaling (24), and increases glucose uptake (25), via up-regulated translocation of glucose transporter GLUT-4 to the plasma membrane (26, 27).

Closely related to aspirin, sodium salicylate is a non-steroidal anti-inflammatory drug (NSAID) with two distinct molecular modes of action; at low doses sodium salicylate inhibits the classical NSAID targets, cyclooxygenase-1 (28-30) and –2 (28, 31), thus blocking prostaglandin formation, but at high doses sodium salicylate blocks the action of nuclear factor kappa B (NF-κB) (32) and its upstream activator IκB kinase β (IKK-β) (33). Via reduced signalling through the IKK-β pathway, sodium salicylate treatment lowers blood glucose concentrations (34), and improves insulin resistance (35), and restores normal insulin sensitivity in mice with diet-induced obesity (36).

Rosiglitazone (Avandia, GlaxoSmithKline) is an insulin-sensitizing agent of the thiazolidinedione class of drugs that also includes pioglitazone (Actos, Takeda/Eli Lilly) (37). The thiazolidinediones are highly selective and potent agonists for the nuclear receptor peroxisome proliferator activated receptor–gamma (PPARG) (38), strongly implicated in female reproduction (39). Following rosiglitazone activation of PPARG, a heterodimeric complex with the retinoid X receptor (RXR) forms and binds to PPAR response elements (PPREs) located in promoter regions of target genes (40), thus altering transcription. Recent investigation using genome-wide screening has produced a comprehensive list of genes containing PPREs that are potentially regulated by the PPARG/rosiglitazone complex (41). Among these genes are many related to lipid metabolism, including those involved in fatty acid transport and lipid clearance from the circulation (apolipoproteins and lipoprotein lipase), fatty acid transport through plasma membranes (CD36 and SCARB1), fatty acid oxidation (acyl-CoA oxidase), mitochondrial uncoupling (uncoupling proteins (UCP)-1, UCP-2, and UCP-3), lipogenesis (acyetyl-CoA carboxylase, fatty acid synthase), transcription
factors involved in lipid metabolism control (sterol-regulatory element-binding protein 1) (reviewed in (42)), as well as other genes related to glucose metabolism (43). A number of studies have reported on the effects of rosiglitazone treatment in obese rodents, frequently reporting changes in bodyweight, plasma lipid profile, blood glucose levels, and circulating insulin levels (44, 45).

The effects of diet-induced obesity on aspects of female reproductive function (ovulation, fertilization and embryonic development to the blastocyst stage) were assessed. Obese animals were also treated with one of the three insulin sensitiseris. Comparing the effects of each drug on metabolic status and ovarian gene expression, as well as upon later reproductive outcomes, identified the pathway most important for these processes. In order to focus on the peri-conception effects of these drugs, the treatment timeframe was limited to 4 days immediately prior to ovulation and mating (Figure 1), thereby restricting systemic effects of persistent treatment yet elucidating acute effects on the ovarian follicle and oocyte. Following indication of mating, all oocytes were isolated from the oviducts and monitored in vitro, enabling evaluation of oocyte health and precise temporal assessments of developmental competence.

By comparing the effects of each drug on ovarian gene expression, oocyte health and early embryonic developmental competence in DIO mice, we have identified cellular pathways affected by insulin resistance that are important in regulating oocyte potential.
MATERIALS AND METHODS

Animals and diet
All mice were obtained from the University of Adelaide Laboratory Animal Services, Adelaide, Australia. The animal ethics committees of both The Queen Elizabeth Hospital and The University of Adelaide approved all experiments and the animals were handled in accordance with the Australian Code of Practices for the Care and Use of Animals for Scientific Purposes. All mice had free access to water and food and were maintained at The Queen Elizabeth Hospital animal house at 24°C on a 14L:10D illumination cycle. 5-week-old female C57BL/6 mice were housed in groups of 5 and fed either a high fat diet (HFD) containing 22% fat (0.15% cholesterol), 19% protein and 49.5% carbohydrate (SF00-219 Specialty Feeds, Glen Forrest, Australia) or a matched control diet (CD) containing only 6% fat, 19% protein and 64.7% carbohydrate (SF04-057 Specialty Feeds, Glen Forrest, Australia) for 16 weeks. Body weights were determined weekly. Male mice were maintained on standard rodent chow, were 10-16 weeks old at time of mating, and were proven fertile.

Insulin sensitizer treatment
Female mice fed the HFD were randomly allocated to either the HFD + vehicle group, the HFD + AICAR group, the HFD + sodium salicylate group or the HFD + rosiglitazone group (all groups n=15). Four days before the conclusion of the 16-week feeding period, they were injected intraperitoneally once daily with 30mg/kg bodyweight/day AICAR (Toronto Research Chemicals, Canada, in 0.9% saline), 50mg/kg bodyweight/day sodium salicylate (Sigma Aldrich, St Louis MO, in sterile water (46)), 10mg/kg bodyweight/day rosiglitazone (Avandia, GlaxoSmithKline, in 10% DMSO (47)) or vehicle (10% DMSO). Bodyweight was recorded immediately prior to the first dosage of drug or vehicle (day -4) and immediately prior to the final dose of drug or vehicle (day -1) to indicate bodyweight flux resulting from drug administration.

Tissue and zygote collection
After 16 weeks of control diet, or 16 weeks of high fat diet, including 4 days of drug vehicle treatment, two female mice (mixed experimental groups) were caged with one male mouse for a maximum of 8 nights. Females were checked daily at 0800 for the presence of a post-coital vaginal plug. On day of presence of vaginal plug, females were deemed to be at day 1 of pregnancy, and tissues collected at 1300 that day. Any females in which a plug was not observed over the 8 day period of cohabitation (10/75 mice), were housed individually for a further 7 days to allow conceptions that may have occurred to progress to a developmental stage where visual inspection of the uterine horns could confirm pregnancy. From these, 1 female was found to have implantation sites, and 1 was found to have implantation and resorption sites, indicating 2 plugs had been missed out of 75 animals. The number of days post-coitus was estimated. Prior to killing by cervical dislocation, blood was collected, allowed to clot at room temperature and centrifuged at 4000 rpm for 10 minutes and serum removed. Ovulated oocytes were collected immediately from the oviductal ampulla into G-MOPS media and treated with hyaluronidase (0.5mg/ml, Sigma, bovine testes, type IV) to facilitate removal of the surrounding cumulus cells. Abdominal and retroperitoneal adipose tissue, ovary and liver were dissected, weighed and snap frozen in liquid nitrogen.

Metabolite and endocrine measurements
Samples for analysis of fed plasma insulin levels were analyzed by a Sensitive Rat Insulin RIA Kit (Linco Research, Inc. Missouri USA), with a sensitivity of 0.02 ng/ml, and an intraassay coefficient of 3.13%. Fed blood glucose levels and the circulating lipid profile of each mouse were determined from samples of serum utilising a Roche Cobas Mira automated sample system. Cholesterol levels were measured using the Cholesterol (CHOL-PAP) assay kit (Roche), and the mean coefficient of variation was less than 2.7%. Triglycerides were measured using the triglycerides (TRIG) assay kit (Roche) and the mean coefficient of variation was less than
2.6%. Each of these also used the C.f.a.s. Calibrator and the Precinorm U and Precipath U Quality Controls (Roche). Free fatty acids were measured using the NEFA-C Free Fatty Acid assay kit (NovoChem) and quality controls: QCS 1 and 2 (Bio-Rad, Australia). The mean coefficient of variation was less than 4.6%. All assays have been validated for use in the mouse. Mice were not fasted to avoid detrimental physiological impact of short-term starvation on hormone production, oocyte quality or fertilized zygote survival.

**mRNA preparation and real-time RT-PCR**

Total cellular RNA was isolated from liver and ovary using a Tri Reagent (Sigma Aldrich, St Louis, MO) protocol. RNA concentration and purity were determined using NanoDrop Spectrophotometer (NO-100, Biolab). 500ng RNA was reverse transcribed using random primers (Roche, Castle Hill, Australia) and a Superscript™ II RNase H Reverse Transcriptase (Invitrogen, Carlsbrand, CA) preamplification system for first strand cDNA synthesis according to the manufacturer’s instructions. For each reverse transcription, a control was performed in which all incubations and buffers were identical, but no Superscript RT enzyme was added, verifying the absence of contaminating genomic DNA in PCR reactions. Complementary DNA templates were then subjected to fluorometric semi-quantitative real time PCR using the Corbett Rotor-Gene™ 6000 (Corbett Life Sciences) real-time rotary analyser with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Ribosomal protein L19 (Rpl19) was used as an internal control for every sample. All primers were designed using Primer Express™ software and synthesized by GeneWorks (Thebarton, South Australia, Australia): Rpl19 – F 5’-TTCCCGAGTACAGCACCTTTGAC-3’, R 5’-CACGGCTTTGGCTTCATTTTAAC-3’; Ppar – F 5’-CACCATATGGAGTTCATGCTTGTG-3’, R 5’-TTTGTGGATCCGGCAGTTAAG-3’; Cd36 – F 5’-TCATGCCAGTCGGAGACATG-3’, R 5’-TGGTGCCTGTITTTAACCCAGTT-3’; Scarb1 – F 5’-GGTCCCTCAACGGCCAGAAG-3’, R 5’-CACGTTGTCGTTGCTATTGA-3’; Fabp4 – F 5’-TGATGCCTTTGTGGGAACCT-3’, R 5’-ATCCTGCCACTTTCTTGT-3’

**In vitro embryo culture**

Ovulated and denuded oocytes were maintained for 48h in G1 media (48, 49), with assessment for fertilization indicated by first cleavage division occurring after 24h. 2-cell embryos were transferred to EDTA-free G2 media (49, 50), shown previously to provide an optimum environment for growth of the postcompaction embryo. Development of fertilized oocytes was assessed at 9:00 day 3, 16:00 day 4, 9:00 day 5 by assessors blinded to maternal treatment group. Embryos were indicated as “on-time” when normal cell numbers and morphology was observed, (ie: day 3: 4-8 cells, day 4: morula-blastocyst, day 5: expanded or hatching blastocyst), fragmentation was less than 10%, and the zona pellucida was intact (until day 5). Embryos were categorised as not “on-time” if any of these developmental targets were not achieved. Also, embryos exhibiting slow/ceased cell division events (indicating cellular arrest) or overtly accelerated divisions (indicating insufficient or incomplete processing of cellular division) were duly noted as such and consequently categorised as not “on-time” for the remainder of the culturing period, even if their developmental progress later appeared normal.

**Differential nuclear staining**

Expanded and hatching blastocysts surviving at day 5 of culture were subjected to a differential staining protocol for identification of cells within the fetal precursor-inner cell mass (ICM) and placental precursor-trophectoderm (TE) layer following the methods of Hardy et al (51). A blinded assessor then counted red (TE) and blue (ICM) fluorescent cells on an Olympus VANOX AHBT-3 photomicroscope (Faulding Imaging, Mulgrave North, Victoria, Australia).

**Statistical analysis**

Values are reported as mean ± SEM. Statistical differences were determined by ANOVA and Chi-squared analysis using SPSS 13.0 for Windows (SPSS Inc. Chicago, IL). One-way
ANOVA across control diet and high fat diet ± drug treatments was used, with post-hoc analyses of significance made by Tukey’s test. A Student’s t-test was used in the case of comparison of normalized gene expression between control diet or high fat diet and specific drug treatments. For all analyses, P<0.05 was defined as statistically significant.
RESULTS

Insulin sensitizer influences on metabolic and endocrine measurements

Mice fed a high fat diet gained significantly more weight than those on control diet over the course of 16 weeks (Figure 2a). Animals on the high fat diet were assigned to 4 different groups and at the initiation of treatment regimes bodyweight was not significantly different between HFD experimental groups (Figure 2b). Over the 4 treatment days bodyweight was maintained in both CD animals ($\Delta$weight = 0.36 ± 0.35g), as well as in the HFD + vehicle group ($\Delta$weight = -0.26 ± 0.26g) (Figure 2c). Treatment with AICAR or sodium salicylate had no effect on bodyweight compared to vehicle treated animals, however, treatment with rosiglitazone caused significant weight loss ($\Delta$weight = -3.27 ± 0.21g, P<0.001 compared to all other groups).

None of the treatments reduced adipose tissue mass, otherwise elevated by consumption of HFD (Figure 3a). Rosiglitazone treatment reduced liver mass (Figure 3b P=0.048 compared to HFD + vehicle), indicating that the significant weight loss observed with this treatment is likely due to fat mobilization from the liver.

Blood collected at post-mortem was analyzed for circulating lipids, glucose and insulin. Circulating total cholesterol was not significantly different in HFD + vehicle mice (Figure 4a) nor affected by treatment with insulin sensitizers. Circulating Free Fatty Acids were significantly increased by high fat diet feeding, but were not altered in mice treated with insulin sensitizers compared to vehicle (Figure 4b). Triglyceride levels (Figure 4c) were not affected by high fat diet or insulin sensitizers, however, rosiglitazone treatment resulted in the lowest levels (P=0.047, compared to HFD + vehicle). Non-fasting blood glucose and insulin levels were not significantly elevated in response to HFD feeding. Blood glucose levels in high fat diet fed mice were not significantly altered by treatment with AICAR or sodium salicylate (Figure 5a). Rosiglitazone however significantly lowered blood glucose levels compared to vehicle treated mice (P=0.028 vs. HFD + vehicle). Circulating insulin was significantly reduced by both sodium salicylate and rosiglitazone delivery (Figure 5b), compared to vehicle treatment (HFD + vehicle vs. HFD + sodium salicylate or rosiglitazone, P=0.021 and P=0.032 respectively). All blood samples were obtained in the non-fasted state to avoid acute effects of fasting on the fertilized oocytes. Pervious cohorts of mice, which were fasted, showed significantly elevated cholesterol, free fatty acids, blood glucose as well as significant hyperinsulinemia (data not shown) in HFD compared to control diet treatments.

Modulation of ovarian gene expression

To determine if drug treatment was effecting ovarian gene expression, real time RT-PCR was performed on ovarian RNA collected from day 1 pregnant animals (Figure 6). Hepatic RNA was used for comparison as a control tissue in which the effects of each insulin sensitizer have been better characterized. Specifically, the transcription of genes with previously documented nutritional and hormonal regulation was investigated. Expression of the rosiglitazone receptor Pparg was not found to be different between control diet and high fat diet fed mice, in either hepatic or ovarian samples (Figure 6a, b), although expression tended to be higher in ovaries from HFD + rosiglitazone animals. Rosiglitazone treatment increased expression of scavenger receptor Cd36 mRNA within the liver of HFD fed mice (Figure 6c). HFD tended to increase ovarian expression of the Cd36, which was moderately lower in both HFD + AICAR and HFD + sodium salicylate ovaries (Figure 6d). Treatment with rosiglitazone increased expression of Cd36 when compared to control diet ovaries (P=0.037). Consistent with previous reports (52) hepatic expression of scavenger receptor Scarb1, was not influenced by diet (Figure 6e). It was also unaffected by administration of any insulin-sensitizing drug within the liver. However, rosiglitazone lowered ovarian expression of Scarb1 by 61% compared to HFD + vehicle treated ovaries (P=0.003, Figure 6f). Hepatic expression of intracellular lipid transporter Fabp4 (also known as adipocyte P2) was affected by rosiglitazone in a similar way to Cd36, with an up-regulation of transcription (P=0.0005, Figure 6g). This effect was mirrored within the
ovary, although to a greater extent (7.6-fold increase, compared to 3.4-fold increase in the liver, Figure 6h). In general, ovarian expression of each gene reflected the same pattern of changes as those observed in the liver.

**Incidence and rate of ovulation**

Reproductive parameters were next assessed; onset to mating event, ovarian weight, incidence of anovulation and ovulation rate (Table 1). Number of days to plug tended to be longer in the HFD group, with HFD + vehicle females requiring 3.00 ± 0.59 days compared to CD females requiring 1.93 ± 0.4. Ovarian tissue weight was also increased by HFD (P=0.049 vs. CD). Surprisingly, in several mice zero oocytes were present in both oviducts, with this incidence of anovulation highest (6/15 animals) in response to HFD. In mice that did ovulate, HFD increased the ovulation rate (7.0 ± 0.6 oocytes) compared to CD (4.9 ± 0.9 oocytes, P = 0.036). Serum progesterone, although not analyzed in this series of experiments, was measured in previous cohorts of mice and was not influenced by HFD (data not shown).

None of these outcomes (Table 1) were significantly affected by treatment with the insulin sensitizers with the exception that HFD mice treated with sodium salicylate required an extended period of time before mating, 3.92 ± 0.40 days (P < 0.044 vs. CD, HFD + AICAR and HFD + rosiglitazone groups). Sodium salicylate actions associated with the inhibition of cyclooxygenases required for ovulation may be responsible for this observation. Interestingly, with each parameter rosiglitazone treatment resulted in outcomes most similar to those exhibited by CD mice.

**Early embryo development**

Embryonic development in vitro was assessed by blinded, daily evaluation and scoring for correct morphology of the fertilized oocytes. Cleavage rates were not affected by treatments (data not shown). HFD fed mice produced embryos with reduced on-time progression to all developmental milestones assessed (Figure 7); the 4-8 cell stage (P = 0.0001), the morula/early blastocyst stage (P=0.002), compared to embryos produced by CD fed females. Neither AICAR nor sodium salicylate administration affected embryo on-time development. However fertilized oocytes obtained from HFD+ rosiglitazone animals demonstrated significantly improved developmental potential compared to HFD + vehicle oocytes at the 4-8 cell stage (P = 0.001), at the morula/compacted blastocyst stage (P = 0.0003), and at the expanded/hatching blastocyst stage (P=0.004). Overall, there was no discernable difference in developmental dynamics of oocytes obtained from obese, HFD animals that had been treated with pre-ovulatory rosiglitazone.

**Blastomere differentiation**

All embryos surviving to day 5 of culture were subjected to a differential staining protocol to permit counting of cells constituting the trophectoderm layer and the inner cell mass. Embryos derived from HFD fed mice had higher numbers of cells allocated into the trophectoderm layer, and slightly reduced cell numbers within the inner cell mass. Consequently the proportion of inner cell mass, as a percentage of the total embryonic cell number, is smaller (Figure 8). Both AICAR and rosiglitazone administration significantly reduced the number of cells within the trophectoderm layer (P=0.047 HFD + vehicle vs. HFD + AICAR; P=0.007 HFD + vehicle vs. HFD + rosiglitazone, Figure 8a). None of the drug treatments had a dramatic influence on the number of cells within the inner cell mass (Figure 8b). However, there was a statistically significant increase in the percent of cells contained within the inner cell mass (P=0.033 HFD + vehicle vs. HFD + rosiglitazone, Figure 8c).
DISCUSSION

We report that a high fat diet leading to obesity leads to reproductive defects that are initiated within the ovarian environment and manifest as impaired development of the early embryo. Such perturbations of blastocyst development are increasingly understood to result in suboptimal fetal growth as well as to contribute to the fetal origins of adult disease (53, 54). Our findings support evidence of altered granulosa cell phenotype and ovarian dysfunction under conditions of gene mutation-induced obesity (55-58), but expand our understanding to show it persists in wild-type populations in which caloric intake is excessive. The finding that a preovulatory ovarian environment placed within the context of a metabolically compromised individual produces oocytes with a severely impaired capacity to support normal embryo development, is particularly alarming as rates of human obesity in developed nations continue to increase.

We have further demonstrated that the PPARγ pathway, potentially operating at the ovarian level, is intrinsically involved in this interaction. Rosiglitazone is uniquely able to overcome the negative influence of high fat diet consumption on embryonic on-time development. Rosiglitazone treatment also increased the percentage of inner mass cells per embryo, a measurement previously confirmed as indicative of improved fetal outcomes (54, 59). Neither AICAR nor sodium salicylate were able to induce such profound effects, even though they exhibited some expected systemic effects, such as lowering the level of circulating insulin. This suggests that either; a) the metabolic consequences of rosiglitazone (including reducing plasma triglycerides, and lowering elevated blood glucose) or; b) the direct molecular targets of rosiglitazone within ovarian cells are responsible for the observed effects.

The molecular action of rosiglitazone is well established; upon entry into the cell via transmembrane diffusion (as a small and lipophilic molecule) rosiglitazone binds to PPARγ, stimulating formation of regulatory complexes that either up- or down-regulate transcription of target genes. Among the comprehensive list of PPARγ-regulated genes are many genes principally involved in lipid uptake and metabolism, glucose uptake and metabolism, and immune cell responses (41). Systemically insulin resistance in peripheral tissues is ameliorated and circulating levels of lipids are lowered (60, 61). Within ovarian tissue, PPARγ is most highly expressed in the granulosa cells and luteal cells, in the ovaries of rodents and ruminants (62-65). In addition, ovarian macrophages, which surround ovarian follicles and release pro-inflammatory cytokines, have high levels of transcript and protein expression (65). Within the oocyte itself, PPARγ expression seems to be dependent upon species. Although moderate expression has been reported in ruminants (66), oocyte PPARγ expression is low within rodents (63, 65, 67). It is therefore likely that modulation of somatic ovarian cell functions are mediating the improved outcomes observed when mice are treated with rosiglitazone.

In support of this, we identified significant, and specific, modulation of ovarian gene expression; namely, up-regulation of Cd36 and Fabp4, and suppression of Scarb1, genes known to possess PPAR Response Elements (PPREs) in proximal promoter regions (68-70) and to be regulated in response to PPARγ activation (71-73). Within the ovary, high levels of CD36 protein is found in granulosa cells of preantral and early antral follicles, and also within the vascular thecal layer; SCARB1 expression is strongly associated with the HDL-cholesterol ester requirement for production of steroid hormones such as androgen (estradiol precursor) and progesterone; and FABP4 is found predominately within granulosa cells of follicles undergoing atresia (74). FABP4 is known to solubilize lipophilic fatty acids facilitating their intracellular transport, in particular, positioning lipid ligands of PPARγ in close proximity to this nuclear receptor allowing selective enhancement of PPAR transcriptional activity (75). Both CD36 and SCARB1 are involved in selective cholesterol ester uptake from HDL lipoproteins, and SCARB1 is additionally able to bind and uptake unmodified LDL. By up-regulating Cd36 expression, and down-regulating Scarb1 expression, rosiglitazone may specifically increase HDL-uptake potential, and minimize the possibility of native LDL cholesterol ester uptake within the ovary. At the time of sample collection (day 1 of pregnancy) ovarian
function comprises establishment of luteal activity, which includes increased cholesterol uptake. Rosiglitazone-induced modifications to transport protein, and subsequently cholesterol, availability would dramatically influence such functions as progesterone production initiates. Further, activity and expression key molecular regulators of steroidogenesis, including Steroidogenic Acute Regulatory Protein (StAR) and 3β-hydroxysteroid dehydrogenase (3β-HSD) are also both regulated by PPARG activation (76, 77). Precisely how these functions would benefit ovarian follicular function to improve oocyte quality remains to be determined, and close inspection of hormonal synthesis in response to this treatment, and implications for oocyte developmental competence is required.

Overall, PPARG activation may present a promising option in the IVF setting, if pharmaceutical alternatives are required to overcome the influence of a sub-optimal maternal metabolic profile on oocyte health.

Although currently listed as a Pregnancy Category C drug (not tested for use during pregnancy), recent studies have reported no adverse effects of maternal treatment with rosiglitazone during the peri-ovulatory period on embryonic or fetal outcomes (78, 79). In addition, alternative activators of PPARG, including many of the naturally occurring, fatty acid-based ligands may prove useful in circumventing the limitations of rosiglitazone.

In conclusion, this study has demonstrated that rosiglitazone, either through systemic improvements to specific metabolic parameters, or by directly modulating PPARG-regulated gene expression in ovarian cells, is able to reverse deficits in oocyte quality brought about by diet-induced obesity, such that early embryonic developmental competence is greatly improved. It emphasises the important contribution of PPARG-controlled genes in optimal ovarian biology that are consequently key mediators of female reproductive potential.
REFERENCES


34. Baron SH 1982 Salicylates as hypoglycemic agents. Diabetes Care 5:64-71


39. **Minge CE, Robker RL, Norman J** 2008 PPAR Gamma: Coordinating Metabolic and Immune Contributions to Female Fertility. PPAR Research (in press)

40. **Schoonjans K, Martin G, Staels B, Auwerx J** 1997 Peroxisome proliferator-activated receptors, orphans with ligands and functions. Curr Opin Lipidol 8:159-166


52. **Srivastava RA** 2003 Scavenger receptor class B type I expression in murine brain and regulation by estrogen and dietary cholesterol. J Neurol Sci 210:11-18


58. Hamm ML, Bhat GK, Thompson WE, Mann DR 2004 Folliculogenesis is impaired and granulosa cell apoptosis is increased in leptin-deficient mice. Biol Reprod 71:66-72


60. Picard F, Auwerx J 2002 PPAR(gamma) and glucose homeostasis. Annu Rev Nutr 22:167-197


FIGURE LEGENDS

Figure 1. The experimental treatment protocol. Female mice were fed control diet (CD) or high fat diet (HFD) for 16 weeks starting at 5 week of age. 4 days prior to pairing with male, mice fed HFD were treated with AICAR, sodium salicylate, rosiglitazone or vehicle via intraperitoneal injection once daily. Mice were inspected for evidence of mating (vaginal plug) at 0800 hr each morning, and deemed to be at day 1 of pregnancy. Zygotes were removed from the oviduct at 13:00 and maintained in in vitro culture until day 5, when differential stain was performed on blastocysts.

Figure 2. Body weights of mice following feeding with a control diet (CD) or high fat diet (HFD) and in response to treatment with insulin sensitizers or vehicle. (a) Weight gain of mice fed control diet (CD) or high fat diet (HFD) for the 16 weeks preceding drug administration, (b) bodyweight of the CD and 4 HFD groups immediately prior to drug administration, (c) change in bodyweight after 3 days of drug or vehicle treatment. Bars and data points indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. * P<0.05, **P<0.001, Different letters indicate statistically different means, p<0.05.

Figure 3. Tissue weights of mice fed control diet (CD) or high fat diet (HFD) and in response to treatment with insulin sensitizers or vehicle. (a) Adipose tissue (abdominal + retroperitoneal) weight, and (b) liver weight. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, p<0.05.

Figure 4. Circulating lipids in mice fed control diet (CD) or high fat diet (HFD) and in response to treatment with insulin sensitizers or vehicle. (a) Total cholesterol, (b) free fatty acids, and (c) triglycerides. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, p<0.05.

Figure 5. Circulating glucose (a) and insulin (b) in mice fed control diet (CD) or high fat diet (HFD) and in response to treatment with insulin sensitizers or vehicle. Measurements were obtained under non-fasted conditions. Bars indicate mean ± SEM (n=15). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, p<0.05.

Figure 6. Gene expression in liver (a,c,e,g) and ovary (b, d, f, h) from mice fed control diet (CD) or high fat diet (HFD) and in response to treatment with insulin sensitizers or vehicle. Genes analyzed were Pparg (a, b), Cd36 (c, d), Scarb1 (e,f) and Fabp4 (g, h). Each is normalized to ribosomal L19 and expressed as fold change from the control diet group. CD= control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Bars indicate mean ± SEM (n=15). *P<0.05, **P<0.001.

Figure 7. Percentage of cleaved oocytes that reached the 4-8 cell, morula/blastocyst and expanded blastocyst/hatching blastocyst stages on-time. Data shows the % of fertilized zygotes to pass each developmental stage with the actual number of zygotes indicated in the table below. Each female contributed between 1 and 11 oocytes. CD= control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. *P<0.05, **P<0.001
**Figure 8. Cellular composition of embryos.** Number of (a) trophectoderm (TE), and (b) inner cell mass (ICM) cells per embryo. (c) Percentage of inner cell mass per embryo. Bars indicate mean ± SEM (n=21-38 blastocysts/group). CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone. Different letters indicate statistically different means, p<0.05.
Table 1. Ovulation incidence and rate in response to HFD ± insulin sensitizers

<table>
<thead>
<tr>
<th></th>
<th>Days to mate</th>
<th>Ovarian weight (mg)</th>
<th>Oocytes/ovulatory mouse</th>
<th>Incidence of anovulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>1.93 ± 0.47</td>
<td>5.59 ± 0.53</td>
<td>4.9 ± 0.9 (n=11)</td>
<td>4/15 (27%)</td>
</tr>
<tr>
<td>HFD + vehicle</td>
<td>3.00 ± 0.60</td>
<td>7.03 ± 0.60 *</td>
<td>7 ± 0.6 * (n=9)</td>
<td>6/15 (40%)</td>
</tr>
<tr>
<td>HFD + AICAR</td>
<td>2.60 ± 0.45</td>
<td>5.94 ± 0.47</td>
<td>6 ± 0.8 (n=9)</td>
<td>6/15 (40%)</td>
</tr>
<tr>
<td>HFD + SS</td>
<td>3.92 ± 0.40 *</td>
<td>6.15 ± 0.28</td>
<td>6.3 ± 0.9 (n=10)</td>
<td>5/15 (33%)</td>
</tr>
<tr>
<td>HFD + Rosi</td>
<td>1.92 ± 0.27</td>
<td>6.14 ± 0.35</td>
<td>5.6 ± 0.7 (n=11)</td>
<td>4/15 (27%)</td>
</tr>
</tbody>
</table>

CD = control diet, HFD = high fat diet, SS = sodium salicylate, Rosi = rosiglitazone, * P < 0.05 vs. CD.

Table 1.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

(a) Plasma glucose (mmol/L)

(b) Plasma insulin (ng/ml)
Figure 6.

(a) Hepatic Pparg

(b) Ovarian Pparg

(c) Hepatic Cd36

(d) Ovarian Cd36

(e) Hepatic Scarb1

(f) Ovarian Scarb1

(g) Hepatic Fabp4

(h) Ovarian Fabp4
Figure 7.
Figure 8.