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Exposure to rosiglitazone, a PPARγ agonist, in late gestation reduces the abundance of factors regulating cardiac metabolism and cardiomyocyte size in the sheep fetus

Short title: Rosiglitazone and the fetal heart

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Abstract

Aims: It is unknown whether cardiomyocyte hypertrophy and the transition to fatty acid oxidation as the main source of energy after birth is dependent on the maturation of the cardiomyocytes' metabolic system, or on the limitation of substrate availability before birth. This study aimed to investigate whether intrafetal administration of a PPARγ agonist, rosiglitazone, during late gestation can stimulate the expression of factors regulating cardiac growth and metabolism in preparation for birth, and the consequences on cardiac contractility in the fetal sheep at ~140d gestation.

Methods: The mRNA expression and protein abundance of key factors regulating growth and metabolism were quantified using qRT-PCR and Western blotting, respectively. Cardiac contractility was determined by measuring the Ca^{2+} sensitivity and maximum Ca^{2+} activated force of skinned cardiomyocyte bundles.

Results: Rosiglitazone treated fetuses had a lower cardiac abundance of insulin signaling molecules, including IRβ, IRS-1, phospho-IRS-1(Tyr895), PI3K regulatory subunit p85, PI3K catalytic subunit p110α, phospho-PDPK-1(Ser241), Akt-1, phospho-Akt(ser273), PKCζ, phospho-PKC(Thr410), AS160, phospho-AS160(Thr642) and GLUT-4. Additionally, cardiac abundance of regulators of fatty acid β-oxidation, including AdipoR1, AMPKα, phospho-AMPKα(Thr172), phospho-ACC(Ser79), CPT-1 and PGC-1α was lower in the rosiglitazone treated group. Rosiglitazone administration also resulted in a decrease in cardiomyocyte size.

Conclusions: Rosiglitazone administration in the late gestation sheep fetus resulted in a decreased abundance of factors regulating cardiac glucose uptake, fatty acid β-oxidation and

cardiomyocyte size. These findings suggest that activation of PPARγ using rosiglitazone does not promote the maturation of cardiomyocyte, rather, it may decrease cardiac metabolism and compromise cardiac health later in life.

Key words: programming, insulin, fatty acid, glucose transporter, adiponectin, mononucleated, binucleated, contractility, fetus, pregnancy.

Glossary:

ACC	Acetyl CoA Carboxylase
AdipoR1	Adiponectin Receptor 1
Akt	Protein Kinase B
AMPK	AMP-Activated Protein Kinase
ANP	Atrial Natriuretic Peptide
AS160	Akt substrate 160kDa
BCA	Bicinchoninic Acid
CDK-4	Cyclin Dependent Kinase 4
CPT-1	Carnitine Palmitoyltransferase-1
FAT/CD36	Fatty Acid Translocase
FATP1	Fatty Acid Transport Protein 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT-1	Glucose Transporter type-1
GLUT-4	Glucose Transporter type-4
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
IGF	Insulin-like Growth Factor
IGF-1R	Insulin-like Growth Factor 1 Receptor
IGF-2R	Insulin-like Growth Factor 2 Receptor
IR	Insulin Receptor
IRS-1	Insulin Receptor Substrate-1
PDH	Pyruvate Dehydrogenase
PDK-4	Pyruvate Dehydrogenase Kinase-4
PDPK-1	3-Phosphoinositide-dependent Protein Kinase 1

PGC1α	PPARγ-coactivator 1 alpha
PGK1	Phosphoglycerate Kinase 1
PI3K	Phosphatidylinositol 3-Kinase
PKCζ	atypical Protein Kinase C zeta
PPARα	Peroxisome Proliferator Activated Receptor alpha
PPARγ	Peroxisome Proliferator Activated Receptor gamma
PVDF	Polyvinylidene Difluoride
qRT-PCR	quantitative Real Time Reverse Transcription-PCR
TBS-T	Tris-Buffered Saline with 1% Tween-20

Introduction

Early growth of the heart is associated with proliferation of mononucleated cardiomyocytes. In mid gestation, these mononucleated cells become binucleated cardiomyocytes, which contribute to increasing cardiac mass by hypertrophy (5, 17). In the human and sheep, the endowment of cardiomyocytes present in the adult heart is largely determined before birth (42). In the fetal heart, lactate and glucose are~~is~~ the main sources of energy, while after birth, there is a switch to fatty acid β-oxidation (9, 21). It is not known whether the dominance of glucose as the main fuel source in the fetal cardiomyocyte is a consequence of the relatively limited availability of fatty acids in the fetal circulation or rather as a consequence of the immaturity of key enzyme systems present within the fetal cardiomyocyte. It is also unclear whether the maturation of cardiomyocytes is linked to cardiac metabolism, however several factors are known to impact on cardiac maturation and metabolism. For example, glucocorticoids are essential in the maturation of key fetal organ systems, including the lung, gut and heart in late gestation (10). In rats, glucocorticoid infusion increases the abundance of the transcription factor Peroxisome Proliferator Activated Receptor gamma (PPARγ) leading to increased ATP production (26). Additionally, PPARγ may regulate cardiac insulin signalling, as it has been shown that cardiac specific PPARγ knockout mice have decreased phosphorylation of Protein kinase B (Akt), which is a key insulin signalling molecule (8). Rosiglitazone, a PPARγ agonist, increases plasma adiponectin concentration, which is a key regulator of cardiac fatty acid β-oxidation (1). Rosiglitazone also upregulates adiponectin mRNA expression in perirenal fat in sheep (29) and increases cardiac adiponectin and Adiponectin Receptor 1 (AdipoR1) in cultured cardiomyocytes from adult rats and mice (7, 41). Furthermore, rosiglitazone administration in adult rats induces cardiac hypertrophy (8). Thus one possibility is that an upregulation of PPARγ in late gestation may induce changes in

factors that regulate insulin dependent cardiac glucose uptake, fatty acid β-oxidation and cardiac hypertrophy in fetal cardiomyocytes in preparation for the transition to extrauterine life.

Cardiac glucose uptake in the fetus is maintained through the activity of the insulin independent Glucose Transporter type-1 (GLUT-1) (13). In postnatal life, however, cardiac glucose uptake is regulated by the insulin dependent (GLUT-4), through the activation of the Insulin Receptor (IR), Insulin Receptor Substrate-1 (IRS-1), Phosphatidylinositol 3-Kinase (PI3K), 3-Phosphoinositide-dependent Protein Kinase 1 (PDPK-1) and/or Akt. Activation of PDPK-1 results in the phosphorylation and activation of the atypical Protein Kinase C zeta (PKCζ), while phosphorylation of Akt results in the phosphorylation and activation of the Akt substrate 160kDa (AS160). Phosphorylated PKCζ and AS160 each play a major role in the translocation of the GLUT-4 to the plasma membrane to facilitate glucose uptake (38).

Cardiac fatty acid uptake is facilitated by Fatty Acid Translocase (FAT/CD36) and Fatty Acid Transport Protein 1 (FATP1) (36). Fatty acid oxidation, however, is regulated by the activation of AdipoR1 by adiponectin binding, leading to the phosphorylation, and hence activation of AMP-Activated Protein Kinase (AMPK), which in turn phosphorylates Acetyl CoA Carboxylase (ACC) resulting in its inhibition (32, 34). ACC catalyses the production of malonyl CoA, which inhibits the action of Carnitine Palmitoyltransferase-1 (CPT-1) in facilitating fatty acid transport into the mitochondria (20). Fatty acid β-oxidation in the heart is also regulated by PGC1α and PPAR alpha (PPARα), which stimulate mitochondrial biogenesis and fatty acid β-oxidation by increasing the transcription of regulators such as CPT-1 (39). Pyruvate Dehydrogenase Kinase-4 (PDK-4) also plays a role in promoting

cardiac fatty acid β-oxidation by inhibiting glucose oxidation through inhibition of the Pyruvate Dehydrogenase complex (PDH) (37).

Insulin-like Growth Factor-1 (IGF-1) and IGF-2, which act through the IGF-1 receptor (IGF-1R), play an important role in cell growth and metabolism through activation of downstream signalling pathways (6). IGF-2 receptor (IGF-2R) is a clearance receptor, function to degrade IGF-2, therefore limiting its action on IGF-1R in normally grown fetuses (18). However, recent studies have shown that activation of IGF-2R signalling leads to pathological cardiac hypertrophy during late gestation in the sheep fetus (40), indicated by increased expression of the marker of hypertrophy, Atrial Natriuretic Peptide (ANP) (30). Additionally, IGF-1 also regulates proliferation through the activation of the Cyclin Dependent Kinase 4 (CDK-4) and Cyclin D1 complex, which is inhibited by the CDK inhibitor, p27 (24). The expression of CDK-4 is stimulated by the transcription factor c-myc (15).

We hypothesise that activation of PPARγ with intrafetal rosiglitazone infusion will stimulate cardiac insulin dependent glucose uptake and fatty acid β-oxidation, thus stimulating cardiac maturation and growth. In this study, we have therefore determined the effect of PPARγ activation using rosiglitazone infusion to the sheep fetus for ~16 days in late gestation on the mRNA expression and protein abundance of factors regulating cardiac glucose uptake, fatty acid β-oxidation, cardiomyocyte proliferation and hypertrophy, as well as cardiomyocyte parameters in late gestation at ~140d gestation. We have also determined both Ca^{2+} sensitivity and maximum Ca^{2+} -activated force in small bundles of chemically skinned cardiac muscle, as an indication of cardiac function.

Materials and methods

Animals, surgery and rosiglitazone administration

All procedures were approved by the Institute for Medical and Veterinary Science Animal Ethics Committee.

Pregnancies were confirmed in 14 adult Merino ewes by ultrasound scanning in early gestation. Surgery was performed between 123 and 126d gestation using aseptic techniques. General anesthesia was induced by intravenous injection of sodium thiopentone (1.25g, Pentothal; Rhone Merieux, Pinkenba, Qld, Australia) and maintained with 1.5-2.5% isoflurane (Fluothane; ICI, Melbourne, Vic, Australia) in oxygen.

Ethanol was diluted in water to make a sterile 15% ethanol (vol/vol) solution. Rosiglitazone (30 mg, generously donated by GlaxoSmithKline, Brentford, UK) was dissolved in sterile 15% ethanol (15 mg/ml) and then injected into a 2-ml Alzet osmotic pump (DURECT Corp., Cupertino, CA) under sterile conditions. Rosiglitazone was administered directly to the fetus with Alzet osmotic pumps, which were inserted subcutaneously over the scapula at surgery as previously described (29). Fetuses assigned to the control group (vehicle) also had Alzet osmotic pumps inserted containing 15% ethanol. The solution was released from the osmotic pumps at an average rate of 60µl/d for both rosiglitazone and control groups, according to the manufacturer's specifications regarding the estimated flow rate of the pumps (DURECT Corp., Cupertino, CA). Based on this flow rate, and the amount of drug initially loaded into each pump, ~~This~~ this regimen delivered a~~provided an estimated~~ dose of ~3.6mg/fetus/day of rosiglitazone. This resulted in ~~ (calculated according to the amount of rosiglitazone loaded into the pumps and flow rate of the pumps), resulting in~~ a plasma concentration of ~25ng/ml,

across ~~or 7.14ng/ml/kg (25ng/ml divided by average fetal sheep weight of 3.5kg)~~ (3). ~~This plasma concentration is comparable to those seen in adults treated with an oral dose of 8mg/day, which results in a plasma concentration of ~598ng/ml or 7.97ng/ml/kg (598ng/ml divided by average adult weight of 75kg) (9)~~ the infusion period and was sufficient to activate PPARγa target genes in adipose tissue, liver and skeletal muscle. Further, we have reported previously that this regime resulted in accumulation of rosiglitazone in the fetus throughout the infusion period (3).

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Blood sampling, post mortem and tissue collection

Fetal arterial blood (0.5 ml) was collected daily from the time of surgery to post mortem for determination of fetal blood gases PO_2 and PCO_2 using an ABL 520 analyzer (Radiometer, Copenhagen, Denmark) (29).

Between 137 and 140d gestation, ewes were humanely killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd., Peakhurst, NSW, Australia). Timing of tissue collection was determined to allow rosiglitazone infusion for 16 ± 1d. Singleton and twin fetuses from the control (n=12) and rosiglitazone treated (n=9) groups were delivered by hysterectomy and weighed. All organs were dissected and weighed, and samples of heart muscle (left ventricle) were snap frozen in liquid nitrogen and stored at −80°C. The remainder of the heart was perfused through the aorta with heparin and saturated potassium chloride, to prevent blood clotting and to arrest the heart in diastole. Cardiomyocytes were enzymatically isolated from the heart as previously described (27) and fixed in 1% paraformaldehyde (Table 1) and stored until determination of the percentage of mononucleated cardiomyocytes and cardiomyocyte size.

Quantitative real-time RT-PCR (qRT-PCR)

RNA was extracted from ~50mg of left ventricle tissue using Trizol reagent (Invitrogen) (Table 1). RNA was purified using the RNeasy Mini Kit (QIAGEN). cDNA was synthesised using the purified RNA and Superscript 3 reverse transcriptase (Invitrogen) with random hexamers. The expression of mRNA transcripts of glucose transporters (GLUT-1 and GLUT-4), cardiac lipid metabolism factors (Adiponectin, AdipoR1, AdipoR2, CD36, FATP, PPARα, PGC1α and PDK-4), cardiac growth factors (IGF-1, IGF-2, IGF-1R and IGF-2R), proliferative factors (p27, Cyclin D1, CDK-4 and c-myc), cardiac hypertrophy markers (ANP) and the housekeeper genes Hypoxanthine Phosphoribosyltransferase 1 (HPRT1), Phosphoglycerate Kinase 1 (PGK1) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (33) was measured by quantitative Real Time Reverse Transcription-PCR (qRT-PCR) using the Sybr Green system in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Normalised expression of the target genes was calculated using DataAssist Software v3.0 (Applied Biosystems) (14).

Primer sequences were validated for use in sheep in this (Table 2) or in prior studies (23, 28, 29). Each amplicon was sequenced to ensure the authenticity of the DNA product and a dissociation melt curve analysis was performed after each run to demonstrate amplicon homogeneity. Each qRT-PCR reaction well contained: 5µl Sybr Green Master Mix (Applied Biosystems), 2µl primer (forward and reverse), 2µl molecular grade H_2O and 1µl of cDNA (50ng/µl). The cycling conditions consisted of 40 cycles of 95°C for 15min and 60°C for 1min.

Quantification of protein abundance

The protein abundance of factors regulating cardiomyocyte proliferation and hypertrophy, glucose and fatty acid metabolism and cardiac contractility were determined using Western Blotting (31). Briefly, left ventricle samples (~50mg) (Table 1) were sonicated in 800µl lysis buffer (50mM Tris HCL pH 8.0, 150mM NaCl, 1% NP-40, 1mM Na_3VO_4, 30mM NaF, 10mM $Na_4P_2O_7$, 10mM EDTA, 1 protease inhibitor tablet) and centrifuged at 12,000*g* at 4^{o}C for 15min to remove insoluble material. Protein content of the clarified extracts was quantified using micro Bicinchoninic Acid (microBCA) protein assay. Prior to Western Blot analysis, samples (10µg protein) were subjected to SDS-PAGE and stained with Coomassie blue reagent (Thermo Fisher Scientific, Rockford, IL, USA) to ensure equal loading of the proteins. Equal volumes and concentrations of protein were subjected to SDS-PAGE. The proteins were transferred onto a PolyScreen® Polyvinylidene Difluoride (PVDF) hybridization transfer membrane (PerkinElmer, Waltham, MA, USA) using a semi-dry blotter (Hoefer Inc, Holliston, CA, USA). The membranes were blocked with 5% BSA in Tris-Buffered Saline with 1% Tween-20 (TBS-T) at room temperature for 1h and then incubated overnight with primary antibody against IRβ, PKCζ, GLUT-1, PPARα, CPT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); IGF-1R, phospho-IRS-1 (Tyr895), p110α, Akt1, Akt2, total phospho-Akt (Ser473), PDPK-1, phospho-PDPK1 (Ser241), phospho-PKCζ (Thr410), AS160, phospho-AS160 (Thr642), total AMPK, total phospho-AMPK (Thr172), PGC1α, ACC, phospho-ACC (Ser79) (Cell Signalling, Danvers, MA, USA); IRS-1, p85 (Merck Milipore, Billerica, MA, USA); AdipoR1 (Epitomics, Burlingame, CA, USA); GLUT-4, PDK-4, ANP (Abcam, Cambridge, UK) and IGF-2R (BD Transduction laboratories, San Jose, CA, USA). Membranes were washed and bound antibody detected using anti-rabbit or anti-mouse (Cell Signalling) horseradish peroxidase-conjugated secondary IgG antibodies at room temperature for 1h. Enhanced chemiluminescence reagents SuperSignal® West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and

ImageQuant™ LAS 4000 (GE Healthcare, Rydalmere, NSW, Australia) was used to detect the protein:antibody complexes. AlphaEaseFC (Alpha Innotech Corporation, Santa Clara, CA, USA) was utilised to quantify the optical density of the specific bands of the target proteins (40).

Determination of proportion of mononucleated cardiomyocytes and cardiomyocyte size

Cardiomyocytes were stained with methylene blue (ProSiTech, Thuringowa, Qld, Australia) and examined using an Olympus VANOX-T microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The relative proportion of mononucleated and binucleated cardiomyocytes was determined by counting a total of 300 cardiomyocytes. To determine cardiomyocyte size, the length and width of 50 mononucleated and 50 binucleated cardiomyocytes were assessed using AnalySIS software (Software Imaging System, Gulfview Heights, SA, Australia) (40).

Cardiac contractility studies

Bundle isolation: Under a dissecting microscope, small bundles of cardiomyocytes (Table 1) of ~300 μm diameter were isolated from the left ventricle and then attached between a force transducer (AE801 Memscap, Skoppun, Norway) and stationary pin with fine suture silk. The bundle was then briefly immersed in a high-EGTA physiological solution (*solution 1*; see below). We have shown in a previous study that stretching bundles by 130% of the resting length resulted in the production of optimum force to Ca^{2+} activation consistent with the approach of other studies (35). In this study, the bundle was therefore stretched by 120% of its slack length to produced ~90% of optimum maximum Ca^{2+}-activated force. Bundles were then chemically skinned in *solution 1* containing 2% Triton X-100 for 30min (35) (see below). The output of the transducer was acquired and digitized by a PowerLab/8Sp

(ADInstruments, Castle Hill, NSW, Australia) data-acquisition system and the subsequent force responses recorded onto both a paper chart recorder (Kipp Zonnen, Bohemia, NY, USA) and computer using PowerLab Chart v4.1 computer software (ADInstruments).

Force-calcium relationship: The standard composition of the skinned fibre solutions used were (mM): (a) *Solution 1* - Hepes, 90; EGTA, 50; total Mg^{2+}, 10.3; total ATP, 8; creatine phosphate (CP), 10; (b) *Solution 2* - Hepes, 90; EGTA, 50; total Ca^{2+}, 48.5; total Mg^{2+}, 8.12; total ATP, 8; CP, 10; (c) *Solution 3* - Hepes, 90; EGTA, 0.05; $HDTA^{2-}$ (1,6-diaminohexane-*N*,*N*,*N*_,*N*_-tetraacetic acid), 50; total Mg^{2+}, 8.6; total ATP, 8; CP, 10. All solutions contained (mM): K^+, 126; Na^+, 36; azide, 1; free Mg^{2+}, 1 and the pH and osmolality were 7.10 ± 0.01 and 295 mmols kg^{-1}, respectively.

All bundles were chemically skinned in *solution 1* containing 2% Triton-X 100 for 30min. This procedure destroys all membranes, leaving only the contractile apparatus intact. Skinned bundles were then washed in fresh *solution 1* for 5min and then equilibrated in a weakly buffered (2 mM) EGTA solution by combining proportions of *solutions 1* and *3*. The force-pCa relationship was then determined by activating each bundle in solutions of increasing free Ca^{2+}, created by combining *solutions 1* and *2* in various ratios ($pCa = \log_{10}[Ca^{2+}]$; 7.3 to 5.5); the precise pCa in each activation ratio was subsequently measured by using an Orion Ca^{2+}-sensitive electrode. Bundles were maximally activated by exposure to *solution 2* (pCa ~4.5). The maximum Ca^{2+}-activated force responses in bundles were normalized to the cross-sectional area of the bundle (mN/mm^2) for comparison. Cross-sectional area was determined by the equation area πr^2, assuming the muscle bundle had a cylindrical form and taking the average diameter across the fiber bundle. Submaximal force relative to the maximum Ca^{2+}-activated force was used in determination of the force-pCa relationship. For each fiber

bundle, the relative force produced for each free [Ca^{2+}] was plotted by use of GraphPad Prism v4.01 (GraphPad Software, San Diego, CA, USA) and a sigmoidal dose-response curve (Hill equation: $Y=min + (max - min)/(1 + 10^{((LogEC50 - X) \times n))})$ was fitted. Parameters Max (pCa 4.5) and Min (pCa 7.0) of the fitted curve were set to 100 and 0%, respectively. From each resulting curve the pCa required to produce 50% (pCa_{50}) of maximum Ca^{2+}-activated force and the Hill coefficient (n) were measured and averaged as reported in previous studies (35).

Statistical Analyses

All data are presented as mean ± SEM. Two-way ANOVA was performed using the Statistical Package for the Social Sciences Software (SPSS Inc, Chicago, IL, USA), and showed no effect of fetal number, thus data from singletons and twins were combined and Student's unpaired t-tests was used to determine the effects of rosiglitazone compared to controls on cardiac mRNA expression and protein abundance and to compare contractility parameters. A probability level of 5% ($P<0.05$) was considered significant.

Results

There was no effect of rosiglitazone administration on fetal weight at ~140d gestation (control, 4.65±0.15kg; rosiglitazone, 4.83±0.17kg). There was also no effect of rosiglitazone administration on mean fetal arterial PO_2 (control, 22.5±0.6mmHg; rosiglitazone, 21.5±1.0mmHg) and PCO_2 (control, 49.9±0.7mmHg; rosiglitazone, 49.5±0.5mmHg) in late gestation.

Impact of rosiglitazone on the mRNA expression and protein abundance of factors regulating cardiac glucose uptake in late gestation

Rosiglitazone administration during late gestation decreased the cardiac protein abundance of IRβ ($P<0.05$), IRS-1 ($P<0.05$), phospho-IRS-1 (Tyr895) ($P<0.05$), PI3K (p85) ($P<0.05$), PI3K (p110α) ($P<0.05$) , phospho-PDPK-1 (Ser241) ($P<0.05$), Akt1 ($P<0.05$), phospho-Akt (Ser273) ($P<0.001$), PKCζ ($P<0.05$), phospho-PKCζ (Thr410) ($P<0.01$), AS160 ($P<0.05$), phospho-AS160 (Thr642) ($P<0.05$) and GLUT-4 ($P<0.01$) (Table 3). The cardiac abundance of GLUT-1, however, was increased ($P<0.05$) in rosiglitazone treated fetuses compared to controls (Table3). The protein abundance of PDPK-1 (Table 3) and mRNA expression of GLUT-1 and GLUT-4 were not different in rosiglitazone treated fetuses compared to controls (Table 3).

Impact of rosiglitazone on the mRNA expression and protein abundance of factors regulating cardiac fatty acid β-oxidation in late gestation

The cardiac protein abundance of AdipoR1 ($P<0.01$), AMPK ($P<0.05$), phospho-AMPK (Thr172) ($P<0.05$), ACC ($P<0.01$), phospho-ACC (Ser79) ($P<0.05$), CPT-1 ($P<0.05$), PDK-4 ($P<0.05$) and PGC-1α ($P<0.05$) (Table 4) was decreased in rosiglitazone treated fetuses compared to controls. There were no differences, however, in the mRNA expression of cardiac PPARγ, adiponectin, AdipoR1, AdipoR2, CD36, FATP1, PPARα and PGC1α between groups (Table 4).

Impact of rosiglitazone on the mRNA expression and protein abundance of factors regulating cardiac proliferation and hypertrophy and cardiac parameters in late gestation

There was no effect of rosiglitazone on the mRNA expression of cardiac IGF-1, IGF-2, IGF-1R, IGF-2R, c-myc, CDK-4, Cyclin D1, p27 and ANP (Table 5). There was also no difference in the protein abundance of IGF-1R, IGF-2R and ANP in the rosiglitazone treated

fetuses compared to controls (Table 5). There was, however, a decrease in the absolute length of the mononucleated ($P<0.05$) and binucleated ($P<0.05$) cardiomyocytes (Table 6). The absolute and relative heart weight and absolute width of the mononucleated and binucleated cardiomyocytes, as well as the percentage of mononucleated cardiomyocytes were not changed in rosiglitazone treated fetuses compared to controls (Table 6).

Impact of rosiglitazone on cardiac contractility parameters in late gestation

There was no difference in the Ca^{2+} sensitivity of the contractile apparatus (Figure 1) and maximum Ca^{2+}-activated force between control and rosiglitazone groups (Table 7).

Discussion

In this study, we aimed to determine whether activation of PPARγ with intrafetal rosiglitazone infusion could stimulate cardiac insulin dependent glucose uptake and fatty acid β-oxidation. Interestingly, we have shown that rosiglitazone administration during late gestation resulted in decreased protein abundance of key insulin signalling molecules (Figure 2), which may lead to a decrease in cardiac glucose uptake in postnatal life. This finding is in contrast to the known effect of rosiglitazone in improving whole body insulin sensitivity and glucose uptake in the heart and skeletal muscle in adult humans and mice with type 2 diabetes (12, 19, 25). In addition, rosiglitazone treated fetuses also had a decrease in the protein abundance of key regulators of cardiac fatty acid β-oxidation, (Figure 3), which may have a detrimental effect in postnatal life, as the cardiomyocytes are more reliant on fatty acid β-oxidation to produce energy. This finding is in contrast to studies in adults in human, rats and mice, whereby rosiglitazone increased cardiac adiponectin and AdipoR1 expression (1, 7, 41). However, rosiglitazone resulted in similar decrease in the mRNA expression of AdipoR1 and protein abundance of GLUT-4 and phospho-AMPK (Thr172) in diabetic rats treated with

3mg/kg/day of rosiglitazone compared to untreated diabetic rats (11). Our findings showed that rosiglitazone administration in late gestation fetuses resulted in a different effect than in adults, but similar to when administered to adult diabetic rats. ~~Furthermore, i~~It is interesting that we found a decrease in the abundance of the insulin signalling and fatty acid β-oxidation molecules in this study despite no change in the maternal and fetal glucose and free fatty acid concentration in this cohort of animals~~, shown in the previous study~~ (29). ~~.~~ We have previously shown that intrafetal infusion of rosiglitazone resulted in decreased plasma insulin concentrations in late gestation (29), and it is therefore possible that this resulted in the observed decrease in the abundance of the insulin signalling factors. We speculate that the decrease in the abundance of the cardiac regulators of fatty acid β-oxidation may be a consequence of limited availability of fatty acids *in utero* and/or as a negative response to the increased adiponectin expression in the fetal perirenal adipose tissue, which is the main source of plasma adiponectin (29). ~~We have previously shown that intrafetal infusion of rosiglitazone resulted in decreased plasma insulin concentration (29), and this may lead to the observed decrease in the abundance of the insulin signalling factors.~~

We have also shown that rosiglitazone administration did not change the Ca^{2+} sensitivity of the contractile apparatus and maximum Ca^{2+}-activated force. There was, however, increased cardiac GLUT-1 protein abundance in rosiglitazone treated fetuses. This finding shows that the decrease in the abundance of insulin signalling and fatty acid β-oxidation molecules may not affect cardiac function in late gestation fetuses, which is consistent with the knowledge that fetal cardiomyocytes are dependent on glycolysis (21) from glucose uptake facilitated by GLUT-1. Interestingly, rosiglitazone treated fetuses had reduced absolute mononucleated and binucleated cardiomyocyte length, in the absence of any differences in absolute or relative heart weight. This finding is in contrast to a study in adult rats administered with

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rosiglitazone, which resulted in cardiac hypertrophy (8), but consistent with reports of the antihypertrophic effect of PPARγ in PPARγ knockout mice (22). Furthermore, rosiglitazone and pioglitazone interact with numerous 'off-target' proteins involved in lipid and glucose metabolism (16). Additionally, administration of thiazolidinediones (TZDs) in adult mice limits cardiac lipid accumulation following a high fat diet, but with a decrease in PPARγ expression and other factors regulating cardiac fatty acid β-oxidation (2). This finding leads the authors to speculate that TZDs may exert this effect through a cardiac PPARγ independent mechanism. Findings from this and other studies (2, 16) and the opposing effect between the impact of rosiglitazone administration and PPARγ knockout on hypertrophy in adult rats and mice (8, 22), therefore raise the possibility that the effect of rosiglitazone on cardiomyocyte growth and metabolism may be a consequence of indirect binding or the 'off target' effects of rosiglitazone. Furthermore, these findings raise concerns regarding the specificity of TZDs such as rosiglitazone as PPARγ 'specific' agonist.

In addition, a decrease in cardiomyocyte length in the absence of a reduction in absolute or relative heart weight may suggest an increase in the number of cardiomyocytes in the heart. This hypothesis is consistent with the increase in GLUT-1 abundance, which may result in increased substrate availability for glycolysis, which is the major source of energy for proliferating cardiomyocytes (21). However, we were not able to measure cardiomyocyte number in this cohort because the tissue was not collected appropriately for non-biased assessment of this parameter (4). ~~Another limitation of this study is the gender bias in the~~We also cannot exclude the possibility that there were differences in the cardiac response to rosiglitazone exposure between males and females, and as such the small differences in the relative number of males and females between the ~~protein quantification assay (~~control~~s~~ and rosiglitazone treated groups used in~~)~~ protein quantification assay and the contractility assays

~~(Ca^{2+} activated force; rosiglitazone treated group), which is male dominate~~needs to be considered when interpreting the results. ~~d. Therefore, i~~It is possible, therefore, that the decrease in the abundance of the insulin signalling and fatty acid β-oxidation molecules found in this study is only applicable for males and the lack of change in the contractility (Ca^{2+} activated force) study may be due to the male dominance in the rosiglitazone treated group. Furthermore, it is worth noting that there was a variation of 3 days in the ~~timing~~e ~~during~~ gestational age at which ~~of when the~~ rosiglitazone ~~was administered~~exposure commenced. It is possible that this~~. This disparity~~ may have ~~an effect on the~~impacted on the response of the cardiomyoctes to ~~magnitude of the~~ rosiglitazone treatment, ~~as the cardiomyocytes are rapidly maturing during late gestation~~ since the period of rosiglitazone exposure may have coincided with subtly different stages in their development (5, 17). ~~Although, we have previously shown that there was no difference in the percentage of mononucleated cardiomyocytes between 132-134d and 137-141d gestation (27). Therefore, this disparity in the timing of rosiglitazone administration may cause a variation within the data sets, however it is unlikely to alter the findings in this study.~~

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Perspective and significance

Rosiglitazone administration during late gestation resulted in decreased abundance of cardiac insulin signalling molecules and regulators of fatty acid β-oxidation, as well as a decrease in cardiomyocyte size, with no effect on measures of cardiac contractility. These findings suggest that stimulation of PPARγ using rosiglitazone in late gestation is not adequate to stimulate cardiac insulin-dependent glucose uptake and fatty acid β-oxidation, but it may result in adverse effects for cardiac health in later life. However, it is important to note that findings from this and other studies (2, 8, 16, 22) also suggest that rosiglitazone and other

TZDs may not specifically act as PPARγ agonists, and that the potential adverse cardiometabolic effects may not necessarily due to the activation of cardiac PPARγ.

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Conflict of interest

None

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600 **Figure captions**

601

602 **Figure 1.** Representative traces of the force-pCa relationship from chemically skinned
603 bundles of fetal cardiomyocytes from the left ventricles of control (●) and rosiglitazone
604 treated (▲) animals. The pCa and Hill coefficients respectively for control fetuses were 6.19
605 and 1.80 and for rosiglitazone treated fetuses were 6.24 and 1.31.

606

607 **Figure 2.** Summary diagram of the impact of rosiglitazone administration on protein
608 abundance of factors regulating cardiac glucose uptake in late gestation sheep fetus.

609

610 **Figure 3.** Summary diagram of the impact of rosiglitazone administration on protein
611 abundance of factors regulating cardiac lipid metabolism in late gestation sheep fetus.

612

613 **Table 1. Number of animals from each treatment group used in each set of analyses.**

Measurements	Control n=12	Rosiglitazone n=9
Cardiomyocyte measures	7 males = 4, females = 3	5 males = 3, females = 2
mRNA expression	5 males = 5, females = 0	7 males = 5, females = 2
Protein abundance	5 males = 5, females = 0	7 males = 5, females = 2
Contractility (Ca^{2+} activated force)	7 males = 3, females = 4	7 males = 5, females = 2
Contractility (Ca^{2+} sensitivity)	10 males = 6, females = 4	7 males = 4, females = 3

614

Formatted Table

615 **Table 2. Primer sequences for qRT-PCR.**

Gene name	Sequence	Accession no.
HPRT1	F: 5' GCTGAGGATTTGGAGAAGGTGT 3'	NM_001034035.1
	R: 5' GGCCACCCATCTCCTTCAT 3'	
PGK1	F: 5' ACTCCTTGCAGCCAGTTGCT 3’	NM_001034299
	R: 5' AGCACAAGCCTTCTCCACTTCT 3'	
GAPDH	F: 5' CCTGGAGAAACCTGCCAAGT 3'	DQ152956.1
	R: 5' GCCAAATTCATTGTCGTACCA 3'	
p27	F: 5' AAACCCAGAGGACACGCATTTGGT 3'	NM_001100346.1
	R: 5' TTTGAGGAGAGGAATCATCTGCGG 3'	
Cyclin D1	F: 5' GCCGAGAAGCTGTGCATTTAC 3'	NM_001046273.1
	R: 5' CCAGGACCAGCTCCATGTG 3'	
CDK-4	F: 5' AGGCTTGCCAGTGGAGACCATAAA 3'	NM_001037594.1
	R: 5' GGTGAACGATGCAGTTGGCATGAA 3'	
c-myc	F: 5' CTACAGATGCCCACAATCTGCACT 3'	NM_001174109.1
	R: 5' TGGTATGGTTTCATCTGGGAAGGC 3'	
ANP	F: 5' ATCACCACGAGCTTCCTCCTCTTT 3'	NM_001160027.1
	R: 5' ATACTTGTGAGGGCACAGCCTCAT 3'	
AdipoR1	F: 5' ACACTCCCTGGGCAATAAACTCCA 3'	BC102259
	R: 5’ TTCTGAAGTCCCAGTCCATCGCTT 3’	
AdipoR2	F: 5' TCTCATGGCTGTTCCACACAGTCT 3'	BC110019
	R: 5' AGCAAGGTTGCGGGTTACAGTAGA 3'	
CD36	F: 5' TGGTGTGCTAGACATTGGCAAATG 3'	BC103112.1
	R: 5' TGTTGACCTGCAGCCGTTTTGC 3'	
FATP1	F: 5' AGCCTGGTCAAGTTCTGTTCTGGA 3'	NM_001033625.2
	R: 5' AGAAGAGTCGATCATCCATGCCCT 3'	
PDK-4	F: 5' GCACCAACGCCTGTGATGGATAAT 3'	NM_001101883.1
	R: 5' AGCATCAGTTCCGTATCCTGGCAA 3'	

616

Table 3. Impact of rosiglitazone on the mRNA expression and protein abundance of factors regulating glucose uptake in heart muscle in late gestation.

Gene expression (MNE)	Control	Rosiglitazone
GLUT-1	0.050 ± 0.002	0.051 ± 0.006
GLUT-4	0.14 ± 0.02	0.13 ± 0.01
Protein abundance (Au x 10^2)		
IRβ	848 ± 91	572 ± 44*
IRS-1	384 ± 63	239 ± 23*
phospho-IRS-1 (Tyr895)	1018 ± 57	860 ± 88*
PI3K (p85)	199 ± 9	158 ± 11*
PI3K (p110α)	456 ± 9	355 ± 32*
PDPK-1	628 ± 93	475 ± 65
phospho-PDPK-1 (Ser241)	301 ± 29	207 ± 23*
Akt1	253 ± 25	169 ± 21*
phospho-Akt (Ser273)	1818 ± 228	234 ± 55***
PKCζ	1004 ± 101	696 ± 72*
phospho-PKCζ (Thr410)	952 ± 106	337 ± 71**
AS160	216 ± 38	105 ± 19*
phospho-AS160 (Thr642)	253 ± 9	76 ± 30**
GLUT-4	245 ± 17	185 ± 18*
GLUT-1	50 ± 8	76 ± 6 *

Data presented as mean ± standard error of mean. MNE, mean normalised expression; Au, arbitrary units. * P<0.05, ** P<0.01, *** P<0.001. Immunoblots of proteins with altered abundance shown in Supporting Figure.

Table 4. Impact of rosiglitazone on the mRNA expression and protein abundance of factors regulating lipid metabolism in heart muscle in late gestation.

Gene expression (MNE)	Control	Rosiglitazone
PPARγ	0.016 ± 0.003	0.015 ± 0.002
Adiponectin	0.005 ± 0.001	0.004 ± 0.001
AdipoR1	0.32 ± 0.10	0.20 ± 0.02
AdipoR2	1.40 ± 0.51	0.75 ± 0.13
CD36	4.52 ± 0.46	4.32 ± 0.46
FATP1	0.13 ± 0.02	0.13 ± 0.02
PPARα	0.22 ± 0.06	0.18 ± 0.03
PGC1α	0.81 ± 0.19	0.63 ± 0.06
Protein abundance (Au x 10^2)		
AdipoR1	477 ± 89	191 ± 16**
AMPK	570 ± 35	445 ± 31*
phospho-AMPK (Thr172)	432 ± 88	193 ± 42*
ACC	279 ± 31	172 ± 16**
phospho-ACC (Ser79)	310 ± 54	193 ± 23*
CPT-1	99 ± 12	59 ± 10*
PDK-4	157 ± 38	66 ± 13*
PGC1α	304 ± 45	112 ± 41*

Data presented as mean ± standard error of mean. MNE, mean normalised expression; Au, arbitrary units. * P<0.05, ** P<0.01. Immunoblots of proteins with altered abundance shown in Supporting Figure.

628 **Table 5. Impact of rosiglitazone on the mRNA expression and protein abundance of**
629 **factors regulating proliferation and hypertrophy, and markers of hypertrophy in heart**
630 **muscle in late gestation.**

Gene expression (MNE)	Control	Rosiglitazone
IGF-1	0.12 ± 0.01	0.09 ± 0.02
IGF-2	12.0 ± 1.4	13.3 ± 1.6
IGF-1R	0.58 ± 0.05	0.57 ± 0.04
IGF-2R	1.8 ± 0.1	1.8 ± 0.2
p27	0.31 ± 0.06	0.30 ± 0.02
Cyclin D1	0.021 ± 0.003	0.018 ± 0.003
CDK-4	0.18 ± 0.04	0.16 ± 0.03
c-myc	0.23 ± 0.03	0.23 ± 0.03
ANP	0.32 ± 0.09	0.27 ± 0.07
Protein abundance (Au x 10^2)		
IGF-1R	540 ± 74	377 ± 48
IGF-2R	529 ± 14	565 ± 76
ANP	167 ± 18	161 ± 10

631 Data presented as mean ± standard error of mean. MNE, mean normalised expression; Au,
632 arbitrary units.
633
634

635 **Table 6. Impact of rosiglitazone on heart and cardiomyocyte growth in heart muscle in**

636 **late gestation.**

Heart and cardiomyocyte measures	Control	Rosiglitazone
Absolute heart weight (g/kg)	32.9 ± 1.4	34.0 ± 1.9
Relative heart weight (g/kg)	7.1 ± 0.3	6.9 ± 0.2
Percentage of mononucleated cardiomyocytes (%)	50.5 ± 1.4	54.1± 4.0
Mononucleated cardiomyocyte length (mm)	60.3 ± 1.7	53.2 ± 1.0*
Mononucleated cardiomyocyte width (mm)	10.0 ± 0.6	10.8 ± 0.4
Binucleated cardiomyocyte length (mm)	77.7 ± 2.3	68.0 ± 1.2*
Binucleatedcardiomyocyte width (mm)	10.8 ± 0.6	11.6 ± 0.4

637 Data presented as mean ± standard error of mean. * P<0.05.

638

Table 7. Impact of rosiglitazone on the contractile apparatus of small bundles of fetal sheep heart tissue.

	pCa_{50}	Hill Coefficient	Force/cross sectional area (mN/mm^2)
Control	6.07 ± 08	1.92 ± 0.17	7.10 ± 1.57
Rosiglitazone	6.12 ± 0.1	1.50 ± 0.14	5.60 ± 0.90

Data presented as mean ± standard error of mean.