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Impact of Maternal Overnutrition on Gluconeogenic Factors and Methylation of the Phospho*enol*pyruate Carboxykinase Promoter in the Fetal and Postnatal Liver

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Abstract

Background:

Exposure to maternal obesity or hyperglycaemia increases the risk of obesity and poor glucose tolerance in the offspring. We hypothesized that maternal overnutrition in late pregnancy would result in lower methylation in the promoter region of the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK-C; PCK1) and higher expression of hepatic gluconeogenic factors in the fetal and postnatal lamb.

Methods:

Ewes were fed 100% (n=18) or ~155% (n=17) of energy requirements from 115 days gestation and livers collected at ~140 days gestation or 30 days postnatal age.

Results:

Maternal overnutrition resulted in a decrease in hepatic expression of the mitochondrial form of PEPCK (PEPCK-M; PCK2) but not PEPCK-C or glucose-6-phosphatase (G6PHOS) before and after birth. Hepatic expression of peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1), peroxisome proliferator-activated receptor α (PPARα), PEPCK-C, G6PHOS and 11β hydroxysteroid dehydrogenase type 1 (11βHSD1), but not PEPCK-M, was higher in the postnatal compared to the fetal lamb. The level of PCK1 methylation was paradoxically ~2 fold higher in the postnatal compared to the fetal liver.

Conclusions:

Maternal overnutrition programs a decrease in hepatic PEPCK-M in offspring and as ~50% of total hepatic PEPCK is PEPCK-M, the longer term consequences of this decrease may be significant.

Introduction

In pregnancies complicated by maternal diabetes mellitus, gestational diabetes or mildly impaired glucose tolerance, offspring are at risk of developing obesity and glucose intolerance (1-3). It has therefore been proposed that exposure to maternal and hence fetal hyperglycaemia may result in permanent metabolic changes within insulin sensitive tissues and the programming of an increased body fat mass, glucose intolerance and insulin resistance in later life (4,5).

Exposure to maternal overnutrition during the last 30-40 days of gestation in the pregnant ewe results in fetal hyperglycaemia and an increase in fasting plasma glucose concentrations and in body fat deposition in the postnatal lamb (6). It is not clear, however, whether the metabolic consequences of exposure to maternal overnutrition are solely due to the increase in body fat mass in the offspring or whether they represent the outcome of programmed changes in insulin sensitive tissues, such as the liver, in addition to those changes which occur in adipose tissue to increase fat deposition.

Hepatic peroxisome proliferator-activated receptor gamma co-activator 1 (PGC-1), induces fatty acid oxidation in the liver by co-activating the transcription factor peroxisome proliferator-activated receptor α (PPARα) and also induces expression of the hepatic gluconeogenic enzymes, phospho*enol*pyruvate carboxykinase (PEPCK-C; PCK1) and glucose-6-phosphatase (G6PHOS). PEPCK is the rate-limiting enzyme which regulates hepatic gluconeogenesis and it has been shown that exposure to chronic hypoxemia, hypoglycemia and glucocorticoids increase hepatic PEPCK and gluconeogenesis in the fetal sheep (7-10). There is also an increase in hepatic gluconeogenic enzyme activity with increasing gestational age which is dependent on the normal pre-partum rise in fetal cortisol (7).

The enzyme11β hydroxysteroid dehydrogenase type 1 (11βHSD1) catalyses the inter-conversion of active cortisol and inert 11-dehydrocortisol (11). 11βHSD1 is highly expressed in liver, where

the reaction direction is 11β-reduction, potentially resulting in an increase in intra-hepatic cortisol. Suppression of hepatic 11βHSD1 expression and enzyme activity results in a reduced expression of hepatic PEPCK, suggesting that 11βHSD1 plays an important role in maintaining expression of key glucocorticoid-regulated hepatic transcripts (12). Whilst exposure to excess prenatal glucocorticoids or poor maternal nutrition can permanently program abnormal glucose metabolism in the offspring (13), it is not known whether maternal overnutrition has an effect on expression of either the glucocorticoid receptor (GR) or 11βHSD1 in the liver before or after birth.

PEPCK is present in two isoforms in the liver, cytosolic PEPCK (PEPCK-C: *PCK1*) and mitochondrial PEPCK (PEPCK-M: *PCK2*). It is well established that mRNA and protein abundance of the cytosolic or inducible form of PEPCK are highly correlated and the role of PEPCK-C in gluconeogenesis as well as its transcriptional regulation in development has been extensively investigated (14-17). While 50% of total PEPCK activity in the livers of most mammals, is comprised of PEPCK-M, there is relatively little known about the role of PEPCK-M during development (14,17).

Epigenetic modification of key genes plays a role in metabolic programming through a range of mechanisms including methylation of CpG sites in the promoter regions of key genes including *PCK1* (17,18). While maternal undernutrition results in a decrease in the level of methylation in the *PCK1* promoter and a concomitant increase in PEPCK-C expression in the fetal baboon liver (19), there have been no studies on the impact of maternal overnutrition on the methylation of the *PCK1* promoter in the liver of either the fetus or offspring. In this study, we have therefore investigated the effects of maternal overnutrition during late gestation in the sheep on the expression of PGC-1, PPARα, PEPCK-C, PEPCK-M, G6PHOS, glucose transporter-1 (GLUT-1), GR and 11βHSD1 and on the level of methylation of the PCK1 promoter at 3 CpG sites (-49, -58 and -88 relative to the transcription start site) in the liver of the fetal and postnatal lamb.

Results

Maternal overnutrition and relative fetal and lamb liver weight

There was no difference in fetal arterial PO_2 (Control, 22.8 ±.6 mm Hg; WF, 21.8 ± 0.4 mm Hg), PCO_2 (Control, 49.9 ± 0.7 mm Hg; WF, 51.0 ± 0.9 mm Hg), or pH (Control, 7.39 ± 0.002; WF, 7.39 ± 0.005) between the WF and Control groups throughout late gestation. Maternal plasma glucose concentrations were 3.5± 0.2 mmoll-1 and 3.0 ± 0.2 mmoll-1in the WF and Control groups respectively in late gestation. Mean plasma glucose concentrations were higher in the WF group in both fetuses and lambs (Table 2). Plasma insulin concentrations were higher in fetuses, but not in lambs, in the WF group compared to Controls (Table 2). There was no impact of exposure to maternal overnutrition on the plasma cortisol concentrations in either fetuses or lambs (Table 2).

There was no difference between the relative liver weight in fetuses in the WF and Control groups at 139-141 days gestation (Control, 28.0 ± 2.3 g/kg; WF, 26.0 ± 1.4 g/kg). In the postnatal lambs, however, relative liver weight was significantly greater in the lambs of the WF compared to the Control group at 30 days postnatal age (WF, 21.7 ± 0.59 g/kg; Control, 19.4 ± 0.57 g/kg, $P<0.05$).

Maternal overnutrition and hepatic gene expression in the fetal and postnatal lamb

Hepatic expression of PEPCK-M mRNA was lower in the fetal and postnatal lambs in the WF group compared to Controls (Figure 1a). Whilst there was also a trend ($P<0.06$) for hepatic PEPCK-C mRNA expression to be lower in the fetuses of the WF group, there was no effect of exposure to maternal overnutrition on hepatic PEPCK-C mRNA expression in the postnatal lamb (Figure 1b). There was no effect of exposure to maternal overnutrition, on the hepatic expression of PGC-1, PPARα, G6PHOS, GLUT-1 and 11βHSD1 mRNA either before or after birth

(Figures 2-5). There was also no effect of maternal overnutrition on the hepatic expression of GR either before or after birth (Fetal: Control, 0.50 ± 0.20, WF, 0.62 ± 0.11; Postnatal, Control, 0.44 ± 0.02, WF, 0.43 ± 0.02), or G6PHOS mRNA in the postnatal lamb (Control, 0.032 ± 0.005, WF, 0.041± 0.005).

The levels of expression of PEPCK-C, PGC-1, PPARα and 11βHSD1 mRNA levels were significantly higher, whereas GLUT-1 mRNA expression was lower, in the liver of the postnatal lamb compared to the fetus, independent of the level of maternal nutrition (Figures 1b, 2-5). G6PHOS mRNA levels were not detectable in the fetus but increased after birth in both the Control and WF groups. There was no difference in GR mRNA expression between the fetuses and postnatal lambs (Fetal; 0.56 ±0.21, Postnatal 0.43 ±0.02).

Furthermore, there was no relationship between the hepatic expression of these genes and the plasma concentrations of glucose, insulin or cortisol concentrations in either the fetus or postnatal lamb.

Relationships between hepatic PGC-1, PPARα and gluconeogenic gene expression in the sheep fetus and postnatal lamb

There was a direct relationship between the hepatic expression of PGC-1 and PEPCK-M mRNA ($y= 6.39x - 0.04$; $r^2= 0.34$, $P =0.016$, $n=14$) in the fetal but not the postnatal cohort. In contrast, there was no relationship between hepatic PGC-1 and PEPCK-C mRNA expression before birth. In the postnatal cohort, however, both PEPCK-C mRNA ($y= 18.32x + 3.15$; $r^2= 0.60$, $P<0.00001$, $n=19$) and G6PHOS mRNA expression ($y= 0.34x + 0.01$; $r^2=0.38$, $P= 0.004$, $n=19$) were directly related to hepatic PGC-1 mRNA expression.

***PCK1* promoter methylation**

The level of methylation at each of the 3 CpG sites in the PCK1 promoter was relatively low (~3-10%) in the late gestation sheep fetus (Table 3). There was no impact of maternal overnutrition on the level of methylation at any CpG site in PCK1 in the liver of either the late gestation sheep fetus or postnatal lamb (Figure 5). The level of methylation at each site increased significantly, however, after the transition from fetal to postnatal life (Figure 5).

Discussion

Maternal overnutrition and liver growth in the offspring

Whilst previous studies have shown that exposure to maternal global undernutrition or low protein diets result in altered liver growth and morphology (27-32), this study has found that there is an impact of maternal overnutrition in late pregnancy on liver size in early postnatal life. Interestingly there is also evidence that gestational diabetes is associated with an increase in liver size in the human fetus in mid gestation (33). Previous studies in the rodent have shown that high fat feeding during pregnancy results in an increase in liver weight (34) and in liver triglyceride content in the offspring (34,35). Similarly, experimental induction of gestational diabetes in the rodent results in postnatal obesity and in changes in liver fatty acid composition and very-low-density lipoprotein (VLDL) lipid concentrations (36). Exposure to maternal overnutrition during late pregnancy results in an increase in subcutaneous body fat mass in the postnatal lamb but this occurs in the absence of an increase in circulating free fatty acid concentrations (37). Whilst an increase in lipid deposition in the liver appears unlikely to explain the increase in liver size in WF lambs, an increase in liver triglyceride content requires further investigation. An alternative explanation may be that an increase in fetal substrate supply programs an up-regulation of the expression of hepatic growth factors to induce hypertrophy or hyperplasia of hepatocytes after the transition to the postnatal nutritional environment.

Maternal overnutrition and hepatic gluconeogenic gene expression

An increase in maternal and fetal nutrition resulted in a significant suppression in PEPCK-M mRNA expression and a trend towards a decrease in PEPCK-C mRNA expression in the fetal, but not postnatal liver. These data highlight that the expression of the mitochondrial and cytosolic forms of PEPCK in the fetal liver are each sensitive to an increase in the prevailing glucose and/or insulin concentrations in late gestation. Interestingly the decrease in hepatic

PEPCK-M, but not PEPCK-C, mRNA expression persisted in postnatal lambs which had been exposed to maternal overnutrition in late gestation. One possibility is that the suppression of PEPCK-M mRNA expression in the postnatal liver is related to the presence of higher circulating glucose concentrations in the lambs of the WF group. There was no difference, however, in the level of PEPCK-M expression between the fetal and postnatal liver despite the markedly higher plasma glucose concentrations in postnatal compared to fetal life. Exposure to maternal overnutrition in late gestation may therefore program a persistent suppression of PEPCK-M expression in the offspring. PEPCK-M plays a role within the hepatic cell in balancing the cytosolic redox state during gluconeogenesis from lactate by synthesizing P-enolpyruvate directly in the mitochondria. This ensures that only one molecule of NADH is produced during the production of glucose from lactate (17). Thus a persistent suppression of PEPCK-M may have deleterious consequences for the hepatocyte in later life. Whilst PEPCK-C expression may be altered by a number of epigenetic modifications including DNA and chromatin methylation (17), nothing is currently known about factors which may result in a persistent down regulation of hepatic PEPCK-M expression.

Transition to postnatal nutrition and gluconeogenic gene expression

Consistent with previous studies in other tissues, GLUT-1 mRNA expression was higher in the fetal than postnatal liver (38). Interestingly, while it has been shown that glucose infusion into the pregnant ewe and resultant fetal hyperglycaemia result in a suppression of hepatic GLUT-1 expression (38), we found no effect of the moderate increase in fetal glucose induced by increased maternal nutrition on hepatic GLUT-1 expression either before or after birth.

There was a different impact of the transition from placental to enteral nutrition on the hepatic expression of PEPCK-M and PEPCK-C. In contrast to PEPCK-M, the level of PEPCK-C expression was significantly higher in the liver of the postnatal lamb compared to the fetus.

Similarly, G6PHOS mRNA was expressed in the postnatal lamb liver in contrast to the negligible level of hepatic expression of G6PHOS mRNA in fetal life. It is well established that exposure to increased glucocorticoids induces hepatic PEPCK-C and gluconeogenesis in the fetal sheep (7-10). In the present study, hepatic 11βHSD1 mRNA expression was increased in postnatal compared to fetal life. Hepatic PGC-1 expression was also higher in the postnatal lamb compared to the fetus and there was the emergence of a relationship between hepatic PGC-1 and PEPCK-C mRNA expression in the lamb after birth. Thus in contrast to PEPCK-M, the transition from the fetal to the postnatal nutritional environment has a significantly greater impact on the regulation of PEPCK-C and G6PHOS than does exposure to maternal overnutrition in late gestation.

Epigenetic changes in the *PCK1* promoter after transition to postnatal life

Intriguingly, in the current study we found that the increase in hepatic PEPCK-C expression which occurred after birth was associated with an increase in the level of methylation at 3 CpG dinucleotides (-88, -58 and -49) upstream of the transcription start site. In contrast, it has been reported that the *PCK1* gene is heavily methylated in the fetal rat liver and relatively under methylated in the adult liver (39). Whilst an increase in methylation at the 3 sites in the *PCK1* promoter would be expected to result in a decrease in the binding of transcription factors, including the cAMP response element-binding (CREB) protein, it is of note that the 3 CpG sites lie outside of the 'glucocorticoid regulatory unit' in *PCK1*. The increase in methylation at these 3 CpG dinucleotides in the *PCK1* promoter region may result in a suppression of 'constitutive' *PCK1* expression to allow for the glucocorticoid induction of *PCK1* which is important as the fetus makes the transition from continuous placental to intermittent enteral nutrition.

There was no evidence that the epigenetic state of the *PCK1* promoter was altered by exposure to either maternal overnutrition or prevailing high circulating glucose concentrations in fetal or

postnatal life. There is evidence from studies in the fetal baboon at 0.9 of gestation, that the level of methylation of a number of CpG sites (-82, -30, -5, +31, +99, +105) in the *PCK1* gene was reduced after exposure to maternal undernutrition and that this decrease was associated with an increase in hepatic PEPCK-C mRNA expression (19). It appears therefore that maternal undernutrition and the transition from fetal to postnatal nutrition may each play different roles in determining the epigenetic state of the *PCK1* gene.

Summary

Exposure to either a restriction or an oversupply of fetal nutrients can result in long term consequences for glucose tolerance and insulin sensitivity in later life (40,41). We have investigated the impact of maternal overnutrition and separately the transition from placental to enteral nutrition on the factors which regulate hepatic gluconeogenesis, the expression of the cytosolic and mitochondrial isoforms of PEPCK and the epigenetic status of *PCK1*. We have shown that there is a differential impact of exposure to prenatal overnutrition on PEPCK-M and PEPCK-C expression in the postnatal liver. Further work is required to understand the mechanism by which maternal overnutrition programs a decrease in hepatic *PCK2* expression and the metabolic consequences of a decrease in PEPCK-M expression in the postnatal animal.

Material and Methods

Animals and Feeding Regimen

All procedures were approved by the University of Adelaide Animal Ethics Committee. Merino ewes were mated and pregnancy confirmed by ultrasound scanning in early gestation. From 90 days gestation ewes were acclimatized to a Control diet which consisted of 1 kg lucerne chaff (85% dry matter, metabolisable energy (ME) content = 8.3 MJ/kg) and 300 g concentrated pellets containing: straw, cereal, hay, clover, barley, oats, lupins, almond shells, oat husks and limestone (89% dry matter, ME content = 11.6 MJ/kg; Ridley Agriproducts Sheep Nutrition Ration, Murray Bridge, South Australia, Australia). The diet was calculated to provide 100% of the energy requirements for the maintenance of a pregnant ewe bearing a singleton fetus or twin fetuses as appropriate, as specified by the Ministry of Agriculture, Fisheries and Food, UK (20).

Surgery was then performed on these ewes between 103 and 113days gestation (term = 147 +/- 3 days) using aseptic techniques. General anaesthesia was induced by intravenous injection of sodium thiopentone (1.25 g i.v., Pentothal, Rhone Merieux, Pinkenba, Queensland, Australia) and maintained with 2.5 - 4% halothane (Fluothane, ICI, Melbourne, Victoria, Australia) in oxygen. Vascular catheters were implanted in a jugular vein and carotid artery of the ewe and fetus, and in the amniotic cavity, as previously described (21). During surgery intramuscular injections of antibiotics (2 ml Procaine penicillin 250 mg/ml, Dihydrostreptomycin 250 mg/ml, procaine hydrochloride 20 mg/ml, Lyppards, Adelaide, South Australia, Australia or 0.1 ml/kg Terramycin 100, 100 mg/ml oxytetracycline hydrochloride, Pfizer, New South Wales, Australia) were administered to each ewe and fetus. All catheters were filled with heparinised saline and the fetal catheters exteriorised through an incision in the ewe’s flank. Before and after surgery the ewes were housed in individual pens in animal holding rooms with a 12 h: 12 h light/dark cycle. If there was any evidence of a decline in fetal well-being after surgery, as indicated by a

decline in fetal oxygenation, ampicillin (5ml) was administered to that fetus via the amniotic catheter for a period of 4 days.

At 115 days gestation, i.e. prior to the commencement of the rapid fetal growth phase in late gestation (22), ewes were randomly assigned to either a Control (n=14) or Well Fed (WF) (n= 16) group. Between 115 and 124 days gestation, Control ewes were provided with 14.0 ± 0.4 g of lucerne chaff and 6.5 ± 0.4 g of pelleted concentrate per kg bodyweight and WF ewes were provided with 22.1 ± 0.8 g lucerne chaff and 10.4 ± 0.7 g pelleted concentrate per kg bodyweight each day. The feed allowance of all ewes was proportionately increased by 15% every 10 days (20).

Fetal Blood Sampling

Between 113 and 139 days gestation, maternal (5.0 ml) and fetal (3.0 ml) arterial blood samples were collected three times per week prior to feeding at 0900 h. Fetal arterial blood (0.5 ml) was collected three times per week for determination of fetal blood gases (PO_2, PCO_2), oxygen saturation, pH, hematocrit, and haemoglobin using an ABL 520 analyser (Radiometer, Copenhagen, Denmark).

Collection of Fetal Tissues

Between 139 and 141 days gestation, ewes from the Control group (n=6) and the WF group (n=8) were killed with sodium pentobarbitone (Virbac Pty Ltd., Peakhurst, New South Wales, Australia). Fetal sheep were delivered by hysterectomy, weighed and killed by decapitation. Livers were weighed and samples were collected, snap frozen in liquid N_2 and stored at –80° C. All fetuses were singletons.

Lamb Protocols and Tissue Collection

The remaining Control (n=8; 4 carrying twins, 4 singletons) and WF (n=8; 1 carrying twins, 7 singletons) ewes lambed spontaneously at term. Both twins from each ewe were used in this study. After lambing, all ewes were provided with 1 kg lucerne chaff and 500 g pelleted concentrate once daily. If all feed was consumed before 1500 h, an additional 1 kg of chaff was provided. After birth, each ewe and her lamb(s) were housed in an individual pen in a facility maintained at 20-22 ° C and a 12h light: 12h dark light cycle. The day of birth was designated as day 1. Venous blood samples were collected between 0800 h – 1300 h after a 2 h fast on days 1-5 and every 3 days thereafter until day 30. All blood samples were centrifuged at 1500 g for 10 min and plasma stored at -20°C.

At 30 days of age, lambs were killed with an overdose of sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, New South Wales, Australia). Livers were dissected out and weighed and samples were collected and snap frozen in liquid N_2 and stored at –80° C.

Plasma Glucose

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland) (21). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter assay coefficients of variation were both < 5%.

Plasma Insulin

Plasma insulin concentrations were measured using a radioimmunoassay (Rat insulin kit, Linco Research, Inc., Missouri, USA), which was validated for use with sheep plasma (6). The sensitivity of the assay was 0.01 ng/ml and the intra and inter assay coefficients of variance were both <10%.

Plasma Cortisol

Cortisol was extracted from fetal plasma using dichloromethane as previously described (23). Fetal cortisol concentrations were then measured using an Orion Diagnostica Radioimmunoassay kit (Orion Diagnostica, Turku, Finland) previously validated for fetal sheep plasma (24). The inter-assay coefficient of variation was 20% and the intra-assay coefficient of variation was < 10%.

RNA Extraction

RNA from the dorsal lobe of the liver (≈30mg) was extracted using Trizol reagent (Invitrogen Australia Pty Limited, Mount Waverley, Victoria,Australia) and chloroform. RNA was treated with 50% ethanol and run through a purification process using the RNeasy Mini Kit (QIAGEN Pty Ltd Australia, Doncaster, Victoria, Australia). The quality and concentration of the RNA was determined by measuring absorbance at 260 and 280 nm, and RNA integrity was confirmed by agarose gel electrophoresis. cDNA was then synthesised using the purified RNA (≈ 5µg), Superscript III Reverse Transcriptase (Invitrogen Australia Pty Limited, Mount Waverley, Victoria, Australia) and random hexamers.

Quantitative Real Time Reverse Transcription-PCR (qRT-PCR)

The relative expression of PGC-1, PPARα, PEPCK-M, PEPCK-C, G6PHOS, GLUT-1, GR and 11βHSD1 mRNA transcripts were measured by qRT-PCR using the SYBR Green system in an ABI prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, California). (25). For each transcript qRT-PCR was performed using specific primers (Table 1). Each amplicon was designed to be approximately 200 bp in length, was sequenced to ensure the authenticity of the DNA product and qRT-PCR melt curve analysis was performed to demonstrate amplicon homogeneity. Controls containing no reverse transcriptase were also used.

For the qRT-PCR measurements, the primer concentrations were equivalent for all genes and the amplification efficiencies were 0.981-0.999. A constant amount of cDNA equating to 10ng of total RNA was used for each qRT-PCR measurement and three technical replicates were performed in duplicate for each gene.

Each qRT-PCR reaction (5µl total volume) contained: 2.5 µl 2x SYBR Green master mix (Applied Biosystems, Foster City, California, USA); 0.25 µl of each primer giving a final concentration of 450 µl, 1.0 µl of molecular grade H2O and 1.0 µl of a 1:10 dilution of the stock template. The cycling conditions consisted of 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. At the end of each run, a dissociation melt curve was obtained.

The abundance of each mRNA transcript was measured and its expression relative to that of Ribosomal Protein Large Subunit P0 (RPLP0) was calculated using Q-gene qRT-PCR analysis software. There were no differences in the expression of the housekeeper gene between the Control and Well Fed groups in either the fetal or postnatal cohort.

Combined Bisulphite Restriction Analysis

DNA methylation within the *PCK1* promoter region was analysed using combined bisulphite restriction assay (COBRA) (26). Briefly, approximately 2 µg of DNA from the ventral lobe of fetal and lamb liver was subjected to bisulphite conversion (EpiTect; QIAGEN Pty Ltd Australia, Doncaster, Victoria, Australia). PCR was then performed on 100 ng of bisulphite-converted DNA using primers (Forward: 5’ TAAAGGTTTGTTATGGTTGGTTTAG 3’; Reverse: 3’ CTAACCTTTAAATTCCAAAAAAA 5’) and conditions that amplified methylated and unmethylated templates with no bias. The amplicon contained three CpG sites at -49, -58 and -88 where +1 denotes the transcription start site. COBRA was performed using restriction endonucleases that cleave only those amplicons derived from methylated templates. The *PCK1* amplicon was digested with 20 U of TaqI (Thermo Fisher Scientific, Scoresby, Victoria,

Australia), MaeII and DpnII (New England Biolabs, Ipswich, Massachusetts, USA), which digests methylated templates at -49, -58 and -88 respectively. The intensity of uncut and cut fragments was quantified using the ExperionTM Automated Electrophoresis System (Bio-Rad, Hercules, California, USA). The percentage of methylation was estimated by measuring the ratio of cut to total PCR product.

Statistical Analyses

Data are presented as the mean ± SEM. The fetal cohort comprised 6 Control fetuses (4 males and 2 females) and 8 fetuses from WF ewes (3 males and 5 females). The postnatal lamb cohort comprised 12 Control lambs (6 males and 6 females) and 9 lambs in the WF group (3 males and 6 females). There were 14 singleton and no twins in the fetal group and 11 singletons and 10 twins in the postnatal lamb group. Using multifactorial ANOVA we found no significant effect (main effect or interaction with treatment group) of being a twin or of the sex of the lamb on the expression of any hepatic mRNA level and therefore data were combined for subsequent analysis. Two way Analysis of Variance (ANOVA) was then used to determine the main effects of maternal nutritional treatment (Control vs. WF) and developmental age (fetal vs. postnatal) and their interaction on birth weight, liver mass, and hepatic gene expression and the level of *PCK1* methylation. Simple linear regression analyses were used to determine relationships between postnatal measures of fat mass, plasma nutrient and hormone concentrations and gluconeogenic gene expression. Plasma glucose and insulin and cortisol concentrations across postnatal week 1 – 4 were averaged for correlation analyses unless stated otherwise. Partial correlation analysis was used to control for the effects of maternal intake or mean plasma glucose levels where appropriate. A probability of 5% ($P<0.05$) was taken as the level of significance in all analyses.

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Figure Legends:

Figure 1. (a) PEPCK-M mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups. * denotes a significant difference when compared to the Control group (P<0.05). (b) PEPCK-C mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups. ** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.001). † denotes that the level of difference between the WF and Control group was at the P= 0.06 level

Figure 2. (a) PPARα mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups. ** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.01) (b) PGC-1 mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups ** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.01).

Figure 3. GLUT1 mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups ** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.001).

Figure 4. 11βHSD1 mRNA expression in fetal and lamb livers in Control (open bars) and WF (closed bars) groups. ** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.01).

Figure 5. The level of methylation at each of the 3 CpG sites in the *PCK1* promoter in the Control (open bars) and WF (closed bars) fetal sheep and lambs.** denotes a significant difference between one month of age when compared to 139-141d gestation (P<0.01).