Impact of maternal overnutrition on gluconeogenic factors and methylation of the phospho*enol*pyruvate carboxykinase promoter in the fetal and postnatal liver

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BACKGROUND: Exposure to maternal obesity or hyperglycemia increases the risk of obesity and poor glucose tolerance in the offspring. We hypothesized that maternal overnutrition in late pregnancy would result in (i) lower methylation in the promoter region of the cytosolic form of phospho*enol*pyruvate carboxykinase (PEPCK-C; *PCK1*) and (ii) 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 d gestation, and livers were collected at ~140 d gestation or 30 d postnatal age.
RESULTS: Maternal overnutrition resulted in a decrease in hepatic expression of the mitochondrial form of PEPCK (PEPCK-M; *PCK2*) but not of PEPCK-C or glucose-6-phosphatase (G6PHOS) before and after birth. Hepatic expression of peroxisome proliferator–activated receptor γ coactivator 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 lamb compared with that in the fetal lamb. The level of *PCK1* methylation was paradoxically approximately twofold higher in the postnatal liver compared with that in the fetal liver.
CONCLUSION: Maternal overnutrition programs a decrease in hepatic PEPCK-M in the offspring and as ~50% of total hepatic PEPCK is PEPCK-M, the longer-term consequences of this decrease may be significant.

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, hyperglycemia may result in (i) permanent metabolic changes within insulin-sensitive tissues and (ii) the programming of an increased body fat mass, glucose intolerance, and insulin resistance in later life (4,5).

Exposure to maternal overnutrition during the final 30–40 d of gestation in the pregnant ewe results in fetal hyperglycemia and an increase in both fasting plasma glucose concentrations and 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 the changes that occur in adipose tissue to increase fat deposition.

Hepatic peroxisome proliferator–activated receptor γ coactivator 1 (PGC-1) induces fatty acid oxidation in the liver by coactivating the transcription factor peroxisome proliferator–activated receptor α (PPARα); it also induces the expression of the hepatic gluconeogenic enzymes phospho*enol*pyruvate carboxykinase (PEPCK-C; *PCK1*) and glucose-6-phosphatase (G6PHOS). PEPCK is the rate-limiting enzyme that regulates hepatic gluconeogenesis, and it has been shown that exposure to chronic hypoxemia, hypoglycemia, and glucocorticoids increases hepatic PEPCK and gluconeogenesis in fetal sheep (6–9). There is also an increase in hepatic gluconeogenic enzyme activity with increasing gestational age, which is dependent on the normal prepartum rise in fetal cortisol (6).

The enzyme 11β hydroxysteroid dehydrogenase type 1 (11βHSD1) catalyzes the interconversion of active cortisol and inert 11-dehydrocortisol (10). 11βHSD1 is highly expressed in the liver, where the reaction direction is 11β reduction, potentially resulting in an increase in intrahepatic cortisol. Suppression of hepatic 11βHSD1 expression and enzyme activity results in the reduced expression of hepatic PEPCK, suggesting that 11βHSD1 plays an important role in maintaining the expression of key glucocorticoid-regulated hepatic transcripts (11). Although exposure to excess prenatal glucocorticoids or poor maternal nutrition can permanently program abnormal glucose metabolism in the offspring (12), it is not known whether maternal overnutrition has an effect on the expression of either the glucocorticoid receptor (GR) or 11βHSD1 in the liver either 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 the mRNA levels and protein abundance of the cytosolic or inducible form of PEPCK are highly correlated, and the role of PEPCK-C in gluconeogenesis and its transcriptional regulation in development

have been extensively investigated (13–16). Although 50% of total PEPCK activity in the livers of most mammals consists of PEPCK-M, relatively little is known about the role of PEPCK-M during development (13,16).

Epigenetic modification of key genes plays a role in metabolic programming through a range of mechanisms, including methylation of CpG sites (regions where a cytosine nucleotide occurs next to a guanine nucleotide) in the promoter regions of key genes, including *PCK1* (16,17). Although 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 (18), 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 the offspring. In this study, we have, therefore, investigated the effects of maternal overnutrition during late gestation in the sheep on (i) the expression of PGC-1, PPARα, PEPCK-C, PEPCK-M, G6PHOS, glucose transporter-1 (GLUT-1), GR, and 11βHSD1 and (ii) the level of methylation of the *PCK1* promoter at three CpG sites (−49, −58, and −88 relative to the transcription start site) in the liver of fetal and postnatal lamb.

RESULTS

Maternal Overnutrition and Relative Weights of Fetal and Lamb Liver

There was no difference in fetal arterial PO_2 (control, 22.8 ± 0.6 mm Hg; well-fed (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 and 3.0 ± 0.2 mmol/1 in 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 1**). Plasma insulin concentrations were higher in fetuses, but not in lambs, in the WF group compared with those in the controls (**Table 1**). There was no impact of exposure to maternal overnutrition on the plasma cortisol concentrations in either fetuses or lambs (**Table 1**).

There was no difference between the relative liver weights in fetuses in the WF and control groups at 139–141 d gestation (control, 28.0 ± 2.3 g/kg; WF, 26.0 ± 1.4 g/kg). In the postnatal lambs, however, the relative liver weight was significantly greater in the lambs of the WF group compared with that in the lambs of the control group at 30 d 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 with that in the controls (**Figure 1a**). Although 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 on the expression of 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 were significantly higher, whereas GLUT-1 mRNA expression was lower, in the liver of the postnatal lamb, compared with the liver of the fetus, and these were 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 in either the fetus or the 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 in 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, expression levels of both PEPCK-C mRNA ($y = 18.32x + 3.15$; $r^2 = 0.60$, $P < 0.00001$, $n = 19$) and G6PHOS mRNA ($y = 0.34x + 0.01$; $r^2 = 0.38$, $P = 0.004$, $n = 19$) were directly related to hepatic PGC-1 mRNA expression.

Table 1. Mean plasma concentrations of glucose, insulin, and cortisol in fetuses in late gestation (113- to 139-d gestation) and lambs in the first month of postnatal life

	Fetal sheep		Postnatal lamb	
	Control	WF	Control	WF
Mean plasma glucose concentration (mmol/l)	1.44 ± 0.14	1.94 ± 0.11*	5.75 ± 0.17**	6.26 ± 0.11*,**
Mean plasma insulin concentration (μU/ml)	6.78 ± 0.38	8.83 ± 0.92*	1.55 ± 0.22**	1.84 ± 0.35**
Mean plasma cortisol concentration (nmol/l)	6.71 ± 0.38	6.52 ± 0.38	36.60 ± 5.45**	34.06 ± 4.04**

WF, well-fed.

*Denotes a significant difference when compared with the control group. **Denotes a significant difference in fetuses compared with the level in lambs (values are means ± SE, $P < 0.05$).

PCK1 Promoter Methylation

The level of methylation at each of the three CpG sites in the *PCK1* promoter was relatively low (~3–10%) in the late-gestation sheep fetus (**Table 2**). There was no impact of maternal overnutrition on the level of methylation at any of the CpG sites in *PCK1* in the liver of either the late-gestation sheep fetus or the 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

Although previous studies have shown that exposure to maternal global undernutrition or low-protein diets results in altered liver growth and morphology (19–24), this study has found that there is an impact of maternal overnutrition in late pregnancy on the 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 midgestation (25). Previous studies in the rodent have shown that high-fat feeding during pregnancy results in an increase in both liver weight (26) and liver triglyceride content in the offspring (26,27). Similarly, experimental induction of gestational diabetes in the rodent results in postnatal obesity and changes in liver fatty acid composition and very-low-density lipoprotein lipid concentrations (28). 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 (29). Although 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 upregulation of the expression of hepatic growth factors to induce hypertrophy
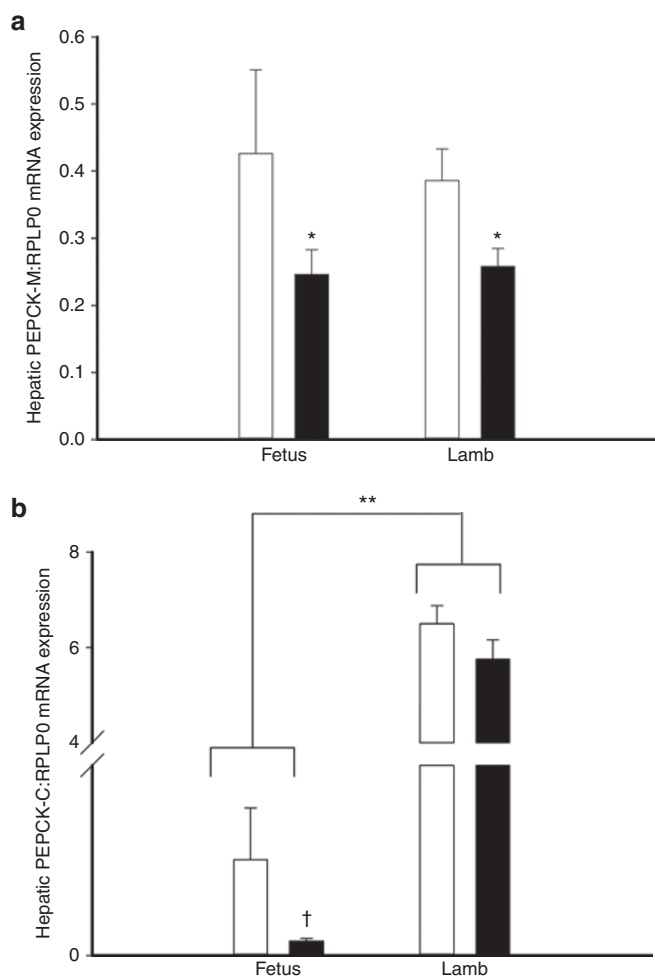

Figure 1. Hepatic mRNA expression of mitochondrial and cytosolic PEPCK in control and well-fed groups. (**a**) PEPCK-M mRNA expression in fetal and lamb livers in control (open bars) and well-fed (WF; closed bars) groups. *Denotes a significant difference when compared with the control group ($P < 0.05$). PEPCK, phospho*enol*pyruvate carboxykinase. (**b**) PEPCK-C mRNA expression in fetal and lamb livers in control (open bars) and WF (closed bars) groups. **Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.001$). †Denotes that the level of difference between the WF and the control groups was at the $P = 0.06$ level.
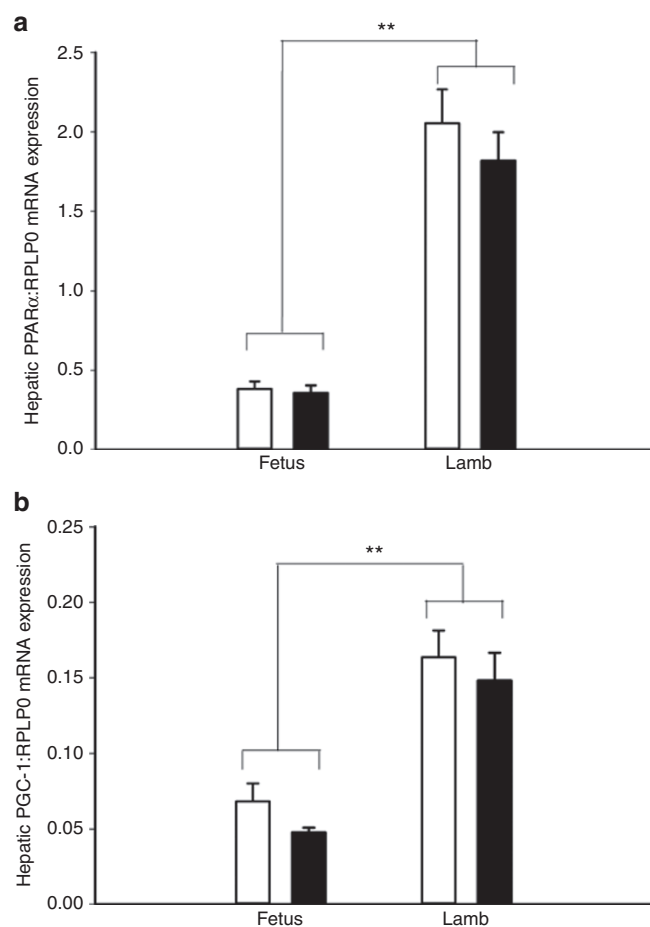

Figure 2. Hepatic mRNA expression of peroxisome proliferator-activated receptor α and peroxisome proliferator-activated receptor γ coactivator in control and well-fed groups. (**a**) Peroxisome proliferator–activated receptor α (PPARα) mRNA expression in fetal and lamb livers in control (open bars) and well-fed (WF; closed bars) groups. **Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.01$). (**b**) Peroxisome proliferator–activated receptor γ coactivator (PGC-1) mRNA expression in fetal and lamb livers in control (open bars) and WF (closed bars) groups. **Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.01$).
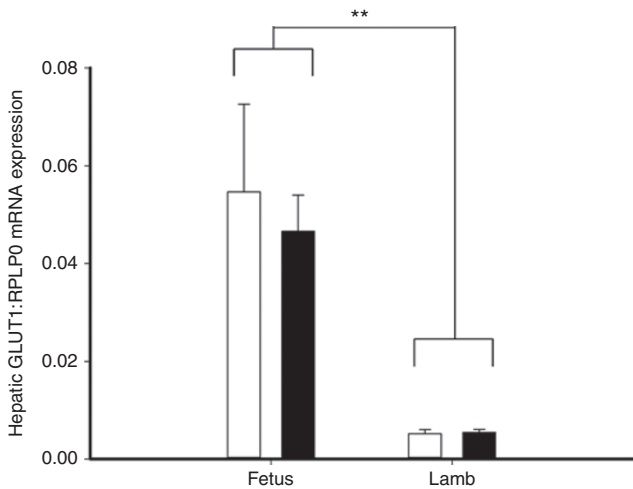
Figure 3. Glucose transporter-1 (GLUT1) mRNA expression in fetal and lamb livers in control (open bars) and well-fed (WF; closed bars) groups. **Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.001$).

Figure 4. 11β Hydroxysteroid dehydrogenase type 1 (11βHSD1) mRNA expression in fetal and lamb livers in control (open bars) and well-fed (WF; closed bars) groups. **Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.01$).
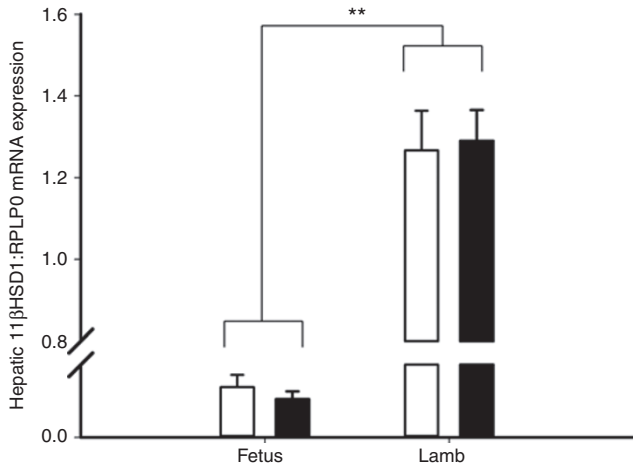
or hyperplasia of hepatocytes after the transition to the postnatal nutritional environment.

Maternal Overnutrition and Hepatic Gluconeogenic Gene Expression

An increase in maternal and fetal nutrition resulted in a significant suppression of PEPCK-M mRNA expression, and a trend toward 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 that had been exposed to maternal overnutrition in late gestation. One possibility is that the suppression of PEPCK-M mRNA expression in
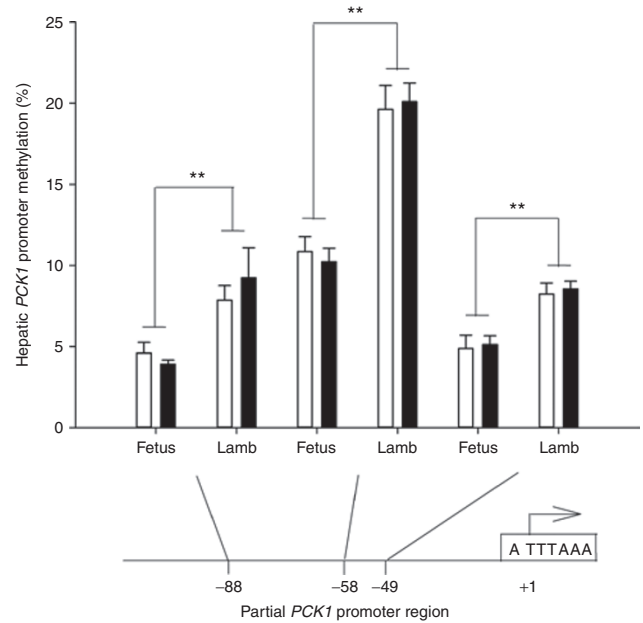

Figure 5. The level of methylation at each of the three CpG sites in the *PCK1* promoter in the control (open bars) and well-fed (WF; closed bars) fetal sheep and lambs.**Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.01$).

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 the postnatal liver despite the markedly higher plasma glucose concentrations in postnatal, compared with that in 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 phospho*enol*pyruvate directly in the mitochondria. This ensures that only one molecule of reduced nicotinamide adenine dinucleotide (NADH) is produced during the production of glucose from lactate (16). Thus, a persistent suppression of PEPCK-M may have deleterious consequences for the hepatocyte in later life. Although PEPCK-C expression may be altered by a number of epigenetic modifications, including DNA and chromatin methylation (16), nothing is currently known about the factors that may result in a persistent downregulation 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 that in the postnatal liver (30). Interestingly, although it has been shown that glucose infusion into the pregnant ewe and resultant fetal hyperglycemia result in a suppression of hepatic GLUT-1 expression (30), 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.

Table 2. Percentage DNA methylation at the −49, −58, and −88 sites of the *PCK1* promoter region in fetuses in late gestation (139- to 141-d gestation) and lambs in the first month of postnatal life

	Fetal sheep		Postnatal lamb	
	Control	WF	Control	WF
PCK1 DNA methylation at −49 (%)	4.61 ± 0.65	3.92 ± 0.25	7.86 ± 0.91**	9.24 ± 1.86**
PCK1 DNA methylation at −58 (%)	4.87 ± 0.83	5.12 ± 0.54	8.24 ± 0.67**	8.56 ± 0.47**
PCK1 DNA methylation at −88 (%)	10.86 ± 0.91	10.22 ± 0.84	19.62 ± 1.47**	20.09 ± 1.15**

PCK1, phospho*enol*pyruvate carboxykinase; WF, well-fed.

**Denotes a significant difference between the level at 1 month of age and that at 139- to 141-d gestation ($P < 0.01$).

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 with the expression of PEPCK-M, the level of PEPCK-C expression was significantly higher in the liver of the postnatal lamb compared with that in the liver of the fetus. Similarly, G6PHOS mRNA was expressed in the postnatal lamb liver, in contrast with 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 (6–9). In the present study, hepatic 11βHSD1 mRNA expression was increased in postnatal lamb, compared with that in fetal life. Hepatic PGC-1 expression was also higher in the postnatal lamb compared with that in the fetus, and there was an emergence of a relationship between hepatic PGC-1 and PEPCK-C mRNA expression in the lamb after birth. Thus, in contrast with 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 three 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 undermethylated in the adult liver (31). Although an increase in methylation at the three sites in the *PCK1* promoter would be expected to result in a decrease in the binding of transcription factors, including the cyclic adenosine monophosphate (cAMP) response element–binding (CREB) protein, it is of note that the three CpG sites lie outside the "glucocorticoid regulatory unit" in *PCK1*. The increase in methylation at these three 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 gestation that the level of methylation of a number of CpG sites (−82, −30, −5, +31, +99, and +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 (18). 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 (32,33). We have investigated the impact of maternal overnutrition and separately the transition from placental to enteral nutrition on the factors that 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.

METHODS

Animals and Feeding Regimen

All procedures were approved by the University of Adelaide Animal Ethics Committee. Merino ewes were mated, and pregnancy was confirmed by ultrasound scanning in early gestation. From 90 d gestation, ewes were acclimatized to a control diet, which consisted of 1 kg lucerne chaff (85% dry matter, metabolizable energy 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, metabolizable energy 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, United Kingdom (34).

Surgery was then performed on these ewes between 103 and 113 d gestation (term = 147 ± 3 d) using aseptic techniques. General anesthesia was given 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, apart from implantation in the amniotic cavity, as previously described (35). During surgery, intramuscular injections of antibiotics (2 ml procaine penicillin (250 mg/ml), dihydrostreptomycin

(250 mg/ml), procaine hydrochloride (20 mg/ml; all from 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 heparinized saline, and the fetal catheters were exteriorized 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 (5 ml) was administered to that fetus via the amniotic catheter for a period of 4 d.

At 115 d gestation, i.e., before the commencement of the rapid fetal growth phase in late gestation (36), ewes were randomly assigned to either control ($n = 14$) or WF ($n = 16$) group. Between 115 and 124 d of gestation, control ewes were provided with 14.0 ± 0.4 g of lucerne chaff and 6.5 ± 0.4 g of pelleted concentrate per kilogram body weight, and WF ewes were provided with 22.1 ± 0.8 g lucerne chaff and 10.4 ± 0.7 g pelleted concentrate per kilogram body weight each day. The feed allowance of all ewes was proportionately increased by 15% every 10 d (34).

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

Collection of Fetal Tissues
Between 139 and 141 d of gestation, ewes from the control group ($n = 6$) and the WF group ($n = 8$) were killed with sodium pentobarbitone (Virbac, 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 ($n = 8$; four carrying twins and four singletons) and WF ($n = 8$; one carrying twins and seven 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 12 h:12 h light/dark cycle. The day of birth was designated as day 1. Venous blood samples were collected between 0800 and 1300 h after 2-h fasting on days 1–5 and every 3 d thereafter until day 30. All blood samples were centrifuged at 1500*g* for 10 min, and the plasma was stored at –20 °C.

At 30 d of age, the lambs were killed with an overdose of sodium pentobarbitone (Virbac). Livers were dissected out and weighed, and samples were 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) (35). The sensitivity of the assay was 0.5 mmol/l, and the intra- and interassay coefficients of variation were both <5%.

Plasma Insulin
Plasma insulin concentrations were measured using a radioimmunoassay (Rat Insulin kit; Linco Research, St Louis, MO), which was validated for use with sheep plasma (37). The sensitivity of the assay was 0.01 ng/ml, and the intra- and interassay coefficients of variation were both <10%.

Plasma Cortisol
Cortisol was extracted from fetal plasma using dichloromethane as previously described (38). Fetal cortisol concentrations were then measured using an Orion Diagnostica Radioimmunoassay kit (Orion Diagnostica, Turku, Finland) previously validated for fetal sheep plasma (39). The interassay coefficient of variation was 20%, and the intraassay coefficient of variation was <10%.

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

Quantitative Real-Time Reverse Transcription–PCR
The relative expression levels of PGC-1, PPARα, PEPCK-M, PEPCK-C, G6PHOS, GLUT-1, GR, and 11βHSD1 mRNA transcripts were measured by quantitative real-time reverse transcription–PCR (qRT–PCR) using the SYBR Green system in an ABI prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA) (40). For each transcript, qRT–PCR was performed using specific primers (**Table 3**). Each amplicon was designed to be ~200 bp in length, sequenced to ensure the authenticity of the DNA product, and qRT–PCR melting curve analysis was performed to demonstrate amplicon homogeneity. Controls containing no reverse transcriptase were also used. For the qRT–PCR measurements, the primer

Table 3. Sequences of real-time PCR primers for hepatic and reference genes

Gene name (GenBank accession no.)	Forward (5′–3′)	Reverse (5′–3′)	Amplicon size (bp)
PGC-1 (AY237127)	5′–tctggaactgcaggcctaactc–3′	5′–gcaagagggcttcagctttg–3′	96
PPARα A (Y369138)	5′–cgtgtgaacatgacctagaag–3′	5′–acgaagggcggattgttg–3′	151
PEPCK-M (Y11484)	5′–tacgaggccttcaactggcgt–3′	5′–agatccaaggcgccttcctta–3′	150
PEPCK-C (NM174737)	5′–aactcacggttctgcactcca–3′	5′–ggtcgtgcatgatgactttgc–3′	115
G6PHOS (NM013098)	5′–gaaggccaagagatggtgtga–3′	5′–tgcagctcttgcggtacatg–3′	137
GLUT1 (U89029)	5′–taaccgcaacgaggagaacc–3′	5′–ccacgcagcttcttcagca–3′	51
GR (NM001114186)	5′–actgccccaagtgaaaacaga–3′	5′–atgaacagaaatggcagacattttatt–3′	230
11βHSD1 (X69561)	5′–gcgccagatccctgtctgat–3′	5′–agccccataccaccttctttt–3′	250
RPLP0 (BT021080)	5′–caaccctgaagtgcttgacat–3′	5′–aggcagatgcatcagcca–3′	227

11βHSD1, 11β hydroxysteroid dehydrogenase type 1; G6PHOS, glucose-6-phosphatase; GLUT1, glucose transporter-1; GR, glucocorticoid receptor; PEPCK, phospho*enol*pyruvate carboxy kinase; PGC-1, peroxisome proliferator–activated receptor γ coactivator 1; PPAR, peroxisome proliferator–activated receptor.

concentrations were equivalent for all genes, and the amplification efficiencies were 0.981–0.999. A constant amount of cDNA equating to 10 ng 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 (5 µl total volume) contained the following: 2.5 µl of 2× SYBR Green master mix (Applied Biosystems), 0.25 µl of each primer giving a final concentration of 450 µl, 1.0 µl of molecular grade H_2O, and 1.0 µl of a 1:10 dilution of the stock template. The cycling conditions consisted of 40 cycles of 95 °C for 15 s and 60 °C for 1 min. At the end of each run, a dissociation 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 the WF groups in either the fetal or the postnatal cohort.

Combined Bisulfite Restriction Analysis

DNA methylation within the *PCK1* promoter region was analyzed using the combined bisulfite restriction assay (COBRA) (41). Briefly, ~2 µg of DNA from the ventral lobe of fetal and lamb liver was subjected to bisulfite conversion (EpiTect; QIAGEN Australia). PCR was then performed on 100 ng of bisulfite-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, MA), which digest 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, CA). 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 six control fetuses (four males and two females) and eight fetuses from WF ewes (three males and five 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 analyses. Two-way ANOVA was then used to determine the main effects of maternal nutritional treatment (control vs. WF), developmental age (fetal vs. postnatal), and their interaction on birth weight, liver mass, hepatic gene expression, and the level of *PCK1* methylation. Simple linear regression analyses were used to determine the relationships between postnatal measures of fat mass, plasma nutrient, and hormone concentrations and the gluconeogenic gene expression. Plasma glucose, insulin, and cortisol concentrations across the postnatal weeks 1–4 were averaged for correlation analyses, unless stated otherwise. Partial correlation analysis was used to control the effects of maternal intake or mean plasma glucose levels where appropriate. A probability of 5% ($P < 0.05$) was considered the level of significance in all analyses.

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