Effects of maternal cold exposure and nutrient restriction on the ghrelin receptor, the GH–IGF axis, and metabolic regulation in the postnatal ovine liver

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Abstract

Maternal cold exposure of pregnant sheep promotes fetal growth, whereas nutrient restriction (NR) can reverse this effect. The present study was designed to establish whether cold exposure induced by winter shearing of the mother at 70 days gestation (term = 147 days), with or without NR (induced by a 50% reduction in maternal food intake from 110 days gestation), has specific effects on mRNA abundance of hepatic genes related to growth and liver energy metabolism that could regulate postnatal body and liver growth. Measurements of hepatic gene expression for the GH secretagogue receptor-1a (GHSR-1A), peroxisome proliferator-activated receptor (PPAR) α , phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase activity together with glycogen content were made in the livers of offspring at 1 and 30 days of age. Maternal NR reduced liver mass at day 1, whereas offspring of cold-exposed mothers had larger livers at day 30 irrespective of maternal diet. Cold exposure resulted in the up-regulation of *GHSR-1A* mRNA abundance and reduced glucose-6-phosphatase activity at 1, but not 30 days of age, whereas *IGF-II* mRNA was decreased at 1 and 30 days. *PPAR* α mRNA abundance was enhanced, while *PEPCK* was reduced in 30-day old offspring of cold-exposed mothers. NR caused reductions in *IGF-I* mRNA and, at 1-day postnatal age, down-regulated *GHR*, while, at 30 days, reduced *GHSR-1A* gene expression and hepatic glycogen content. In conclusion, we have shown that maternal cold exposure and NR have different effects on the hepatic GH–IGF and metabolic axis that may contribute to changes in liver growth over the first month of life.

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Introduction

Ghrelin is a gut-derived endogenous ligand of the growth hormone secretagogue receptor (GHSR; Kojima *et al.* 1999), which functions to stimulate appetite and centrally regulate GH release (Sugino *et al.* 2002). In rats, ghrelin, synthesized by the placenta (Gualillo *et al.* 2001) or mother, has an important role in the control of late gestational fetal growth (Nakahara *et al.* 2006). mRNA expression for both ghrelin and its novel G protein-coupled receptor (GHSR-1A) has been found in a wide variety of fetal and adult organs including the liver which is the main site for glucose synthesis (Gnanapavan *et al.* 2002, Nakahara *et al.* 2006). In large mammals born with a mature hypothalamic–pituitary–adrenal axis after a long gestation (e.g. sheep and humans), the fetal liver matures during late gestation coincident with the commencement of its gluconeogenic capacity (Fowden *et al.* 1998b) and consequent accumulation of glycogen stores that are then mobilized after birth (Clarke *et al.* 1997a). Key hepatic gluconeogenic enzymes including glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK) – the rate limiting enzyme in this

pathway (Fowden *et al.* 1998b) – are concomitantly increased during late gestation. As ghrelin release is regulated, in part, by insulin (Saad *et al.* 2002) and hepatic cells exposed to ghrelin show increased insulin sensitivity via the phosphoinositide 3 kinase signaling pathway (Barazzoni *et al.* 2005), one metabolic function of ghrelin in the fetus may relate to the regulation of liver development and gluconeogenesis over the perinatal period.

The ovine GH–insulin like growth factor (IGF) axis matures between late gestation and early postnatal life (Hyatt *et al.* 2007a). In sheep, like humans, IGFs are major regulators of fetal (Gibson *et al.* 2001) and postnatal growth and development (DeChiara *et al.* 1990, Baker *et al.* 1993, Liu *et al.* 1993, Efstratiadis 1998, Young *et al.* 2001) with a large proportion of circulating IGF-I being produced by the liver (Yakar *et al.* 1999). Plasma IGF-I increases during late gestation and following birth (Butler & Gluckman 1986, Clarke *et al.* 1997b). However, its ontogenic increase during this time is GH independent (Gluckman 1995). In the fetus, plasma ghrelin is inversely related to IGF-I (Kitamura *et al.* 2003) which in turn

is determined by current nutrition. In sheep, chronic undernutrition caused by placental insufficiency decreases hepatic IGF-I expression in late gestational fetuses (Rhoads *et al.* 2000). Furthermore, severely nutrient-restricted late gestational ovine fetuses have a decreased liver weight that may contribute to GH resistance (Bauer *et al.* 1995). Reducing maternal food intake in late gestation sheep can increase maternal plasma cortisol concentrations (Edwards & McMillen 2001). However, such increases do not appear to affect hepatic glucocorticoid receptor (GR) mRNA abundance and therefore hepatic glucocorticoid sensitivity (Hyatt *et al.* 2007c). In rats, the transcription factor peroxisome proliferator-activated receptor (PPAR) α is highly abundant in the liver where it regulates fatty acid oxidation (Panadero *et al.* 2006). Although hepatic PPAR α expression is unaffected by maternal low-protein diets in rats, in sheep PPAR α is nutritionally regulated in fetal adipose tissue (Bispham *et al.* 2005); however, this has yet to be established in the liver.

Further to maternal nutrition, the thermal environment in which the fetus is exposed is also capable of regulating fetal growth and development. For example, maternal cold exposure, induced by winter shearing of pregnant ewes over the final month of gestation, promotes maternal energy utilization and fat oxidation (Symonds *et al.* 1988), enhancing glucose supply to the fetus (Thompson *et al.* 1982) and, thus, increasing birth weight (Symonds *et al.* 1986). By contrast, maternal hyperthermia has the reverse effect (Limesand *et al.* 2005). In sheep, fetal adaptations to maternal cold exposure are accompanied with larger livers at birth and increased hepatic glycogen content (Clarke *et al.* 1997a). Although the immediate effects of maternal cold exposure are beneficial, the postnatal effects on hepatic gene expression of *GHR*, *IGF-I*, *IGF-II*, and ghrelin sensitivity, known regulators of glucose homeostasis and postnatal growth, have yet to be determined.

Our study was designed to examine the hypothesis that the differential effects of cold exposure (enhanced) and nutrient restriction (NR; reduced) on neonatal body and liver weights would affect mRNA abundance of hepatic genes related to growth and liver energy metabolism. We, therefore, investigated the effects of chronic maternal cold exposure induced from mid-gestation (70 days), coincident with the stage of gestation at which placental mass peaks (Ehrhardt & Bell 1995) but before fetal growth accelerates. This was followed by maternal NR (i.e. a 50% reduction in total calorific intake) during late gestation (115 days to term) in order to determine whether the established negative effects of NR in late gestation could be alleviated by previous maternal cold exposure. Gene expression data for potential endocrine regulators of growth and hepatic energy metabolism including components of the GH-IGF axis, GHSR-1A, GR, insulin receptor (IR), PPAR α , PEPCK mRNA, and G6P enzyme activity were assessed in resulting offspring at 1 and 30 days of age in conjunction with plasma concentrations of cortisol, glucose, insulin, non-esterified fatty acid (NEFA), IGF-I, and hepatic glycogen content. This represents the period in which the newborn effectively adapts to the extrauterine environment and concomitantly resets its metabolic response to feeding and growth (Symonds *et al.* 1989, 1992, Hyatt *et al.* 2007c).

Results

Body and liver weight, hepatic glycogen content, plasma hormone and metabolite concentration

Chronic maternal cold exposure from 70 days mid-gestation to term produced significantly heavier offspring at 1 day of age, an adaptation prevented by maternal NR over the final month of gestation (Table 1). Such differences in body weight with cold exposure were not accompanied

Table 1 Effects of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on body and liver weights and hepatic glycogen content in the resulting offspring at 1 and 30 days of age.

	Unshorn		Shorn	
	C	NR	C	NR
1 day of age				
Body weight (kg)	4.74 $\pm$ 0.36	4.27 $\pm$ 0.29	5.46 $\pm$ 0.37 ^{a*}	4.00 $\pm$ 0.21 ^b
Liver weight (g)	115.11 $\pm$ 13.70 ^a	88.07 $\pm$ 6.16 ^b	129.80 $\pm$ 13.62 ^a	77.45 $\pm$ 3.52 ^b
Relative liver weight (g:kg)	23.84 $\pm$ 1.29	20.91 $\pm$ 1.60	23.55 $\pm$ 0.96 ^a	19.45 $\pm$ 0.56 ^b
Hepatic glycogen (mmol) ^c	89.2 $\pm$ 33.0	154.0 $\pm$ 21.23	135.0 $\pm$ 49.0	95.2 $\pm$ 16.0
30 days of age				
Body weight (kg)	15.74 $\pm$ 0.86	14.60 $\pm$ 0.47	17.16 $\pm$ 0.81 [*]	16.70 $\pm$ 0.66 [*]
Liver weight (g)	372.25 $\pm$ 19.38	355.85 $\pm$ 15.89	434.00 $\pm$ 30.0 [*]	414.83 $\pm$ 21.69 [*]
Relative liver weight (g:kg)	23.75 $\pm$ 0.68	24.42 $\pm$ 0.91	25.48 $\pm$ 1.86	24.81 $\pm$ 0.79
Hepatic glycogen (mmol) ^c	1002.7 $\pm$ 136.1	758.2 $\pm$ 246.4	988.8 $\pm$ 144.7 ^a	471.6 $\pm$ 93.4 ^b

Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their standard errors; $n=7-9$ per group. Different superscript letters indicate an effect ($P<0.05$) of diet within the same shearing group. Superscript asterisk indicates an effect ($P<0.05$) of maternal cold exposure within the same nutritional group. ^cmmol of glucose stored as glycogen.

by any direct change in absolute or relative liver weight which was reduced in nutrient-restricted offspring, irrespective of maternal shearing (Table 1). By 1 month of age, offspring of cold-exposed mothers were heavier than controls and possessed larger livers irrespective of prenatal calorific intake. However, differences in liver weight were removed when expressed relative to whole body weight. Hepatic glycogen content although comparable between intervention groups at birth was significantly reduced in NR offspring at 30 days of age but only in lambs born to cold-exposed mothers. By contrast, there were no significant effects of maternal cold exposure or late gestation nutrition on plasma concentrations for cortisol, glucose, NEFA, insulin, or IGF-1 at either sampling age (e.g. US-C: IGF-I – 1 day 9.6 ± 1.0 ; 30 days 58.9 ± 3.6 nmol/l; cortisol – 1 day 176 ± 13 ; 30 days 68 ± 11 nmol/l). However, the expected age-related increase in both hepatic glycogen content and plasma IGF-1 together with a decrease in plasma cortisol concentrations over the first month of life was observed.

GHR, GHSR-1A, and GR mRNA abundance

Strikingly, maternal cold exposure up-regulated mRNA abundance of *GHSR-1A* at 1 day of age, an adaptation prevented by late gestational maternal NR (Fig. 1a). This adaptation was transient and no longer present at 30 days of age. However, at 30 days of age *GHSR-1A* mRNA abundance was significantly affected by late gestation maternal undernutrition in that NR offspring born to unshorn and shorn mothers had lower *GHSR-1A* mRNA levels (Fig. 1b). Hepatic *GHR* mRNA abundance was transiently decreased at 1 day of age in nutrient-restricted offspring irrespective of maternal cold exposure (Table 2). By day 30 of postnatal life, hepatic *GHR* mRNA levels were comparable between all groups. By contrast, hepatic *GR* mRNA abundance, although similar at 1 day of age, was significantly increased by 30 days of age in offspring born to cold-exposed mothers that had been NR in late gestation.

Hepatic IGF-I, IGF-II, IGF-IR, and IGF-IIR mRNA abundance

IGF-I mRNA abundance was down-regulated by late gestation maternal NR at day 1 of postnatal age, a finding that persisted to 30 days of age in offspring born to cold-exposed mothers, during which time, hepatic *IGF-I* mRNA was also decreased in offspring born to cold-exposed mothers that were also NR in late gestation (Table 3). *IGF-IR* was unaffected by NR over the first month of life but was up-regulated in 30-day old control lambs in response to maternal cold exposure. By contrast, maternal cold exposure decreased hepatic *IGF-II* mRNA abundance at both sampling ages. Furthermore, late gestation maternal NR reduced

Figure 1 Effects of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on hepatic mRNA abundance for GH secretagog receptor (*GHSR*)-1a in the resulting offspring at (a) 1 and (b) 30 days of age. Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their standard errors; $n=7-9$ per group. $P<0.05$ indicates mean value significantly different within the same shearing group (i.e. effect of maternal nutrient restriction). Superscript asterisk indicates an effect ($P<0.01$) of maternal cold exposure within the same nutritional group.

hepatic *IGF-II* mRNA abundance in 30-day old offspring born to unshorn mothers. No maternal effects were observed on hepatic *IGF-IIR* mRNA (Table 3).

IR, PPAR α , PEPCK mRNA abundance, and G6P enzyme activity

There was no effect of maternal calorific intake on mRNA abundance for *IR*, *PPAR α* , or *PEPCK* in the livers of offspring born to unshorn or cold-exposed mothers at 1 day of age (Table 4). At this age, only G6P activity was reduced in offspring of cold-exposed mothers irrespective of maternal NR. However, by 30 days of age, *IR* was increased in offspring whose mothers were cold exposed and NR. Hepatic *PPAR α* mRNA abundance was increased in offspring born to cold-exposed mothers. By contrast, *PEPCK* mRNA was reduced in offspring born to cold-exposed mothers, an adaptation that was unaffected by late gestation nutrient intake.

Discussion

We have shown markedly differential adaptations upon neonatal body weights to maternal cold exposure and late gestational NR. In this investigation, we have

Table 2 Effects of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on hepatic mRNA abundance for growth hormone (GHR) and glucocorticoid (GR) receptor in resulting offspring at 1 and 30 days of age.

	Unshorn		Shorn	
	C	NR	C	NR
1 day of age				
GHR:18S (% of ref)	39.5±3.6 ^a	20.4±6.1 ^b	42.4±6.9 ^a	26.7±6.6 ^b
GR:18S (% of ref)	99.0±22.9	110.5±13.0	97.3±7.0	127.9±24.1
30 days of age				
GHR:18S (% of ref)	51.1±6.6	34.8±5.4	48.1±7.0	36.5±7.7
GR:18S (% of ref)	121.4±10.9	136.6±10.9	92.7±13.9 ^a	178.4±20.5 ^b

Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their standard errors; $n=7-9$ per group. Different superscript letters indicate an effect ($P<0.05$) of diet within the same shearing group.

focused on the liver of the resultant newborns as this tissue represents a substantial endogenous energy reserve that can be rapidly mobilized after birth to sustain immediate neonatal survival and is highly sensitive to the maternal nutrient environment (Clarke *et al.* 1997a). Furthermore, liver tissue plays a critical role in enabling the newborn to effectively adapt to the pronounced change in metabolic demands at birth with its ability to commence endogenous glucose production to ensure that postnatal growth and development is maximized.

A major finding of our study was that newborn offspring born to cold-exposed mothers exhibited an increase in hepatic *GHSR-1A* mRNA abundance that was abolished by maternal NR. This up-regulation of *GHSR-1A* was coupled with increased body weight at 1 day of age. Restricting maternal nutrition during late gestation reduced offspring liver weight although this was not accounted for by hepatic glycogen content that was comparable between nutritional groups at 1 day of age. However, reduced liver weight in NR lambs occurred in conjunction with a down-regulation of hepatic *GHR* and *IGF-I* mRNA abundance. By 30 days of postnatal age, the reduction in *IGF-I*

mRNA abundance with late gestation NR only persisted if it was combined with maternal cold exposure. Furthermore, liver weight was no longer reduced by previous exposure to maternal NR in 1-month old lambs, although hepatic glycogen content was significantly reduced in 30-day old NR lambs born to cold-exposed mothers. Additionally, the increased body weights of 30-day old offspring born to cold-exposed mothers were accompanied by increased hepatic *IGF-IR* and *PPAR α* , while both *IGF-II* and *PEPCK* were reduced. Taken together, such adaptations emphasize the plasticity of ovine liver development and energy supplies in early life, which are likely to contribute to longer term changes on hepatic function and metabolism together with later growth of the offspring (Hyatt *et al.* 2007c).

Maternal cold exposure and offspring body weight

The increased birth weight of lambs born to control cold-exposed mothers was accompanied by a proportional increase in all internal organs including liver weight and total perirenal fat mass (Butt *et al.* 2006). Interestingly, offspring born to mothers that were cold exposed and NR

Table 3 Effects of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on hepatic mRNA abundance for insulin like growth factor (IGF)-I and -II ligands and receptors (R) in resulting offspring at 1 and 30 days of age.

	Unshorn		Shorn	
	C	NR	C	NR
1 day of age				
IGF-I:18S (% of ref)	117.4±39.4 ^a	45.4±8.4 ^b	87.6±11.2 ^a	6.6±0.6 ^b
IGF-IR:18S (% of ref)	56.3±13.0	82.8±23.1	74.2±8.5	77.7±7.7
IGF-II:18S (% of ref)	160.6±38.3	128.6±34.3	84.2±9.1*	79.8±8.7*
IGF-IIR:18S (% of ref)	158.6±17.8	153.3±46.6	146.9±11.1	136.6±5.4
30 days of age				
IGF-I:18S (% of ref)	137.5±40.5	97.7±22.0	101.8±16.5 ^a	6.1±1.3* ^b
IGF-IR:18S (% of ref)	29.3±11.4	36.9±11.7	74.2±9.3*	53.5±18.4
IGF-II:18S (% of ref)	183.2±24.0 ^a	107.3±18.7 ^b	84.5±6.9*	78.1±14.7*
IGF-IIR:18S (% of ref)	158.7±28.8	200.5±25.9	133.9±8.5	153.3±17.0

Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their standard errors; $n=7-9$ per group. Different superscript letters indicate an effect ($P<0.05$) of diet within the same shearing group. Superscript asterisk indicates an effect ($P<0.05$) of maternal cold exposure within the same nutritional group.

Table 4 Effects of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on hepatic mRNA abundance for insulin receptor (IR), peroxisome proliferator-activated receptor (PPAR) α , phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6P) activity in resulting offspring at 1 and 30 days of age.

	Unshorn		Shorn	
	C	NR	C	NR
1 day of age				
IR:18S (% of ref)	101.5 $\pm$ 39.6	68.5 $\pm$ 10.4	88.0 $\pm$ 27.3	172.9 $\pm$ 80.0
PPAR α :18S (% of ref)	90.1 $\pm$ 17.8	99.5 $\pm$ 13.9	105.9 $\pm$ 17.9	151.7 $\pm$ 25.0
PEPCK:18S (% of ref)	99.2 $\pm$ 13.3	134.6 $\pm$ 19.0	114.7 $\pm$ 11.1	102.2 $\pm$ 20.3
G6P activity (% of ref)	0.52 $\pm$ 0.13	0.55 $\pm$ 0.09	0.21 $\pm$ 0.08*	0.22 $\pm$ 0.04*
30 days of age				
IR:18S (% of ref)	63.6 $\pm$ 9.9	67.0 $\pm$ 4.5	50.6 $\pm$ 7.6 ^a	102.1 $\pm$ 23.4 ^{a,b}
PPAR α :18S (% of ref)	68.3 $\pm$ 15.4	73.4 $\pm$ 13.6	150.1 $\pm$ 20.8*	105.0 $\pm$ 13.9*
PEPCK:18S (% of ref)	136.3 $\pm$ 19.1	137.5 $\pm$ 12.7	55.0 $\pm$ 15.7*	102.3 $\pm$ 13.7*
G6P activity (% of ref)	0.30 $\pm$ 0.01	0.35 $\pm$ 0.02	0.27 $\pm$ 0.02	0.30 $\pm$ 0.01

Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their S.E.M.; $n=7-9$ per group. Different superscript letters indicate an effect ($P<0.05$) of diet within the same shearing group. Superscript asterisk indicates an effect ($P<0.05$) of maternal cold exposure within the same nutritional group.

were the lightest and thus possessed the smallest livers and least amount of adipose tissue although this difference was not statistically significant. By 1 month of age, however, these lambs were significantly larger than their unshorn counterparts and had significantly lower glycogen reserves when compared with control lambs born to cold-exposed mothers. The mechanism responsible for larger lambs being born to cold-exposed mothers is thought to relate to maternal plasma glucose concentrations (Symonds *et al.* 1992). Previous studies have proposed that maternal cold exposure increases birth weight by preventing the late gestational decrease in maternal plasma glucose, which is most apparent in nutrient-restricted mothers (Symonds *et al.* 1988). Furthermore, it is hypothesized that cold-exposed lambs would have had larger placentas that might have partitioned nutrients in favor of fetal growth, although this remains to be determined.

Ghrelin and GHSR-1A

Increased hepatic *GHSR-1A* mRNA abundance in newborn offspring born to cold-exposed, control fed mothers may have been mediated by an increase in nutrient supply to the fetus (Symonds *et al.* 1990), which suggests a potential increase in plasma ghrelin concentrations (Murata *et al.* 2002). However, the extent to which the proposed increase in plasma ghrelin may be mediated by changes in both the acylated and deacylated forms of ghrelin (Hosoda *et al.* 2000, Nakahara *et al.* 2006) remains unknown and an area for future research. Further work is needed to confirm plasma ghrelin concentrations in the sheep. Hypothesized increased circulating concentrations of ghrelin are predicted to affect both insulin action and hepatic gluconeogenesis (Murata *et al.* 2002, Saad *et al.* 2002, Gauna *et al.* 2005).

In the present study, all nutrient-restricted offspring were able to restore their liver mass by 1 month of age but possibly by different mechanisms depending on the maternal metabolic and endocrine environment that potentially impacted on insulin and cortisol sensitivity. In this regard, increased hepatic *IR* and *GR* mRNA in 30-day old lambs born to cold-exposed nutrient-restricted mothers would be predicted to enhance the metabolic effects of both plasma insulin and cortisol despite comparable plasma concentrations of each (Symonds *et al.* 1992) together with similar IGF-I concentrations. Furthermore, 30-day old lambs born to cold-exposed mothers had significantly reduced hepatic PEPCK and increased PPAR α with S-NR lambs also having reduced hepatic *IGF-I* mRNA abundance. Taken together, these findings suggest that S-NR lambs although smaller at birth with reduced liver weights caught up to S-C counterparts by 1 month of age. At that time liver weights were no longer reduced; however, they did have significantly reduced hepatic glycogen stores suggesting that either reduced gluconeogenic capacity (decreased PEPCK in shorn offspring) or glucose produced is used to promote increased growth and therefore not converted into glycogen for storage in the liver.

The present study supports the concept that energy balance is a major determinant of tissue ghrelin sensitivity, i.e. GHSR-1A abundance that further interacts with fetal growth through the regulation of gene expression of hepatic growth factors. During late gestation, the fetal liver matures with regards to its functional capacity, i.e. onset of gluconeogenesis and expression of GH-IGF axis (Fowden 1995). Such developmental processes may, potentially, occur in synchronization with increased plasma ghrelin concentrations seen in early human development (Soriano-Guillen *et al.* 2004). In the present study, hepatic GHSR-1A was differentially influenced by

maternal cold exposure and NR. Taken together, our findings support the proposal that ghrelin, alongside IGF-I, plays an important role in maturation after birth in sheep as well as humans (Soriano-Guillen *et al.* 2004).

The hepatic GH-IGF axis

Previous sheep studies have demonstrated the GH-IGF axis to be functional in the late gestational fetus and that plasma GH and IGF-I concentrations respond accordingly to specific stimuli such as maternal NR (Oliver *et al.* 1993) and changes in placental glucose supply (Gluckman *et al.* 1987). In the present study, while the determination of plasma GH levels was not possible (limited plasma supplies), we have shown that maternal cold exposure and NR differentially affect the development of the hepatic GH-IGF axis, in that, despite a transient decrease in GHR mRNA abundance in the NR neonate, hepatic *IGF-I* mRNA was reduced in S-NR offspring over the first month of postnatal life. Such an adaptation may reflect a prior decrease in late gestational fetal plasma IGF-I concentrations, which is known to be highly sensitive to maternal, and thus fetal, nutrition (Bassett *et al.* 1990). However, further studies are essential to confirm the effect of maternal cold exposure combined with late gestation maternal undernutrition upon fetal plasma IGF-I concentrations. In sheep, maturation of the GH-IGF axis occurs during late gestation and involves the development of feedback and interaction between IGF, their receptors, and GH in the preparation for birth (Fowden *et al.* 1998a). The development of the ovine fetal and juvenile GH-IGF axes appears to be highly sensitive to the maternal nutritional and metabolic environment (Bauer *et al.* 1995, Hyatt *et al.* 2007c). Surprisingly, reduced hepatic *IGF-I* mRNA abundance seen in the present study was not accompanied by any parallel reduction in hepatic IGF-IR or IGF-IIR, indicating that the potentially decreased hepatic IGF-I secretory capacity did not further alter the ability of liver to respond to or synthesize IGF-I. This is reflected in comparable plasma IGF-I concentrations between groups at both 1 and 30 days of age.

Hepatic *IGF-II* mRNA, but not its receptor, was persistently down-regulated up to 30 days of age as a result of maternal cold exposure, irrespective of maternal diet. In the developing fetus plasma, IGF-II is the predominant IGF promoting fetal growth. However, following birth, its concentration declines rapidly as IGF-I takes over as the main growth factor (Li *et al.* 1998, Dupont & Holzenberger 2003). Consequently, the ontogenic decline in IGF-II may be accelerated in offspring born to cold-exposed mothers. However, further work is warranted to confirm this hypothesis. Plasma cortisol is another factor that influences the clearance of IGF-II (Li *et al.* 1998). Short-term cold exposure has been shown to stimulate plasma cortisol

concentrations (Cabello 1983). However, maternal cold exposure from mid-gestation in the present study had no effect on offspring plasma cortisol concentrations. Furthermore, previous studies have also demonstrated that maternal NR over the final month of gestation transiently increases maternal, but not fetal, plasma cortisol concentrations (Edwards & McMillen 2001), a finding that was consistent with the present study with respect to offspring plasma cortisol concentrations. The cold-exposed reduction in *IGF-II* gene expression could potentially mediate changes in hepatic function in these offspring as indicated by reduced G6P activity observed at 1 day of age. To this extent, in addition to insulin (Lopez *et al.* 1999), glycogen synthesis has been shown to be regulated by IGF-II in rat hepatocytes (Menuelle *et al.* 1995). PEPCK, however, is the rate-limiting enzyme for gluconeogenesis, which is regulated in turn by the uptake of gluconeogenic precursors (Girard 1986).

In the mother, cold exposure and NR have opposite effects on maternal glucose production and, therefore, fetal glucose supply (Thompson *et al.* 1982, Symonds *et al.* 1986). Such decreased hepatic *PEPCK* mRNA abundance in offspring of cold-exposed mothers could be a transitional response to changes in amino acid supply, which are maintained even during prolonged periods of undernutrition (Robinson & Symonds 1995). It could also reflect changes in substrate supply or handling as this was accompanied by raised hepatic PPAR α that in the neonatal rat promotes fatty acid oxidation (Panadero *et al.* 2006). In neonatal sheep, however, fat is rapidly oxidized within brown adipose tissue and oxidation is much lower (i.e. negligible rates) compared with rats (that are born immature at birth and are altricial) in which the ratio of glucose requirements compared with its supply in milk is several fold greater than in the sheep which are precocial (Girard 1990). Interestingly, in the present study longer term hepatic adaptations were associated with decreased insulin sensitivity, as indicated by a reduction in IR; the latter being inversely related to insulin (Pandini *et al.* 2002). Any physiological differences in carbohydrate and lipid metabolism in the postnatal period would not be expected to be accompanied by significant changes in plasma concentrations of glucose or NEFA because, as discussed above, these endocrine responses markedly change during the postnatal period as the newborn adapts to the extra-uterine environment (Symonds & Lomax 1992).

In conclusion, we have demonstrated different effects of maternal cold exposure and NR on the gene expression of a number of growth factors and/or their receptors, particularly the GHSR-1A in the postnatal liver. The increase in GHSR-1A in newborn offspring born to normally fed cold-exposed mothers could reflect their increased nutrient supply and thus energy balance, thereby promoting hepatic gluconeogenic potential. Critically, maternal cold exposure can potentially

reverse some of the hepatic effects of NR on the newborn at day 1 postnatal age. Thus, the catabolic compromise in the hepatic GH-IGF and metabolic axes of the neonate induced by NR during fetal development can be prevented, in part, by maternal cold exposure.

Materials and Methods

Animals

Thirty-three twin-bearing multiparous Bluefaced Leicester crossed Swaledale sheep, of similar body weight and of known mating date, were group-housed indoors at mid-gestation (68 ± 2 days) under natural daylight conditions as previously described (Pearce *et al.* 2005). Fifteen sheep were shorn (S; i.e. cold exposed) at 70 days gestation and the remaining 18 sheep were left unshorn (US). These animals were further divided into control (C) and NR groups, at 115 ± 2 days, resulting in four groups: unshorn control (US-C, $n=9$), unshorn NR (US-NR, $n=9$), shorn control (S-C, $n=8$), and shorn NR (S-NR, $n=7$). The control animals were fed straw *ad libitum* and a fixed amount of concentrate, which was calculated to sufficiently meet the metabolizable energy requirements (in accordance with fetal number and stage of gestation), throughout the study (Agricultural Research Council 1980). NR mothers consumed 50% of this amount during the final 4 weeks of gestation, as previously described (Pearce *et al.* 2005).

At term, all offspring were delivered naturally with no intervention and birth weight recorded. On day 1 of postnatal life, one twin from each mother was randomly selected to be blood sampled and then humanely euthanized (pentobarbital sodium via the jugular vein: 200 mg/kg Euthatal: RMB Animal Health UK) for tissue sampling. The remaining twin was ewe reared (i.e. raised as a singleton) until blood and tissue sampling at 30 days postnatal age. After giving birth normally at term, all mothers were housed with their offspring and fed a diet of hay *ad libitum* together with a fixed amount of concentrate sufficient to fully meet the mothers' own metabolizable requirements plus that required to maintain lactation. At 30 days of age, all remaining twin lambs were humanely euthanized and the livers rapidly dissected, weighed, and a representative portion (i.e. 20 g from the same position of the right lobe from each animal) placed in liquid nitrogen and stored at -80°C until further analysis. All procedures were performed with the necessary institutional

ethical approval as designated under the UK Animals (Scientific Procedures) Act, 1986.

Laboratory analyses

mRNA detection

Total RNA was isolated from a central region of the right lobe of the liver using Tri-Reagent (Sigma). In order to maximize sensitivity, a fully validated two-tube approach to RT-PCR was adopted, as previously described (Hyatt *et al.* 2007b, 2007c). RT-PCR analysis used ovine-specific oligonucleotide primers designed specifically to cross an exon-intron boundary thus reducing the potential for genomic DNA contamination as previously described (Hyatt *et al.* 2007b, 2007c). In addition, oligonucleotide primers were designed specifically to *GHSR-1A*, *IR*, *PEPCK*, and *PPAR α* (see Table 5 for optimized PCR conditions utilized). Agarose gel electrophoresis (2.5%) and ethidium bromide staining confirmed the presence of both the target gene and 18S (internal standard, classic II 18S; Ambion, Warrington, UK) at the expected sizes (Table 5). Consistency of lane loading for each sample was verified from the measurement of 18S rRNA, which was comparable between all intervention groups and sampling ages (Fig. 2). Densitometric analysis was performed on each gel by image detection using a Fujifilm LAS-1000 cooled charge-coupled device camera (Raytek Scientific Ltd, Sheffield, UK) to determine mRNA abundance for each gene. Results are expressed as a ratio of 18S and normalized to a reference sample (liver tissue taken from a 1-day old lamb) present on all gels and was unaffected by either the animals age or maternal environment (Fig. 2). All RT-PCR analyses were conducted in duplicate with appropriate positive and negative controls and a range of molecular weight markers and has previously been shown to give comparable results with those obtained by real-time PCR (Hyatt *et al.* 2007c). The resultant PCR product was extracted (QIAquick gel extraction kit, catalog number 28704), sequenced, and results cross-referenced against the GenBank database (<http://www.ncbi.nlm.nih.gov/>) to further confirm specificity of the target gene.

Hepatic glucose-6-phosphatase activity

G6P activity was determined by measuring the rate of phosphate production from G6P, as previously described (Fowden *et al.* 1993). All samples were measured in triplicate.

Table 5 Optimized PCR conditions for ovine-specific semi-quantitative RT-PCR oligonucleotide primers.

Primer set	Size (bp)	Annealing temperature ($^\circ\text{C}$)	Cycle no.	Sequence
<i>GHSR-1A</i>	155	57.4	33	F: CTA CTT CGC CAT CTG CTT CC R: GAG GGT CGG TAC CAT TCT CA
<i>IR</i>	150	60.1	25	F: CTG CAC CAT CAT CAA CGG AA R: CGT AAC TTC CGG GAA GAA GGA
<i>PPARα</i>	113	60	30	F: TTT CAC AAG TGC CTT TCC G R: AGG TCA TGT TCA CAC GTA AGG
<i>PEPCK</i>	201	58.9	30	F: GTA TGT CAT CCC GTT CAG CA R: ACA GGC CCA GTT GTT AAC CA

GHSR-1A, growth hormone secretagogue receptor-1A; *IR*, insulin receptor; *PPAR α* , peroxisome proliferator-activated receptor α ; *PEPCK*, phosphoenolpyruvate carboxykinase.

Figure 2 Confirmation of the absence of any effect of maternal cold exposure induced from mid-gestation (70 days) and late gestational nutrient restriction (110 days to term) on 18S hepatic mRNA abundance in the resulting offspring at (a) 1 and (b) 30 days of age. Offspring were born to shorn and unshorn mothers that were fed either a control (C) or nutrient-restricted (NR) diet; full dietary details are provided in Materials and Methods. Values are means with their standard errors; $n=7-9$ per group.

Hepatic glycogen content

Hepatic glycogen content was measured using the method of Keppler & Decker (1984), as previously described (Keppler & Decker 1984).

Hormone and metabolite analysis

Plasma cortisol and insulin concentrations were measured by RIA (Symonds *et al.* 1986, Gardner *et al.* 2006). Plasma IGF-I concentrations were determined by ELISA (Heasman *et al.* 2000), while offspring plasma glucose and NEFA concentrations were assessed using spectrophotometric methods as previously described (Gardner *et al.* 2005).

Statistical analyses

Statistical analysis was performed using SPSS software package (version 14.0; SPSS Inc., Woking, Surrey). Data were firstly subjected to a Kolmogorov–Smirnov normality test to confirm normal distribution, thereby determining that parametric statistical tests were appropriate. Mean body weight and liver weight as well as gene abundance, glycogen content, and enzyme activity were analyzed using a factorial (2×2) univariate general linear model (GLM) test. The GLM tests assessed the main effect of cold exposure and maternal diet, i.e. late gestational NR together with any potential interactions

(i.e. maternal cold exposure $\times$ late gestation NR). A statistically significant difference was inferred by $P < 0.05$. Results are presented as means and standard errors.

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