Ontogeny and nutritional programming of mitochondrial proteins in the ovine kidney, liver and lung

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Abstract

This study investigated the developmental and nutritional programming of two important mitochondrial proteins, namely voltage-dependent anion channel (VDAC) and cytochrome c, in the sheep kidney, liver and lung. The effect of maternal nutrient restriction between early and mid-gestation (i.e. 28- to 80-day gestation, the period of maximal placental growth) on the abundance of these proteins was also examined in fetal and juvenile offspring. Fetuses were sampled at 80 and 140 days of gestation (term ~147 days), and postnatal animals at 1 and 30 days and 6 months of age. The abundance of VDAC peaked at 140 days of gestation in the lung, compared with 1 day after birth in the kidney and liver, whereas cytochrome c abundance was greatest at 140 days of gestation in the liver, 1 day after birth in the kidney and 6 months of age in lungs. This differential ontogeny in mitochondrial protein abundance between tissues was accompanied with very different tissue-specific responses to changes in maternal food intake. In the liver, maternal nutrient restriction only increased mitochondrial protein abundance at 80 days of gestation, compared with no effect in the kidney. In contrast, in the lung mitochondrial protein, abundance was raised near to term, whereas VDAC abundance was decreased by 6 months of age. These findings demonstrate the tissue-specific nature of mitochondrial protein development that reflects differences in functional adaptation after birth. The divergence in mitochondrial response between tissues to maternal nutrient restriction early in pregnancy further reflects these differential ontogenies.


Introduction

Mitochondria play a major role in regulating energy supply and related processes in most tissues. This role is particularly important at birth when the function of many fetal tissues has to adapt to the thermal, gaseous and metabolic challenges of the extraterine environment (Gnanalingham et al. 2005b, 2006). In some but not all tissues, mitochondrial protein abundance would be expected to peak around birth, coincident with their pronounced increase in metabolic activity (Mostyn et al. 2004). The most widely studied mitochondrial proteins in the newborn have been the uncoupling proteins, which have a pronounced tissue specificity (Symonds et al. 2004, Gnanalingham et al. 2006). In precocious newborn, which have to establish metabolic and thermo-regulatory independence at birth, uncoupling protein abundance in both adipose tissue and the lung peaks soon after parturition (Clarke et al. 1997, Gnanalingham et al. 2005c) and are critical in enabling the onset of non-shivering thermogenesis and lung maturation.

Other mitochondrial proteins whose abundance can peak at birth include voltage-dependent anion channel (VDAC) and cytochrome c and as such these proteins have a potential role in enabling the newborn to effectively adapt to the extraterine environment (Gnanalingham et al. 2006). This could involve the tissue-specific remodelling that enables the onset of postnatal function including the maintenance of fluid balance, gluconeogenesis and respiration (Barac-Nieto & Spitzer 1988, Fowden et al. 1998, Gnanalingham et al. 2006). VDAC is located in the outer mitochondrial membrane (Colombini 1979) and may be responsible for the release of cytochrome c from the inter-membrane space, one process that has been implicated in the chain of events culminating in apoptosis (Crompton 1999). Both of these mitochondrial proteins are highly abundant in the kidney, liver and lung of the newborn sheep (Yakubu et al. 2007). They do, however, have tissue-specific locations that may explain their potentially very different functions between organs (Yakubu et al. 2007). In the kidney, both VDAC and cytochrome c are located within the tubules, in the liver each is present within the epithelial lining, whereas, in the lung, VDAC is located in the alveoli and cytochrome c in the bronchioles. The
extent to which VDAC and cytochrome c may exhibit different pre- and postnatal ontogenies depending on their tissue location is not known and is the main aim of the present study.

One factor that has a primary role in regulating mitochondrial protein abundance during development is maternal food intake (Mostyn et al. 2003). Depending on the timing, maternal nutrient restriction can have differential effects on mitochondrial protein abundance in both adipose tissue and the lung that can persist into later life (Gnanalingham et al. 2005a, 2005c). This effect may be mediated in part by changes in fetal glucocorticoid action (Gnanalingham et al. 2005a, 2005c) which is up-regulated in a range of tissues including the kidney, liver and lung following maternal nutrient restriction targeted over the period from uterine attachment and maximal placental growth, i.e. 28–80 days of gestation (Whorwood et al. 2001). These adaptations in the fetus increase with gestational age and may be mediated by changes in maternal plasma cortisol through gestation (Bispham et al. 2003) in conjunction with placental sensitivity to glucocorticoids (Gnanalingham et al. 2007). The extent to which this further impacts on mitochondrial protein abundance during development in these tissues has yet to be examined and was a further aim of our study. Interestingly, the kidney of nutrient-restricted offspring appears to be protected from the adverse effects of later obesity (Williams et al. 2007). It is therefore important to have a clear understanding of the developmental changes that occur in this and related tissues under conditions in which its function is normal.

It is not only the rise in cortisol that is important in determining the rapid appearance of mitochondrial proteins after birth, but also the subsequent endocrine changes after the postnatal period (Symonds 1995). Consequently, in the lung, the large decrease in glucocorticoid action between 1 and 6 months of age is paralleled by a decline in uncoupling protein 2 (UCP2) abundance (Gnanalingham et al. 2005c). It has yet to be established whether the abundance of other mitochondrial proteins may be similarly affected, or whether this varies between tissues. For example, in white adipose tissue, there is an increase in glucocorticoid action after birth that is paralleled by a rise in UCP2 abundance (Gnanalingham et al. 2005a). A further aim of our study was, therefore, to determine the magnitude of change in VDAC and cytochrome c during juvenile life together with the extent to which this may be related to changes in glucocorticoid receptor (GR) abundance.

Results

Ontogeny of mitochondrial protein abundance

The kidney

In the kidney, the relative abundance of VDAC did not change between 80 and 140 days of gestation, but increased after birth to peak at 1 day of age, before gradually declining up to 6 months of age. A comparable pattern of developmental changes were observed for cytochrome c with the modification that the rate of decrease after birth was delayed by 1 month. As a consequence, the abundance of cytochrome c was higher in the juvenile than the fetal kidney. These ontogenic changes in mitochondrial protein abundance were not directly related to the large increase in organ weight that occurs between mid-gestation and 6 months of age (Fig. 1). In particular, there was no increase in total mitochondrial protein content of the kidney after 30 days of age.

The liver

There was a pronounced increase in the abundance of VDAC within the liver from mid-gestation up to 1 day after birth (Fig. 2). This was followed by a substantial decrease up to 30 days of age with a smaller decline up to 6 months. In the case of cytochrome c, its abundance increased between 80 and 140 days of gestation but did not change further immediately after birth. A decrease was then observed to 1 and 6 months of age. In contrast to the kidney, there was a greater increase in liver weight between 1 and 6 months of age that was followed by a proportionately greater rise in its total mitochondrial protein content.

The lung

In contrast to the kidney and liver, there was a marked divergence in ontogeny of VDAC and cytochrome c in the lung. A peak in VDAC abundance was thus observed at 140 days of gestation with a large decrease soon after birth that continued up to 6 months of age (Fig. 3). Cytochrome c abundance, however, remained

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Figure 1 Developmental ontogeny of kidney (a) weight, (b) mitochondrial protein content, (c) voltage-dependent anion channel (VDAC) and (d) cytochrome c abundance in the sheep. Values are means with their standard errors and n = 6 at each sampling point. Different letters denote statistically significant differences (P<0.05).

unchanged through gestation and then increased between 1 and 6 months after birth. Over this period, lung weight increased in conjunction with a proportionately smaller rise in total mitochondrial protein content.

Effect of early to mid-gestational maternal nutrient restriction on mitochondrial protein abundance in the fetal and juvenile kidney, liver and lung

There was no difference in plasma cortisol concentration between nutritional groups when sampled as fetuses or juveniles (e.g. at 180 days after birth: C, 15.4 ± 2.3 (n = 6) and nutrient restricted (NR), 18.2 ± 1.2 (n = 6) mmol/l). The expected increase in plasma cortisol with gestational occurred in all animals irrespective of maternal nutrition (e.g. NR: 80 days, 17.9 ± 2.8 and 140 days, 55.1 ± 8.2 (n = 6) mmol/l). Maternal nutrient restriction had no effect on kidney weight or mitochondrial protein abundance at any sampling age, although GR mRNA abundance was transiently raised in NR fetuses at 140 days of gestation (Table 1). In the liver, although total weight was unaffected by maternal nutrition at all sampling ages, there was a reduction in total mitochondrial protein content at 80 days of gestation in the nutrient-restricted group that was accompanied by an up-regulation in the abundance of both VDAC and cytochrome c (Table 2). There were no differences in mitochondrial protein abundance at either 140 days of gestation or 180 days after birth and as for the kidney the expression of GR was raised in previously nutrient-restricted fetuses near to term.

In the lung, maternal nutrient restriction had no effect on total weight or mitochondrial protein abundance (Table 3). There was also no difference in either VDAC and cytochrome c abundance in the fetal lung at 80 days of gestation but mitochondrial protein abundance was raised in nutrient-restricted fetuses near to term. In the juvenile offspring VDAC, but not cytochrome c, abundance was reduced although the expression of GR mRNA was persistently raised in the lungs of nutrient-restricted fetuses, and offspring, at all sampling ages.

Discussion

We have shown that, as for many of the other mitochondrial proteins such as the UCPs in tissues whose metabolic rate increases rapidly at birth (Clarke et al. 1997, Gnanalingham et al. 2005c), there are pronounced changes in the abundance of both VDAC and cytochrome c in the newborn. These adaptations are likely to be mediated, in part, by the marked increase in circulating plasma cortisol (Fowden et al. 1998) which has a pivotal role in enabling the newborn to effectively adapt to the extrauterine environment (Clarke et al. 1998). What is notable about the ontogenic responses observed is the magnitude of changes in mitochondrial protein abundance between each tissue that may be related to the specificity of location for both VDAC and cytochrome c in the newborn. These adaptations are likely to be mediated, in part, by the marked increase in circulating plasma cortisol (Fowden et al. 1998) which has a pivotal role in enabling the newborn to effectively adapt to the extrauterine environment (Clarke et al. 1998). What is notable about the ontogenic responses observed is the magnitude of changes in mitochondrial protein abundance between each tissue that may be related to the specificity of location for both VDAC and cytochrome c (Yakubu et al. 2007) and the extent to which these are sensitive to maternal nutrient restriction. In this regard, there is a clear divergence in the timing as well as magnitude of mitochondrial adaptation within the kidney, liver and lung that is unrelated to tissue weight.

The kidney

In the kidney, there was no change in VDAC or cytochrome c abundance between mid- and late gestation whereas, with the exception of cytochrome c...
in the lung, these increased substantially with gestation in the other tissues examined. These differences could reflect the fact that the kidney develops structurally much earlier in gestation (Dodic et al. 2002). As such, its metabolic requirements and activity are predicted to be high earlier in gestation and would be accompanied by an appreciable mitochondrial content. Mid-gestation is also the period in which nephrogenesis occurs within the kidney (Wintour & Moritz 1997) that can be accompanied by apoptosis (Camp & Martin 1996).

Consequently, both VDAC and cytochrome c could be involved as VDAC forms the mitochondrial permeability transition pore connecting the inner and outer mitochondrial membranes (Crompton et al. 1998), thereby enabling the release of cytochrome c following apoptosis (Gottlieb 2000).

The time at which the greatest change in abundance of VDAC and cytochrome c occurred in the kidney was between 140 days of gestation and 1 day after birth, which is coincident with the dramatic increase in metabolic rate (Clarke et al. 1994) and concomitant onset of postnatal kidney function. During the neonatal period, as the animal commences liquid feeding, there is a rise in solute turnover within the body that places substantial metabolic demands on the kidney, and this is met, in part, by increased mitochondrial activity. We have established that both VDAC and cytochrome c are located around the kidney tubules (Yakubu et al. 2007) and would be expected to regulate fluid exchange. The decrease in the relative abundance of both mitochondrial proteins between 1 and 6 months of age occurs over a period when kidney growth continues but its mitochondrial content remains unchanged. As such, overall metabolic activity and the rate of solute exchange across the kidney declines (Barac-Nieto & Spitzer 1988). With regard to the primary endocrine mechanisms that regulate mitochondrial development, it is established that the surge in cortisol around the time of birth has a critical role in many tissues including adipose tissue and the lung (Gnanalingham et al. 2005a, 2005c). In the kidney, the rise in glucocorticoids is important in enabling the kidney to adapt from being salt losing to salt conserving (Fowden et al. 1998), a process that may be facilitated by the increase in both VDAC and cytochrome c. Cortisol also regulates the activity of the enzyme 5′-monodeiodinase in the fetal kidney (Forhead et al. 2006), which, in brown adipose tissue at least, can impact of mitochondrial protein abundance (Bianco & Silva 1987). Local production of the metabolically active thyroid hormone, triiodothyronine, together with other factors, must also have a regulatory role within the mitochondria. Indirect evidence for this proposal is provided by the lack of any difference in VDAC or cytochrome c abundance between kidneys sampled from nutrient-restricted or control mothers as fetuses or juveniles, despite increased glucocorticoid action in the nutrient-restricted kidneys of the near term fetus (Whorwood et al. 2001). Interestingly, although raised glucocorticoid action is present at term, it is not apparent in the juvenile offspring when 11β-hydroxysteroid dehydrogenase type 2 (Gopalakrishnan et al. 2005)

Table 1 Effect of maternal nutrient restriction between 28 and 80 days of gestation on mitochondrial protein and glucocorticoid receptor (GR) mRNA abundance in the developing sheep kidney.

<table>
<thead>
<tr>
<th>Sampling age</th>
<th>80 days of gestation</th>
<th>140 days of gestation</th>
<th>180 days after birth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>1.65±0.1</td>
<td>1.46±0.1</td>
<td>10.3±0.5</td>
</tr>
<tr>
<td>Mitochondrial protein content (g)</td>
<td>0.020±0.002</td>
<td>0.019±0.001</td>
<td>0.16±0.03</td>
</tr>
<tr>
<td>VDAC abundance (% of ref.)</td>
<td>38±7</td>
<td>32±9</td>
<td>33±3</td>
</tr>
<tr>
<td>Cytochrome c abundance (% of ref.)</td>
<td>10±3</td>
<td>12±2</td>
<td>9±2</td>
</tr>
<tr>
<td>GR mRNA (% of age matched C)</td>
<td>100±12</td>
<td>105±7</td>
<td>100±10</td>
</tr>
</tbody>
</table>

Values are means with their standard errors and n=6 per group per time point. Significance of NR (*P<0.05, †P<0.01). C, control; NR, nutrient restricted; VDAC, voltage-dependent anion channel.

Table 2 Effect of maternal nutrient restriction between 28 and 80 days of gestation on mitochondrial protein and glucocorticoid receptor (GR) mRNA abundance in the developing sheep liver.

<table>
<thead>
<tr>
<th>Sampling age</th>
<th>80 days of gestation</th>
<th>140 days of gestation</th>
<th>180 days after birth</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>NR</td>
<td>C</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>15.1±1.6</td>
<td>12.6±0.7</td>
<td>134±8</td>
</tr>
<tr>
<td>Mitochondrial protein content (g)</td>
<td>0.29±0.02</td>
<td>0.18±0.02</td>
<td>0.96±0.14</td>
</tr>
<tr>
<td>VDAC abundance (% of ref.)</td>
<td>18±3</td>
<td>24±4</td>
<td>50±3</td>
</tr>
<tr>
<td>Cytochrome c abundance (% of ref.)</td>
<td>17±4</td>
<td>33±2</td>
<td>32±6</td>
</tr>
<tr>
<td>GR mRNA (% of age matched C)</td>
<td>100±14</td>
<td>76±4</td>
<td>100±14</td>
</tr>
</tbody>
</table>

Values are means with their standard errors and n=6 per group per time point. Significant effect of NR (**P<0.05, ††P<0.01). C, control; NR, nutrient restricted; VDAC, voltage-dependent anion channel.
and mitochondrial protein abundance is similarly unaffected. Taken together, our findings suggest that maintained mitochondrial protein abundance during early kidney development could be one factor that contributes to protecting the kidney from the adverse structural effects of later obesity in previously nutrient-restricted offspring (Williams et al. 2007).

The liver

In the liver, the exponential increase in VDAC abundance from 80 days of gestation up to soon after birth closely follows the increase in GR mRNA abundance over the same period (Whorwood et al. 2001). VDAC is located within the epithelial lining (Yakubu et al. 2007) and may have a role in regulating gluconeogenesis (Lemasters & Holmuhamedov 2006). The rise in both VDAC and GR through gestation to peak after birth would, thus, coincide with the onset of glucose production around the time of birth (Fowden et al. 1998), thereby promoting energy production by the liver. In contrast, cytochrome c abundance was not increased after birth. It is possible that in the liver, its role is related more to energy conversion rather than production (Cai et al. 1998). VDAC could have a critical role in ensuring glucose production that is maximised very soon after birth.

Immediately following maternal nutrient restriction targeted between early and mid-gestation, the abundance of VDAC and cytochrome c were both raised despite no change in GR mRNA. Interestingly, this coincides with the stage at which there is a transient increase in gene and protein expression of the prolactin receptor in the livers of nutrient-restricted fetuses (Hyatt et al. 2007b). The prolactin receptor is one endocrine factor that mediates the initial rise in UCP1 in brown adipose tissue of the fetus (Symonds et al. 1998, Symonds & Stephenson 1999). We therefore propose that a similar adaptation may occur in the liver with regard to the early developmental regulation of VDAC. The rise in expression of the GR that occurs with gestation (Whorwood et al. 2001) appears to overcome this adaptation to nutrient restriction. Consequently, near to term hepatic mitochondrial protein abundance subsequently increases irrespective of previous maternal food intake. The absence of any change in either mitochondrial protein abundance in the juvenile liver is therefore not unexpected when GR mRNA abundance was also similar between nutritional groups. It is of interest to note that these findings contrast with effect of nutrient restriction targeted between the time of conception up to 95 days of gestation when a persistent increase in both hepatic VDAC abundance and GR mRNA is seen in the nutrient-restricted offspring in adulthood (Hyatt et al. 2007a). At the same time, liver size is reduced. Taken together, these findings indicate that it is the duration as well as the timing of maternal nutrient restriction that determines the long-term outcome in the liver with respect to mitochondrial sensitivity to glucocorticoids.

The lung

The finding of markedly different developmental ontogenies for VDAC and cytochrome c in the lung is not unexpected (Mostyn et al. 2003) and reflects the very different functions of each protein within the lung (Yakubu et al. 2007). As such, VDAC is primarily located around the lung alveoli, whereas cytochrome c the bronchioles. In the sheep lung, development persists up until term, with the production of terminal air sacs that only become capable of effective gaseous exchange very near to term following exposure to high plasma cortisol coincident with maximal glucocorticoid action (Harding 1994). The increase in cytochrome c after birth within the bronchioles may, thus, be more related to the appreciable lung growth that occurs up to adolescence and concomitant increase in energy conversion within the lung (Harding 1994).

The peak in VDAC abundance within the lung coincides with the maximal glucocorticoid action that is enhanced following maternal nutrient restriction between early and mid-gestation (Gnanalingham et al. 2005c). This was accompanied by an increase in cytochrome c that could be indicative of both metabolic and structural adaptations within the lung. By 6 months of age, however, VDAC abundance was decreased in

<table>
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<th>Table 3 Effect of maternal nutrient restriction between 28 and 80 days of gestation on mitochondrial protein and glucocorticoid receptor (GR) mRNA abundance in the developing sheep lung.</th>
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<td>Sampling age</td>
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<td>Weight (g)</td>
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<td>GR mRNA (% of age matched C) +</td>
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</table>

Values are means with their standard errors and n=6 per group per time point. *Significant effect of NR (*P<0.05). C, control; NR, nutrient restricted; VDAC, voltage-dependent anion channel. + Adapted from Gnanalingham et al. (2005c).
offspring born to nutrient-restricted mothers despite a persistent increase in the expression of the GR. One interpretation of these contrasting responses is that the GR has very different influences on mitochondrial protein abundance within the lung between the perinatal and juvenile period coincident with the substantial decrease in plasma cortisol (Symonds et al. 1989) and loss of GR (Gnanalingham et al. 2005c). This is clearly in contrast to the relationship between the GR and UCP2 in which a persistent up-regulation is observed following previous nutrient restriction despite a decline in both GR and UCP2 with age (Gnanalingham et al. 2005c). It should be noted, however, that UCP2 is present on the inner mitochondrial membrane. It has yet to be determined whether UCP2 is co-located with VDAC in the lung which is clearly not the case for VDAC and cytochrome c (Yakubu et al. 2007). One possible explanation for the divergent responses between UCP2 and VDAC is that it is a compensatory response to prevent excess oxygen species production (Chevillotte et al. 2007) or the maintenance of proton leakage across the mitochondria, which surprisingly is unaffected by loss of UCP2 (Couplan et al. 2002).

In conclusion, we have shown the tissue-specific nature of mitochondrial protein development that is likely to reflect the pronounced differences in functional adaptation after birth. The divergence in mitochondrial response between tissues to maternal nutrient restriction early in pregnancy further reflects these differential ontogenies and may explain the very different longer term outcomes between tissues.

Materials and Methods

Ontogeny of kidney, liver and lung development

For the ontogeny study, a mixture of Welsh Mountain and Border Leicester cross Swaledale sheep was used in order to enable us to study a greater number of time points during development. We have previously established that, with respect to the molecular measurements made in the present study, there are no distinguishable differences between breeds at the same developmental age (Gnanalingham et al. 2005a, 2005c, Yakubu 2005). Kidneys, livers and lungs were sampled from fetuses at 80 and 140 days of gestation (term = 148 days), and sheep after birth at 1, 30, and 180 days (6 months; n = 6 at each sampling point, 30 sheep in total), following euthanasia with an overdose of barbiturate (100 mg/kg pentobarbital sodium: Euthatal; RMB Animal Health, Dagenham, UK). All sheep were born normally at term to mothers that were fed 100% of their total metabolisable energy (ME) requirements (taking into account the requirements for both maternal maintenance and growth of the conceptus in order to produce a 4.5 kg lamb at term; Agricultural Research Council 1980). The tissues were rapidly dissected, weighed and placed in liquid nitrogen and stored at −80 °C until analysed.

Maternal nutritional manipulation of mitochondrial protein abundance

This study was designed to examine the effects of early to mid-gestational nutrient restriction, coinciding with the period of maximal placental growth, on the fetus and offspring and thus utilised singleton-bearing sheep. Thirty-six singleton-bearing Welsh Mountain sheep of similar age (median 3 years) and weight (36.1 ± 0.9 kg; mean ± S.E.M.) were entered into the study and individually housed at 28 days of gestation, as described by Bishpham et al. (2003). Animals were allocated to one of two nutritional groups using stratified randomisation by body weight. They consumed either 60% (i.e., NR) or 150% (i.e. fed to appetite) of their calculated ME requirements (Agricultural Research Council 1980) as determined from their daily food intakes. Food consumption between 28 and 80 days of gestation was 3.2–3.8 MJ/day of ME in the NR group (~60% of ME requirements) or 8.7–9.9 MJ/day of ME in the group fed to appetite (~150% of ME requirements). The amount of feed given to each ewe was increased at 43 and 61 days of gestation to meet the higher energy requirements associated with growth of the conceptus (Agricultural Research Council 1980). The diet comprised chopped hay with an estimated ME content of 7.91 MJ/kg dry matter and a crude protein content (nitrogen × 6.25) of 69 g/kg dry matter and barley-based concentrate with an estimated ME content of 11.6 MJ/kg dry matter and a crude protein content of 162 g/kg dry matter. The proportion of hay to concentrate fed to each animal was ~3:1 with respect to dry weight. All diets contained adequate minerals and vitamins. These were added separately to the diet with equal amounts provided to all sheep and, thus, were sufficient to fully meet their requirements. After 80 days of gestation, all sheep were offered sufficient feed to meet 100% of the ME requirements. They consumed between 6.5 and 7.5 MJ/day of ME, with the amount of feed provided being increased at 100 and 120 days of gestation to meet the increased ME requirements that accompany the increase in fetal weight with gestation. In those sheep allowed to go to term, all gave birth normally and the offspring were weaned at 3 months of age. Throughout lactation, all mothers were fed to requirements with hay provided ad libitum together with 1 kg concentrate.

In order to determine the effect of early to mid-gestational maternal nutrient restriction on fetal tissue development, six sheep within each nutrition group were randomised to tissue sampling at either 80 or 140 days of gestation. Each animal was humanely killed following i.v. administration of 200 mg/kg pentobarbital sodium. An umbilical vein blood sample was taken into a heparinised tube and the fetus was rapidly dissected, weighed and representative portions of each tissue placed in liquid nitrogen and stored at −80 °C until further analysis. The remaining offspring (n = 6 per nutritional group) were sampled at 180 days (6 months) after birth. In those animals sampled at 180 days of age, heparinised blood samples were taken through the day for plasma cortisol analysis (Gopalakrishnan et al. 2005).

All operative procedures and experimental protocols had the required Home Office approval as designated by the Animals (Scientific Procedures) Act (1986).


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**Laboratory analyses**

**Protein detection**

Mitochondria were prepared from ~1 g tissue and protein content determined by the Lowry method (Lowry et al. 1951). Western blotting was utilised to measure the abundance of VDAC and cytochrome c mitochondrial proteins (Mostyn et al. 2003). Identical amounts of protein were loaded (i.e. 10 μg) for each sample. Following electroblotting of the polyacrylamide gel onto a nitrocellulose membrane, Ponceau red staining was used to visually confirm that similar amounts of protein had been transferred before subjecting the membranes to immunodetection (Mostyn et al. 2003). Abundance of cytochrome c was determined using a commercial antibody (sc-7159; Santa Cruz Biotechnology, Santa Cruz, CA USA) at a dilution of 1 in 1000. VDAC abundance was determined using an antibody raised in rabbits to ovine VDAC1, purified from the kidney of a newborn sheep (Mostyn et al. 2003) at a dilution of 1 in 2000. Densitometric analysis was performed using AIDA software (AIDA version 2.0; raytest Isotopenmeßgeräte GmBH) on each membrane following image detection using a Fujifilm LAS-1000 cooled charge-coupled device (CCD) camera (Fuji Photo Film Co. Ltd, Tokyo, Japan). All values were expressed in densitometric units. Specificity of detection was confirmed using non-immune rabbit serum. All gels were run in duplicate and a reference sample for each tissue (from a control sheep sampled at 140 days of gestation) was included on each to allow comparison between gels. It was not possible to make protein measurements for the GR (type 2) due to the lack of availability of an antibody for use in ovine tissues (Hyatt et al. 2007a, 2007b).

**mRNA detection**

Total RNA was isolated from each tissue using Tri-Reagent (Sigma) and the expression of GR (type 2) determined by reverse transcriptase PCR (RT-PCR; Gnanalingham et al. 2005c) and real-time PCR, as described previously (Williams et al. 2007). The analysis used oligonucleotide primers to GR (type 2; Gnanalingham et al. 2005c), generating specific intron-spanning products. For RT-PCR, agarose gel electrophoresis and ethidium bromide staining confirmed the presence of both the product and ribosomal (r)18S and densitometric analysis was performed using a Fujifilm LAS-1000 cooled CCD camera. Consistency of lane loading for each sample was verified, and all results were expressed as a ratio of a reference sample to r18S abundance. For real-time PCR, standards were made using cDNA extracted from the kidney of adult sheep. All analyses were conducted in duplicate. This analysis was only conducted on kidney and liver samples taken from 6-month-old offspring as we have previously published mRNA results from each tissue with regard to samples taken at both 80 and 140 days of gestation (Whorwood et al. 2001, Gnanalingham et al. 2005c), together with the lungs sampled at 6 months of age (Gnanalingham et al. 2005c). These data have also been included to enable a concise comparison with age and between tissues.

**Hormone analysis**

Total cortisol was measured using a commercially available coated tube RIA kit (Coat-a-Count; Diagnostic Products Corp., Ltd, Caerarfon, UK) validated for use with ovine plasma (Bispham et al. 2003). The minimum detection limit for the assay was 0.5 ng/ml, and the intra- and inter-assay (n=5) coefficients of variation were 6 and 9% respectively.

**Statistical analysis**

All data are presented as means ± s.e.m. Statistical analysis with respect to significant differences (P<0.05) between values obtained from the different ages was determined by one-way ANOVA with post hoc Bonferroni correction and, between control and nutrient-restricted groups, by Mann–Whitney U test (SPSS v11.0; SPSS Inc., Chicago, IL, USA).

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