Different effects of maternal parity, cold exposure and nutrient restriction in late pregnancy on the abundance of mitochondrial proteins in the kidney, liver and lung of postnatal sheep

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Abstract

Adaptation to the extrauterine environment at birth relies upon the onset of postnatal function and increased metabolism in the lungs, liver and kidney, mediated partly by activation of mitochondrial proteins such as the voltage-dependent anion channel (VDAC), cytochrome *c* and, in the lung only, uncoupling protein (UCP)2. The magnitude of adaptation is dependent on the maternal metabolic and endocrine environment. We, therefore, examined the influence of maternal cold exposure (MCE) induced by winter shearing of pregnant sheep in conjunction with nutrient restriction (NR; 50% reduction in maternal food intake from 110 days gestation up to term). The effect of parity was also examined, as the offspring of nulliparous mothers are growth restricted compared with multiparous offspring. All sheep were twin bearing. One twin was sampled after birth and its sibling at 30 days. In the lung, both MCE and maternal nulliparity enhanced UCP2 abundance. However, whilst VDAC abundance was decreased in both the offspring of nulliparous mothers and by NR, it was transiently raised by MCE. Kidney VDAC abundance was reduced by MCE and nulliparity, adaptations only influenced by NR in multiparous mothers. Cytochrome *c* abundance was raised by MCE and by NR in multiparous controls and raised in offspring of nulliparous mothers. Liver VDAC and cytochrome *c* abundance were transiently reduced by MCE and persistently lower in offspring of nulliparous mothers. In conclusion, changes in the maternal metabolic environment have marked tissue-specific effects on mitochondrial protein abundance in the lungs, liver and kidney that may be important in enabling the newborn to effectively adapt to the extrauterine environment.

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Introduction

Postnatal survival of mammals is dependent on the newborn's ability to effectively adapt to the extrauterine environment. This process includes the establishment of independent breathing via the lungs as well as the ability to synthesise glucose in the liver and excrete metabolic waste products via the kidneys. Birth is, therefore, associated with a dramatic increase in metabolic activity of these tissues and concomitant up-regulation of mitochondrial protein abundance and function (Mostyn *et al.* 2004). A number of mitochondrial proteins are potentially involved in this process including uncoupling proteins (UCP) and the voltage-dependent anion channel (VDAC; Mostyn *et al.* 2003).

In the lung, the abundance of both UCP2 and VDAC increase around the time of birth, adaptations that are

important in enabling the lung to adapt to the rapid influx of air and increase in its metabolic activity (Mostyn *et al.* 2003, Gnanalingham *et al.* 2005c). UCP2 is a member of the inner mitochondrial membrane carrier subfamily and is highly abundant in a range of tissues (Pecqueur *et al.* 2001, Rousset *et al.* 2003). Its function remains a subject of intense debate (Stuart *et al.* 2001) and it has postulated roles in energy balance (Buemann *et al.* 2001), reactive oxygen species production (Negre-Salvayre *et al.* 1997), apoptosis (Voehringer *et al.* 2000), and macrophage-mediated immunity (Arsenijevic *et al.* 2000). UCP2 shares 56% homology with the brown adipose tissue-specific UCP1 (Erlanson-Albertsson 2003), whose abundance peaks at birth in the sheep and is then no longer detectable after 1 month of postnatal life (Clarke *et al.* 1997b). Both UCP2 mRNA and protein abundance are strongly influenced by the current and previous

maternal nutritional experience in adipose tissue and lung of sheep (Mostyn *et al.* 2003, Gnanalingham *et al.* 2005a, 2005c), an adaptation mediated in part by changes in local glucocorticoid action (Gnanalingham *et al.* 2005a).

Although the abundance of both UCP2 and VDAC in the postnatal lung and adipose tissue are nutritionally regulated (Mostyn *et al.* 2003); it is currently not known if comparable adaptations for VDAC occur in the kidney and liver, tissues that do not possess UCP2. It is also unknown whether mitochondrial protein abundance may be differentially regulated with respect to size at birth, as a result of changes in the maternal environment directly or from manipulating maternal food intake. In order to assess the relative contributions of size at birth and the maternal nutritional environment on the development of these mitochondrial proteins, the present study utilised the following two models of intrauterine growth manipulation, namely maternal parity and maternal cold exposure (MCE).

In contrast, to UCP2, VDAC is located in the outer mitochondrial membrane. It is an anion selective channel considered to be the main pathway for metabolite and ion diffusion (Colombini 1979) and may influence respiration by changes in the permeability of the outer mitochondrial membrane (McEnery *et al.* 1993). VDAC, along with other membrane proteins, is thought to be responsible for the release of cytochrome *c* from the inter-membrane space, a process that has been implicated in the chain of events involved in apoptosis (Crompton 1999, Zalk *et al.* 2005). The exact tissue-specific location of these two mitochondrial proteins has yet to be fully established and was a further aim of the present study.

In summary, our study was designed to determine, first, the effect of maternal parity plus nutrition and any potential interaction between these two variables on the expression and the abundance of mitochondrial VDAC, cytochrome *c* and/or UCP2 in the neonatal kidney, liver and lung. This was accompanied by a second study to investigate the effect of maternal chronic cold exposure induced by winter shearing during mid-pregnancy with nutrient restriction (NR) over the final month of gestation on the same mitochondrial proteins. Given that birth weight is lower in primiparous compared with multiparous mothers (Gardner *et al.* 2007) whereas it is increased by MCE (Symonds *et al.* 1992), it was hypothesised that maternal parity would attenuate the effects of NR on offspring mitochondrial protein abundance. In contrast, chronic MCE induced by winter shearing was hypothesised to overcome the negative effects of NR in late gestation on these mitochondrial proteins. Neonatal development, as determined by body size, organ weight and mitochondrial protein abundance was, therefore, examined at 1 and 30 days of postnatal life. This represents the period over which the newborn effectively adapts to the extrauterine environment and concomitantly establishes respiratory control, metabolic

response to feeding and waste removal (Symonds *et al.* 1989a, 1992) and during which the kidney, liver and lung all have critical roles (Mellor & Cockburn 1986).

Materials and Methods

Study A: effects of maternal parity and NR

Twenty-four (14 nulliparous and 10 multiparous), Border Leicester cross Swaledale sheep of similar body weight and condition score, and of known mating date, were entered into the study. In order to reduce the number of pregnant animals recruited into the study, and to minimise any potential confounding maternal influence on organ development, twin-bearing sheep were used. Following conception, animals were group-housed and fed daily a diet sufficient to fully meet their total metabolic requirements. All nulliparous sheep were 2 years old and had never been previously mated. The multiparous sheep were aged 3–4 years and had all experienced two previous successful pregnancies. This is important because there is little increase in birth weight after a second pregnancy (Gardner *et al.* 2007).

At 110 days gestation, i.e. 1 month prior to predicted date of birth, all animals were individually housed and randomly assigned to one of two nutrition groups. Six nulliparous and five multiparous mothers were allocated to the control (C) group, fed and consumed 100% of total metabolisable energy (ME) requirements for maternal body weight and stage of pregnancy, as described in detail by Mostyn *et al.* (2003). The remaining eight nulliparous and five multiparous mothers were nutrient-restricted, consuming 50% of total ME requirements until term. The diet comprised chopped hay and concentrate and was provided in a 3:1 weight ratio (Mostyn *et al.* 2003), with all animals having continual access to a mineral block and fresh water. At term, all offspring were delivered naturally with no intervention and birth weight recorded. One twin was randomly selected from each mother to be tissue sampled following humane euthanasia by an i.v. injection of barbiturate (200 mg/kg pentobarbital sodium: Euthatal: RMB Animal Health, Dagenham, UK) within 6 h of birth. All mothers were housed with their remaining offspring and fed a diet of hay *ad libitum*, together with a fixed amount of concentrate sufficient to fully meet her own metabolisable requirements plus that required to maintain lactation. The second twin was therefore reared with its mother and, thus, was raised as a singleton until 30 days postnatal age when it was similarly tissue sampled. Again, each animal was euthanized, their organs rapidly dissected, weighed, a representative portion of each placed in liquid nitrogen and stored at -80°C until further analysis. In the case of the liver, this comprised the same area of the right lobe from each animal. All procedures were performed with the

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

Study B: effects of MCE and NR

Thirty-three twin-bearing multiparous Bluefaced Leicester crossed Swaledale sheep of similar body weight and condition score and of known mating date were group housed indoors under natural day light conditions at mid gestation (68 ± 2 days) in December as previously described (Pearce *et al.* 2005). Fifteen were shorn (S; i.e. cold-exposed) at 70 days gestation and 18 remained unshorn (U). These animals were further divided into control (C) and NR groups, at 115 ± 2 days, resulting in four groups: unshorn control (UC $n=9$), unshorn nutrient-restricted (UNR $n=9$), shorn control (SC $n=8$) and shorn nutrient-restricted (SNR $n=7$). The C animals were fed straw *ad libitum* and a fixed amount of concentrate, which was calculated to sufficiently meet the ME requirements (in accordance to fetal number and stage of gestation), throughout the study. NR mothers again consumed 50% of this amount during the final 4 weeks of gestation (Agricultural Research Council 1980). After giving birth normally at term, all mothers and offspring were fed and sampled as described above for study A.

Study C: tissue-specific histological location of VDAC and cytochrome c

In order to establish the cellular location of both VDAC and cytochrome *c* within the kidney, liver and lung representative histological samples were taken from each tissue from both a late gestation fetus and an adult sheep ($n=4$). This enabled us to determine whether the relative distribution of each protein differs with development. All sheep used for this analysis were fed the same diet as described above and each animal consumed sufficient feed to fully meet their total metabolic energy requirements. For each tissue, serial sections were prepared from paraffin-embedded tissue samples and immunocytochemistry for each protein performed using the same antibodies as described below at antiserum at a range of dilutions from 1:100 to 1:1000 using the same techniques as described by Dandrea *et al.* (2001). Following incubation with enzyme conjugated second antibody and chromogen substrate, sections were examined by light microscopy. The specificity of the procedure was confirmed by the absence of binding when adjacent sections were incubated with rabbit serum from an unimmunised rabbit in place of rabbit anti-ovine primary antibody. It was not able to perform the same analyses with respect to the location of UCP2 in the lung due to the lack of available antibodies (Gnanalingham *et al.* 2005f).

Laboratory analyses

Protein detection

Mitochondria were prepared from 0.5 g frozen tissue as described previously (Budge *et al.* 2000) and the protein concentration of each mitochondrial preparation, as well as the initial tissue homogenate, determined (Lowry *et al.* 1951) and used to calculate total organ protein. Western blotting was carried out to measure the abundance of VDAC, cytochrome *c* and UCP2 in mitochondrial preparations. Ten micrograms of mitochondrial protein from each tissue was loaded onto a 12% SDS-polyacrylamide gel and subjected to electrophoresis. Proteins were then transferred to a nitrocellulose membrane by electroblotting. Densitometric analysis of Ponceau red staining of all membranes confirmed that equal amounts of protein were transferred before immunodetection (Mostyn *et al.* 2003). The abundance of VDAC abundance was determined using an antibody raised in rabbits to ovine VDAC (Mostyn *et al.* 2003) at a dilution of 1 in 2000. Abundance of cytochrome *c* was determined using a commercial antibody (Santa Cruz, CA, USA) at a dilution of 1:1000 whilst UCP2 was measured (Mostyn *et al.* 2003), using the same antibody as described by Pecqueur *et al.* (2001) raised against human UCP2, at a dilution of 1:10 000. Due to the very limited amount of this antibody available, it was only possible to determine UCP2 abundance in mitochondria prepared from lungs of 1-day old offspring. There are currently no other antibodies that are able to convincingly detect ovine UCP2 (Gnanalingham *et al.* 2005f). Specificity of detection was confirmed using non-immune rabbit serum. All gels were run in duplicate and included a range of molecular weight markers and an appropriate reference sample. For the liver and kidney, this reference was an ovine mitochondrial protein sample from a newborn sheep born to a multiparous well-fed mother whilst for UCP2, the peptide to which the antibody was raised against was used. Densitometric analysis for VDAC detection was performed on each gel following image detection using a Fujifilm LAS-1000 cooled CCD camera (Fuji Photo Film Co. Ltd, Tokyo, Japan) and all values were expressed in densitometric units.

Dry weight analysis

Lung dry weights were determined by freeze-drying a representative portion of each tissue.

Statistical analyses

Data were first subjected to a Kolmogorov–Smirnov normality test. As data were found to conform to a normal distribution, parametric statistical tests were performed. Separate statistical analysis with respect to the effects between the groups of parity and NR or cold exposure and

NR were carried out using a general linear model test (GLM). Mean organ weight and mitochondrial protein abundance were each analysed using a univariate GLM test to investigate the independent effect of parity (or cold exposure), late gestational NR and potential parity (or cold exposure) $\times$ diet interactions. A statistically significant difference was inferred by $P < 0.05$. All statistical analyses were performed using SPSS (Chicago, Illinois, USA) software package (version 11.0). Results are presented as means with their standard errors.

Results

Study A: effect of maternal parity and NR

Birth weight was reduced ($P < 0.001$) in the offspring of nulliparous mothers, irrespective of maternal NR (Nulliparous C: 4.26 ± 0.2 ; Nulliparous NR: 3.54 ± 0.1 ; Multiparous C: 4.73 ± 0.2 ; Multiparous NR: 4.51 ± 0.3 kg). By 1 month age, there was no difference in body weights between parity groups but offspring born to nutrient-restricted mothers weighed less ($P < 0.05$; Nulliparous C: 16.4 ± 1.4 ; Nulliparous NR: 14.3 ± 0.7 ; Multiparous C: 17.2 ± 1.0 ; Multiparous NR: 14.9 ± 0.4 kg).

The lung

At 30, but not 1 day of age, lung fresh and dry and relative weights, were lower in offspring of nulliparous mothers and mitochondrial protein content was raised, whereas lung protein content was lower at 1 and 30 days of age (Tables 1 and 2). Irrespective of parity, maternal NR caused a significant decrease in lung fresh, but not relative, weight, at 1 but not 30 days of age. With respect to the abundance of individual mitochondrial proteins at 1 and 30 days of age, VDAC was significantly lower whilst cytochrome *c* abundance was raised in the lungs of offspring born to

control nulliparous mothers (Fig. 1). This latter adaptation was abolished by maternal NR, but only at 1 day of age. UCP2 abundance was also increased at 1 day of age in offspring of nulliparous mothers, irrespective of maternal food intake. Maternal NR caused a significant decrease in the abundance of VDAC, but not UCP2 (Fig. 1), at 1 day of age. Cytochrome *c* abundance was persistently reduced by maternal NR in offspring of nulliparous, but not multiparous mothers. By 30 days of age, therefore, cytochrome *c* abundance was modestly up-regulated by NR in offspring born to multiparous mothers. At this age, the abundance of VDAC was increased by maternal NR regardless of parity.

The kidney

At 1 day of age mitochondrial protein content was significantly lower in offspring of nulliparous mothers (Table 1). This was accompanied by a reduced total, but not relative, kidney weight although this was only significantly different between NR offspring. By 30 days of age, total and relative kidney weights were lower in offspring born to nulliparous mothers (Table 2), when protein but not mitochondrial protein content was lower in control, but not NR, offspring. The abundance of VDAC was significantly decreased, whilst cytochrome *c* was increased in kidneys of offspring from nulliparous compared with multiparous mothers at both 1 and 30 days of age (Fig. 2). This effect on cytochrome *c* was prevented by maternal NR which resulted in a persistent decreased abundance in the kidneys of the offspring of control-fed mothers. In contrast, maternal NR was accompanied by raised cytochrome *c* abundance in offspring of multiparous mothers at 1 day of age, compared with a decrease at 30 days. NR also caused a reduction in VDAC abundance in offspring of multiparous mothers at both 1 and 30 days of age.

Table 1 Effect of maternal parity and nutrient restriction on organ weight, total protein and mitochondrial protein contents on day 1 of postnatal life.

		Nulliparous		Multiparous	
		C (n=7)	NR (n=7)	C (n=5)	NR (n=5)
Lung	Fresh weight (g)	92 $\pm$ 2.0	76 $\pm$ 2.3*	102 $\pm$ 10.1	83 $\pm$ 9.6*
	Lung:bodyweight ratio	20.6 $\pm$ 3.9	18.7 $\pm$ 3.0	20.1 $\pm$ 4.7	17.8 $\pm$ 3.7
	Dry weight (g)	19.5 $\pm$ 0.6	16.1 $\pm$ 0.6	18.0 $\pm$ 1.5	16.9 $\pm$ 1.2
	Total protein (g)	8.1 $\pm$ 0.8 ^a	6.9 $\pm$ 0.5 ^a	13.0 $\pm$ 1.4 ^b	10.4 $\pm$ 1.2 ^b
	Mitochondrial protein (g)	0.91 $\pm$ 0.36	0.65 $\pm$ 0.11	1.08 $\pm$ 0.12	0.86 $\pm$ 0.08
Kidney	Weight (g)	11.8 $\pm$ 1.2	9.4 $\pm$ 0.6 ^a	13.7 $\pm$ 1.6	11.5 $\pm$ 0.6 ^{ab}
	Kidney:bodyweight ratio	2.8 $\pm$ 0.1	2.8 $\pm$ 0.1	2.9 $\pm$ 0.1	2.7 $\pm$ 0.1
	Total protein (g)	2.92 $\pm$ 0.9	2.97 $\pm$ 0.3	2.89 $\pm$ 0.2	2.29 $\pm$ 0.1
	Mitochondrial protein (g)	0.15 $\pm$ 0.01 ^a	0.16 $\pm$ 0.01 ^a	0.28 $\pm$ 0.02 ^b	0.24 $\pm$ 0.02 ^b
	Weight (g)	85 $\pm$ 7.7 ^a	73 $\pm$ 3.6 ^a	110 $\pm$ 11.3 ^b	92 $\pm$ 4.6 ^b
Liver	Liver:bodyweight ratio	19.7 $\pm$ 2.1	20.8 $\pm$ 1.8	24.1 $\pm$ 3.3	20.4 $\pm$ 2.7
	Total protein (g)	22.3 $\pm$ 2.6 ^a	20.8 $\pm$ 1.4 ^a	34.1 $\pm$ 4.8 ^b	28.1 $\pm$ 1.9 ^b
	Mitochondrial protein (g)	0.83 $\pm$ 0.1 ^a	0.95 $\pm$ 0.1 ^a	1.92 $\pm$ 0.4 ^b	1.52 $\pm$ 0.1 ^b

Organs were sampled from offspring born to nulliparous or multiparous sheep that consumed either 100% (controls, C (n=5–7)) or 50% (nutrient-restricted, NR (n=5–7)) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means $\pm$ S.E.M.; *denotes a significant effect of NR; whilst different superscript letters denote statistically significant differences between nulliparous and multiparous groups; $P < 0.05$.

Table 2 Effect of maternal parity and nutrient restriction on organ weight, total protein and mitochondrial protein contents on day 30 of postnatal life.

		Nulliparous		Multiparous	
		C (n=7)	NR (n=7)	C (n=5)	NR (n=5)
Lung	Fresh weight (g)	187±34.3 ^a	160±19.1 ^a	295±16.5 ^b	232±6.3 ^b
	Lung:bodyweight ratio	12.4±3.6 ^a	11.5±4.3 ^a	17.2±0.9 ^b	15.6±1.2 ^b
	Dry weight (g)	36.3±7.8 ^a	31.5±3.9 ^a	52.3±3.7 ^b	50.8±2.0 ^b
	Total protein (g)	19.3±1.2 ^a	18.2±1.1 ^a	37.4±2.0 ^b	28.1±1.3 ^b
	Mitochondrial protein (g)	1.25±0.1 ^a	0.88±0.2 ^a	0.59±0.1 ^b	0.64±0.1 ^b
Kidney	Weight (g)	31±2.2 ^a	29±1.2 ^a	45±1.7 ^b	36±1.2 ^b
	Kidney:bodyweight ratio	2.0±0.1 ^a	2.0±0.1 ^a	2.6±0.2 ^b	2.4±0.1 ^b
	Total protein (g)	8.9±0.7 ^a	9.1±0.2	11.0±0.4 ^b	9.1±0.5
	Mitochondrial protein (g)	1.78±0.1	1.82±0.1	2.12±0.2	1.77±0.1
	Weight (g)	260±8.3 ^a	265±10.6 ^a	410±20.7 ^b	369±14.7 ^b
Liver	Liver:bodyweight ratio	15.6±2.5 ^a	18.4±3.0 ^a	24.5±1.5 ^b	24.9±2.7 ^b
	Total protein (g)	84±8.9 ^a	79±4.0 ^a	120±8.3 ^b	109±4.8 ^b
	Mitochondrial protein (g)	4.0±0.25 ^a	4.2±0.14 ^a	6.6±0.50 ^b	5.4±0.66 ^b

Organs were sampled from offspring born to nulliparous or multiparous sheep that consumed either 100% (controls, C (n=5–7)) or 50% (nutrient-restricted, NR (n=5–7)) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means±S.E.M.; *denotes a significant effect of NR; whilst different superscript letters denote statistically significant differences between nulliparous and multiparous groups; $P<0.05$.

The liver

Irrespective of postnatal age, total liver weight, plus its protein and mitochondrial protein contents were all lower in offspring of nulliparous compared with multiparous offspring (Tables 1 and 2), and was accompanied with a significant decrease in VDAC and cytochrome *c* abundance (Fig. 3). Relative liver weights were only reduced in offspring of nulliparous mothers at 30 days of age. Maternal NR had no effect on liver weight or composition. In offspring born to nulliparous mothers, VDAC abundance was increased by NR at 1 but not 30 days of age. Cytochrome *c* abundance was decreased by NR in livers of multiparous offspring at 30 days of age only.

Study B: effect of MCE and NR

Birth weight was increased ($P<0.05$) by MCE, an effect blocked by NR (UC: 4.64 ± 0.29 ; UNR: 4.28 ± 0.23 ; SC: 5.51 ± 0.32 ; SNR: 4.06 ± 0.21 kg). By 1 month age there was no effect of either MCE or NR on whole body weights (UC: 16.0 ± 0.8 ; UNR: 14.8 ± 0.6 ; SC: 17.3 ± 0.7 ; SNR: 16.7 ± 0.7 kg).

The lung

Fresh and dry lung weights were unaffected by MCE but at 1 day of age only fresh weight was reduced in NR offspring in conjunction with a lower mitochondrial protein content (Tables 3 and 4). Total mitochondrial protein content was also decreased at 1 day of age in the lungs of offspring born to cold-exposed mothers, whereas the abundance of both UCP2 and VDAC were raised, although the latter effect was blocked by maternal NR (Fig. 4). Cytochrome *c* abundance was unaffected at this age. By 1 month of age, MCE and NR caused a significant increase in VDAC, whereas the rise in

cytochrome *c* abundance with MCE was not seen in NR offspring.

The kidney

At 1 day of age MCE had no effect on kidney weight but caused a significant increase in protein but not mitochondrial protein content (Table 3). This response was prevented by maternal NR. By 1 month of age both total and relative kidney weight, but not protein content was increased by MCE, a response not seen in nutrient-restricted offspring. Maternal NR resulted in a significant reduction in total protein content of the kidney at 1 day of age only. The abundance of VDAC was reduced in the kidneys of offspring born to cold-exposed mothers at 1 and 30 days of age (Fig. 5). At 1 day of age, only, kidneys of offspring born to NR mothers had a lower abundance of VDAC irrespective of MCE. The abundance of cytochrome *c* was increased in the kidneys of offspring born to cold-exposed mothers at 1 day of age. By 30 days of age, its abundance was lower in offspring born to cold-exposed mothers in which no effect of NR was seen. However, in controls, at this age previous maternal NR resulted in a lower cytochrome *c* abundance.

The liver

MCE had no effect on liver weight (or composition) that was reduced in NR offspring, in conjunction with a lower total protein and mitochondrial protein contents only at 1 day of age (Table 3). The abundance of both VDAC and cytochrome *c* were both significantly reduced in cold-exposed offspring at 1 day of age but in all groups were unaffected by maternal NR (Fig. 6). By 1 month of age, total (but not relative) liver weight was increased in the offspring of cold-exposed mothers (Table 4). At this age in offspring of mothers fed to requirements hepatic abundance of both VDAC and cytochrome *c* were

Figure 1 Effects of maternal parity and nutrient restriction during late gestation on the abundance of the mitochondrial proteins voltage-dependent anion channel (VDAC), cytochrome *c* and uncoupling protein (UCP)2 in the postnatal sheep lung. Mitochondria were prepared from lungs sampled from 1- or 30-day-old offspring born to nulliparous (N) and multiparous (M) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=5-7$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.05$.

significantly increased by MCE (Fig. 6). With respect to cytochrome *c*, the same adaptation was observed in offspring born to mothers that were cold-exposed and nutrient-restricted. The abundance of VDAC was also reduced by a combination of MCE and NR.

Figure 2 Effects of maternal parity and nutrient restriction during late gestation on the abundance of the mitochondrial proteins voltage-dependent anion channel (VDAC) and cytochrome *c* in the postnatal sheep kidney. Mitochondria were prepared from kidneys sampled from 1- or 30-day-old offspring born to nulliparous (N) and multiparous (M) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=5-7$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.05$.

Study C: tissue-specific histological location of VDAC and cytochrome *c*

There was no difference in the cellular location for either mitochondrial protein between the late gestational fetus and adult sheep so representative images for each tissue from the adult animal are only illustrated. In the kidney, both VDAC and cytochrome *c* were co-localised to the renal tubules (see example for cytochrome *c* in Fig. 7a). For the liver, these proteins were both co-localised within the epithelial lining (see example for cytochrome *c* in Fig. 7b). With respect to the lung, VDAC was localised within the alveoli, whereas cytochrome *c* was confined to the bronchioles (Fig. 7c).

Discussion

Our study reveals for the first time the pronounced effects that manipulating organ growth in the fetus as a

Figure 3 Effects of maternal parity and nutrient restriction during late gestation on the abundance of the mitochondrial proteins voltage-dependent anion channel (VDAC) and cytochrome *c* in the postnatal sheep liver. Mitochondria were prepared from livers sampled from 1- or 30-day-old offspring born to nulliparous (N) and multiparous (M) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=5-7$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.01$.

consequence of differences in the maternal environment, caused by either maternal parity or cold exposure, on the abundance of the mitochondrial proteins VDAC and cytochrome *c* in kidney, liver and lung and for UCP2 in the lung. Notably, both maternal parity and cold exposure had similar effects on mitochondrial protein abundance despite having opposite effects on birth weight. At the same time, reducing maternal food intake by half over the final month of gestation had only modest effects that were unrelated to the effects of maternal parity and cold exposure on individual organ weights.

The lung

It has recently been established that UCP2 mRNA and protein abundance both peak around the time of birth and may, therefore, have an important role in enabling the lung to effectively adapt to the rapid influx of cold air after commencing breathing (Gnanalingham *et al.*

2005c). An increased abundance of UCP2 in the lungs of offspring of nulliparous and cold-exposed mothers is suggestive of a positive adaptation that is likely to be mediated by changes in the maternal metabolic and hormonal environment through pregnancy. Interestingly, any adaptation of this kind was not dependent on maternal food intake *per se* as increased UCP2 was maintained in offspring born to NR mothers. Indeed, we have previously shown that maternal NR results in a persistent up-regulation of both UCP2 mRNA and protein (Mostyn *et al.* 2003, Gnanalingham *et al.* 2005c) although, in the present study, we were not able to determine protein abundance in lungs sampled from 30-day-old offspring due to insufficient antibody availability. The effect of MCE on UCP2 in the lung is the opposite of the response of the brown adipose tissue-specific UCP1, which has been shown to be down-regulated (Pearce *et al.* 2005).

Both thyroid hormones and glucocorticoids are known to have critical roles in promoting mitochondrial protein abundance in preparation for life after birth (Symonds 1995, Fowden *et al.* 1998) and are, therefore, likely to be important in mediating the observed adaptations in UCP2 (Gnanalingham *et al.* 2005d). Indeed, potential glucocorticoid and thyroid response elements have been identified in the promoter region of human UCP2 (Tu *et al.* 1999). It is known that plasma thyroid hormones, but not cortisol concentrations, are increased in newborn offspring of cold-exposed mothers (Symonds *et al.* 1992, 1995). However, cortisol appears to be more important than thyroid hormones in promoting UCP2 mRNA and VDAC abundance during fetal compromise (Gnanalingham *et al.* 2005e). In this regard, in the fetal, newborn and juvenile sheep lung glucocorticoid receptor and UCP2 mRNA abundance are positively correlated (Gnanalingham *et al.* 2005c). At the same time triiodothyronine administration up-regulates lung UCP2 mRNA abundance 6 h after birth in rat pups, whereas the anti-thyroid drug, propylthiouracil, partially blocks this effect, responses that may be mediated through changes in non-esterified fatty acids (Xiao *et al.* 2004). Clearly, further studies are needed to examine the relative roles of both cortisol and thyroid hormones in the newborn of species born with a mature hypothalamic–pituitary axis.

Increased UCP2 in the lungs from offspring born to both nulliparous and cold-exposed mothers was not accompanied by parallel changes in VDAC abundance. The localisation of UCP2 within the lung has yet to be established but we have shown that VDAC is localised within the alveoli. One interpretation of our finding that the lungs of offspring of nulliparous mothers exhibited persistently lower VDAC abundance is that this could be specifically related to differences in lung alveolarisation. All offspring, with the exception of those born to NR nulliparous mothers, exhibited the expected decrease in VDAC abundance within the lung with postnatal age

Table 3 Effect of maternal cold exposure and nutrient restriction on organ weight, total protein and mitochondrial protein on day 1 of postnatal life.

		Unshorn		Shorn	
		C (n=9)	NR (n=9)	C (n=7)	NR (n=7)
Lung	Fresh weight (g)	105±12.0	80±7.1*	91±3.3	74±4.0*
	Lung:bodyweight ratio	22.2±2.4	18.1±0.9	18.1±1.2	18.8±0.6
	Dry weight (g)	19.0±1.8	16.0±1.2	20.0±1.4	16.0±0.8
	Total protein (g)	13.70±1.5	10.00±1.0	11.63±0.7	8.54±0.5
	Mitochondrial protein (g)	1.13±0.12 ^a	0.82±0.06* ^a	0.80±0.10 ^b	0.67±0.05 ^b
Kidney	Weight (g)	14±1.0	12±0.7	16±1.1	11±0.5
	Kidney:bodyweight ratio	2.8±0.1	2.8±0.1	2.8±0.1	2.7±0.1
	Total protein (g)	2.9±0.2 ^a	2.4±0.1*	3.8±0.5 ^b	2.7±0.2*
	Mitochondrial protein (g)	0.29±0.02	0.25±0.02	0.35±0.03	0.25±0.01
	Weight (g)	120±10.3	86±5.7*	118±3.4	74±4.2*
Liver	Liver:bodyweight ratio	23.4±1.0	20.6±1.3*	23.1±0.9	20.1±1.0*
	Total protein (g)	37.1±5.2	25.7±1.5*	38.0±1.2	23.0±2.2*
	Mitochondrial protein (g)	2.09±0.4	1.58±0.1*	2.85±0.3	1.41±0.2*

Organs were sampled from offspring born to unshorn and shorn sheep that consumed either 100% (controls, C (n=7–9)) or 50% (nutrient-restricted, NR (n=7–9)) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means±s.e.m.; *denotes a significant effect of NR; whilst different superscript letters denote statistically significant differences between shorn and unshorn groups; *P*<0.05.

(Mostyn *et al.* 2003). Interestingly, the rate of loss of VDAC up to 30 days of age was delayed in NR offspring that could be indicative of an extended period of alveolarisation in these animals. These different adaptations were accompanied by the maintenance of cytochrome *c* in offspring born to nulliparous mothers, irrespective of the maternal diet. Cytochrome *c* was shown to be located within the bronchioles where it may have an important role with regard to energy conversion (Kirk & Strange 1998). The extent to which these adaptations may impact on lung function remains to be established but, as discussed below, could be relevant to the previously established effects of MCE on later breathing control (Symonds *et al.* 1993).

MCE caused an increase in VDAC abundance in the lung that was maintained up to 1 month of age and is coincident with the time at which the metabolic stimulus to breathing begins to decline (Andrews *et al.* 1989b). It

is associated with a decrease in breathing frequency and a concomitant rise in the recruitment of laryngeal braking (Symonds *et al.* 1993), the function of which is to extend expiratory time, thereby increasing the time for gaseous exchange within the lung (Andrews *et al.* 1989a) and potentially increasing energy requirements (Symonds *et al.* 1993). Differences in mitochondrial protein responses within the lungs over the period in which there are rapid changes in function are, therefore, not unexpected and are consistent with the very different endocrine regulation of UCP and VDAC during fetal and postnatal development (Gnanalingham *et al.* 2005f).

The kidney

In the kidney both VDAC and cytochrome *c* shared the same cellular localisation namely the tubules. These two

Table 4 Effect of maternal cold exposure and nutrient restriction on organ weight, total protein and mitochondrial protein on day 30 of postnatal life.

		Unshorn		Shorn	
		C (n=9)	NR (n=9)	C (n=7)	NR (n=7)
Lung	Fresh weight (g)	262±15.3	246±9.3	260±7.7	261±9.6
	Lung:bodyweight ratio	17.0±1.6	16.5±1.3	15.2±0.9	15.6±1.1
	Dry weight (g)	56±2.7	48±1.8	51±1.8	52±2.4
	Total protein (g)	33.2±1.9	29.8±1.0	31.6±1.3	33.5±2.3
	Mitochondrial protein (g)	0.53±0.10	0.68±0.10	0.75±0.12	0.69±0.07
Kidney	Weight (g)	42±1.7 ^a	39±1.7*	49±1.2 ^b	43±1.6*
	Kidney:bodyweight ratio	2.0±0.1 ^a	2.0±0.1	2.6±0.2 ^b	2.3±0.1
	Total protein (g)	10.3±0.3	9.9±0.5	11.1±0.5	10.3±0.6
	Mitochondrial protein (g)	0.82±0.10 ^a	1.02±0.10 ^a	1.18±0.04 ^b	1.15±0.10 ^b
	Weight (g)	373±22.7 ^a	366±11.7 ^a	437±25.5 ^b	414±21.7 ^b
Liver	Liver:bodyweight ratio	23.6±2.5	24.4±3.0	25.2±1.7	24.7±2.1
	Total protein (g)	109±7.3	108±5.1	122±5.5	115±5.6
	Mitochondrial protein (g)	6.0±0.84	5.4±0.70	7.1±0.52	5.4±0.54

Organs were sampled from offspring born to unshorn and shorn sheep that consumed either 100% (controls, C (n=7–9)) or 50% (nutrient-restricted, NR (n=7–9)) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means±s.e.m.; *denotes a significant effect of NR; whilst different superscript letters denote statistically significant differences between shorn and unshorn groups; *P*<0.05.

Figure 4 Effects of maternal cold exposure induced from mid-gestation and nutrient restriction during late gestation on the abundance of the mitochondrial proteins voltage-dependent anion channel (VDAC), cytochrome c and uncoupling protein (UCP)2 in the postnatal sheep lung. Mitochondria were prepared from lungs sampled from 1- or 30-day-old offspring born to unshorn (US) and shorn (S i.e. cold-exposed) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=7-9$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.05$.

proteins did, however, show different developmental responses in that a persistent down-regulation of VDAC occurred in offspring born to either nulliparous or cold-exposed mothers that was only accompanied with

Figure 5 Effects of maternal cold exposure induced from mid-gestation and nutrient restriction during late gestation on the abundance of voltage-dependent anion channel (VDAC) and cytochrome c in the postnatal sheep kidney. Mitochondria were prepared from kidneys sampled from 1- or 30-day-old offspring born to unshorn (US) and shorn (S i.e. cold-exposed) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=7-9$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.05$.

increased cytochrome c in the latter group. At the same time, VDAC abundance decreased with postnatal age in all groups. Decreased renal VDAC abundance could relate to the reduction in kidney metabolism that would be expected to accompany the fall in overall metabolic rate with increased age (Symonds *et al.* 1989b). Indeed, an explanation for a reduced metabolic rate in offspring of nulliparous compared with multiparous mothers would be an accelerated loss of UCP1 in their brown adipose tissue (Symonds *et al.* 2004), the major source of additional heat production at birth (Clarke *et al.* 1997a) and, therefore, likely to impact on metabolic rate over the first month of life (Symonds *et al.* 1989b).

The major hormones that regulate mitochondrial protein abundance in adipose tissue and the lung during postnatal development are those secreted from the adrenal and thyroid glands (Gnanalingham *et al.* 2005b, 2006), but their potential impact on mitochondrial development in the kidney has yet to be examined. It is known that maternal NR over the final

Figure 6 Effects of maternal cold-exposure induced from mid-gestation and nutrient restriction during late gestation on the abundance of voltage-dependent anion channel (VDAC) and cytochrome c in the postnatal sheep liver. Mitochondria were prepared from livers sampled from 1- or 30-day-old offspring born to unshorn (US) and shorn (S i.e. cold-exposed) sheep that consumed either 100% (controls, C) or 50% (nutrient-restricted, NR) of total metabolisable energy requirements to maintain maternal metabolism and fetal growth between 110 days gestation and term. Values are means with their standard errors; $n=7-9$ per group and the same superscript letters denote statistically significant differences between groups, $P<0.05$.

month of gestation transiently increases maternal, but not fetal, cortisol (Edwards & McMillen 2001), although neither maternal parity nor cold exposure have any effect on maternal cortisol (Symonds *et al.* 1986, 1988a). In contrast, thyroid hormones are raised in cold-exposed mothers (Symonds *et al.* 1988a) and decrease with parity (Tasende *et al.* 2002), differences that are maintained through lactation (Symonds *et al.* 1990). Hypothyroidism has been shown to delay kidney maturation after birth, resulting in decreased renal cortical Na^+/H^+ transporter activity and brush border membrane NHE-3 protein abundance (Baum *et al.* 1998), blunted maturational increase in both Na^+/H^+ and Cl^-/OH^- exchange activity (Shah *et al.* 2000), and lower rates of sodium-dependent proton secretion (Gupta *et al.* 2004). Differences in maternal and neonatal plasma thyroid hormone concentrations may, therefore, contribute to the maternal effects on mitochondrial protein abundance in the kidney we have found that appear to be confined to the tubular region.

(a) Kidney (x 400 magnification)

(b) Liver (x 200 magnification)

(c) Lung - Voltage dependent anion channel (x 400 magnification)

-Cytochrome c (x 200 magnification)

Figure 7 Representative images showing immunocytochemical detection of cytochrome c in the adult sheep (a) kidney and (b) liver and for voltage-dependent anion channel (VDAC) and cytochrome c in adult the (c) lung.

The liver

In the liver, both VDAC and cytochrome *c* shared the same cellular localisation namely the epithelial lining. Hepatic VDAC and cytochrome *c* abundance was down-regulated in offspring of both nulliparous and cold-exposed mothers, adaptations that persisted up to 1 month of age only in the offspring of nulliparous mothers. These adaptations were unrelated to liver mass and composition, or maternal diet and, in the case of VDAC, are consistent with the reduced renal abundance found in these offspring, suggesting a common endocrine regulation. The persistent decrease in hepatic VDAC and cytochrome *c* abundance of offspring born to nulliparous mothers may relate to a lower organ and/or whole body metabolic rate, as discussed above for the kidney. In addition, it may relate to hepatic gluconeogenic potential, which has an appreciable energy demand (Lemasters & Holmuhamedov 2006) and is raised in cold-exposed mothers (Symonds *et al.* 1988b). Furthermore, the transient decrease in hepatic VDAC and cytochrome *c* abundance in cold-exposed offspring may be due to accelerated loss of these proteins around the time of birth, as both were significantly greater than in controls by 1 month of age.

In conclusion, adaptations in the maternal metabolic environment that act to promote or restrict fetal growth from mid-gestation have pronounced effects on the expression and abundance of mitochondrial VDAC, cytochrome *c* in the kidney, liver and lung, and UCP2 in the lung of neonatal sheep. Interestingly, these adaptations are largely similar between offspring of nulliparous mothers and those born to mothers, who were chronically cold-exposed, suggesting that common mechanisms unrelated to whole body or organ growth are involved. Future studies aimed at elucidating the effects on the maternal and fetal thyroid hormone and cortisol axis under these different conditions would be predicted to further define the precise mechanisms involved. This may be important, particularly in infants whose growth is restricted or enhanced, as optimising mitochondrial function in the newborn may promote metabolic adaptation at birth.

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