Influence of Size at Birth on the Endocrine Profiles and Expression of Uncoupling Proteins in Subcutaneous Adipose Tissue, Lung and Muscle of Neonatal Pigs

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Running title: Size at Birth and Uncoupling Proteins

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Abstract

Epidemiological studies suggest that infants of low birth weight show poor neonatal growth and increased susceptibility to adult diseases such as diabetes and lung disease. UCP 2 and 3 have been implicated in the development of such diseases; pigs provide an ideal model to examine the influence of birth weight due to the natural variance in piglet weight within a litter. This study examined whether birth weight influences the expression of UCP2 and 3 in adipose tissue, skeletal muscle and lung.

Piglets from 11 litters were ranked according to birth weight and 3 from each litter assigned to small (SFD), normal (NFD) or large for dates (LFD) groups. Blood samples and morphometric measurements were taken over the first 14 days life and tissue samples taken on day 7 or 14. Plasma hormone and metabolite concentrations and the expression of UCP2 and 3 mRNA in adipose tissue, skeletal muscle and lung were measured.

UCP2 and 3 expression in adipose tissue were lower in the SFD compared to the LFD group on day 7. UCP3 expression in skeletal muscle was higher than that of adipose tissue. Lung UCP2 and skeletal muscle UCP3 mRNA expression were unaffected by size at birth. Regression analysis indicated that UCP3 expression was differentially associated with IGF-1, leptin and insulin. In conclusion, low birth weight is associated with tissue specific effects on UCP expression. It remains to be established if these subsequently contribute to pathological conditions such as diabetes.

Keywords: mitochondria, postnatal growth, metabolism
Introduction

Epidemiological studies have shown that low birth weight is associated with not only an increase in neonatal mortality, but also an increased risk of cardiovascular, lung and metabolic diseases in later life. These associations are independent of adult weight and lifestyle factors and have become known as the “developmental origins of health and disease” (3, 13, 42). A number of animal studies have been undertaken to complement the large-scale epidemiological trials with molecular evidence for the emergence of later disease such as ischaemic heart disease and non-insulin dependent diabetes melitus (NIDDM). These have included specific dietary restriction during pregnancy in large (6, 49) and small mammals (18) as well as investigating the effect of global undernutrition during pregnancy via carunclectomy (29).

Few studies to date have utilised the pig as a model to examine the effect of birth weight on the development of adult diseases despite the pig providing an excellent model for such investigations. There can be up to a 3 fold difference in body weight amongst litter mates in normally fed sows, thus providing a natural form of fetal growth retardation without the complication of different maternal phenotype. Previous investigations have demonstrated that pigs which have been designated small at birth exhibit significant alterations in the hypothalamic-pituitary axis, glucose tolerance and blood pressure (30-32), elements of which contribute to the development of adult diseases.

A number of factors may be responsible for the nutritional “programming” of organs, tissues and cells including growth factors, enzymes and membrane bound
receptors and mitochondrial uncoupling proteins (UCPs) (26). UCPs are a family of proteins present in the inner mitochondrial membrane and have a number of postulated functions in energy regulation (7). Pigs have recently been shown to express UCP2 and 3 in adipose tissue and skeletal muscle (12, 24).

UCP2 is highly expressed in the organs and cells of the immune system, such as the lung and spleen as well as adipose tissue, whereas UCP3 expression is limited to adipose tissue, heart and skeletal muscle (8). The possible roles of UCP2 and 3 include regulation of fatty acid metabolism, reactive oxygen species (ROS) production and energy expenditure (2, 47). Genetic linkage studies and investigations of polymorphisms have demonstrated alterations in UCP2 and 3 between obese and non-obese humans and roles in the development of diabetes and impaired fatty acid metabolism (1, 15, 41). NIDDM has been linked to a number of adipose tissue and muscle specific genes including UCP2 and 3 (11, 34, 41). However, further work is required to fully understand the roles and regulation of UCP2 and 3.

UCP2-knockout mice are phenotypically normal and exhibit a normal decline in body temperature when exposed to a cold challenge and an increase in body weight when fed a high fat diet (2). However, when infected with toxoplasma gondii they show a significant resistance to the infection, with 100% survival rates compared to 100% mortality rates in the wild type mice (2). This resistance is attributed to increased ROS production in macrophages. Therefore, elevated UCP2 expression may be detrimental in tissues of the immune system and could contribute to impaired lung function in adulthood.
Pilot studies (unpublished) have established that the lung, along with adipose tissue and the spleen, has the highest expression of UCP2 in the pig. The lung is the key immune organ during the first days of life. Given the key role of UCP2 in this system (27), it is possible that altered birth weight may influence the expression of UCP2 in the lung. The immediate postnatal period also corresponds with the transition in the inspired contents of the lung; this represents a time of potential damage from ROS – particularly in premature or intra-uterine growth restricted infants. UCP2 has a number of postulated roles in the control and production of ROS. Any alterations in UCP2 caused by abnormally low or high birth weight could potentially influence the handling and removal of ROS.

UCP3 knockout mice, like UCP2 knockouts, exhibit altered production of ROS (47). UCP3 may protect mitochondria from excess FFA accumulation. During situations when FFA delivery exceeds oxidation, e.g. during fasting, consumption of a high fat diet and exercise, UCP3 levels are raised (17, 38, 40). This has been proposed to allow export of excess FFAs from the mitochondrial matrix where they would otherwise accumulate, resulting in lipid peroxidation leading to mitochondrial, and ultimately, cellular damage (39). Reduced UCP3 expression may, therefore, predispose a tissue to build up FFAs and peroxidative damage.

A number of hormones and metabolites have been implicated in the control of UCPs. These include traditional regulators of metabolism such as the thyroid hormones (19), as well as more recently discovered hormones e.g. leptin (35, 36). However, it is not known if birth weight influences the relationships between these hormones and UCPs in the pig.
Given the postulated roles of UCP2 and 3, it is possible that disrupted expression of these proteins during development could lead to a) altered fatty acid metabolism and therefore ultimately diabetes and obesity and/or b) impaired response to infection via altered ROS regulation. The aim of the study was to investigate the effect of size at birth on the expression of UCPs in adipose tissue, skeletal muscle and lung in newborn piglets. It is hypothesised that piglets with low birth weight will be predisposed to a reduction in UCP2/3 expression in adipose tissue and muscle and increased UCP2 expression in the lung.
Materials and Methods

Animals

Eleven sows of similar body weight and parity were entered into the study. All sows were housed individually in a temperature controlled barn (24°C) and gave birth normally. On the first day of life, piglets were ranked according to birth weight within each litter. Three animals from each litter were assigned to small (SFD n=11), normal (NFD n=11) or large-for-date (LFD n=11) groups and randomly designated for tissue sampling on either day 7 (SFD n=5, NFD n=5, LFD n=5) or day 14 (SFD n=6, NFD n=6, LFD n=6) of postnatal age. Equal numbers of male and female piglets were distributed among groups. On days 0, 4, 7 and 14 of postnatal age, piglets were weighed, colonic temperature taken with an electronic thermometer and morphometric measurements made, including crown to rump length (CRL), girth and head circumference. On days 4, 7 and 14 a venous blood sample was also taken along with measurement of total body electrical conductivity (TOBEC). This is a non-invasive technique designed to estimate fat-free mass and thereby indirectly measures body fat in live animals (4). The model SA-3000 uses electronic circuitry, which drives an oscillating magnetic field to measure the conductivity of the animal. The conductive properties of body fat and lean mass are significantly different, so fat free mass can be quantified. A phantom scan performance test was conducted to verify that the TOBEC system was operating accurately before any measurements were taken. To do this, pigs were placed in an opaque polycarbonate tube with lid and readings were taken with the pig’s nose aligned next to the front end of the measuring coil. The piglets were removed from the chamber between triplicate readings and the individual subject’s TOBEC values were only accepted if the coefficient of variation (cv) of was less than 3%. TOBEC
values are computed using the following formula, which takes into account body size:

\[ \text{Lean mass} = \sqrt{\text{TOBEC} \times \text{CRL}} \]

Measurements such as TOBEC, temperature and blood sampling were carried out at the same time, 9-11am, each day and piglets were sampled post-feeding. It should be noted that as this study was designed to reflect “normal” neonatal development it would not have been possible to separate the piglets from their mothers or control feeding times/volumes at this stage of development.

On the assigned tissue sampling day, piglets were humanely euthanased with an overdose of barbiturate anaesthetic (200 mg kg\(^{-1}\) pentobarbital sodium: Euthatal: RMB Animal Health, UK). The tissues were rapidly dissected, weighed, placed in liquid nitrogen and stored at -80\(^\circ\)C until analysed. All operative procedures and experimental protocols had the required Home Office approval as designated by the Animals (Scientific Procedures) Act (1986).

**Laboratory Procedures**

Plasma concentrations of glucose (CV=5.3%), triglycerides (CV=6.9%) (Sigma Chemical, St. Louis, MO, U.S.A.) and FFA (CV=2.8%) (Wako NEFA-C, Alpha Labs) in plasma were determined enzymatically (37). Total plasma triiodothyronine (T\(_3\)), thyroxine (T\(_4\)), leptin and insulin concentrations were assessed by radioimmunoassay (ICN Pharmaceuticals, Basingstoke, UK and leptin, Linco Research Inc, USA). IGF-1 was measured using an ELISA kit from DRG International Inc, USA.
Total RNA was isolated from adipose tissue (subcutaneous), skeletal muscle (biceps femoris) and lung using Tri-Reagent (Sigma, Poole, UK) as described previously (23). To maximise sensitivity, a two-tube approach to reverse transcription (RT) was adopted, and the conditions used to generate first-strand cDNA were 70°C (5 min), 4°C (5 min), 25°C (5 min), 25°C (10 min), 42°C (1 h), 72°C (10 min), and 4°C (5 min). The RT reaction (final volume, 20 µl) contained: 1 µg total RNA, 5 × cDNA (first-strand) buffer (250 mM Tris-HCl, 40 mM MgCl₂, 150 mM KCl, 5 mM dithioerythritol pH 8.5), 2 mM dNTP’s, 1 × hexanucleotide mix, 10 units RNase inhibitor, and 10 units M-MLV reverse transcriptase. All of these commercially available products were purchased from Roche Diagnostics Ltd (Lewes, UK).

The expression of mRNA for UCP2, 3 and 18S were determined by utilising the following set of cDNA primers to the appropriate porcine gene e.g. UCP2 5’-CTTCTGCGGTTCCTCTGTGT-3’ and 5’-CATAGGTCACCAGCTCAGCA-3’ (Genbank GI4154206); UCP3 5’-GACGTGGTGAAGGTTCGATT-3’ 5’-CGAGTTCATGTACCGGGTCt-3’ (Genbank GI4928051). Intron-spanning products of 641 (UCP2) and 330 (UCP3) base pairs were generated to exclude amplification of genomic DNA. Quantum RNA alternate 18S primers (Ambion, Abingdon, UK) were also used to check for equal loading in order to normalise the samples. 18S was chosen as a “housekeeping” gene as the alternatives e.g. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin have been found to be sensitive to various experimental conditions (5, 33, 44). Mitochondrial markers such as cytochrome c have been used in previous studies investigating UCP abundance to “normalise” data, however, many of these markers are
profundely affected by experimental conditions such as nutrition and age (26) and
would be unsuitable for use in the present study. Briefly, the incubations conditions
were 94°C (2 min) 1 cycle; 94°C (30 s), 60.3°C (30 s), 72°C (1 min) 30 cycles, and
72°C (7 min) 1 cycle. The PCR reaction (final volume, 20 µl) contained 10 × PCR
buffer (100 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3), 500 µM dNTP’s,
1 mM of each UCP2 primer, 3.75 U Taq polymerase. Agarose gel electrophoresis
(2.0%) and ethidium bromide staining confirmed the presence of both UCP2, UCP3
and 18S products of the expected sizes. Cycle number and annealing temperature
were fully optimised for each primer pair and tissue prior to these studies. All gels
were run at least in duplicate with an internal standard run on all gels to facilitate
inter-gel comparisons, for example between adipose tissue and skeletal muscle.
Densitometric analysis was performed on each gel using Advanced image detection
analysis (Aida version 2.31) following image detection using a Fujifilm LAS-1000
cooled CCD camera (Fuji Photo Film Co. Ltd, Tokyo, Japan). Results, in arbitrary
units, are expressed as a ratio of an 18S rRNA internal control and internal
standard.

Statistical analyses

Power calculations using SamplePower 2 dictated that n=6 would have power of
92.4% to yield a statistically significant result. All statistical evaluations were
performed by using SPSS 10.0 for windows using a General Linear Model
procedure followed with Bonferroni Correction post hoc tests, correction for
repeated measures was included if appropriate. The Spearman Rho test was used to
investigate relationships between 2 variables. All values presented are means with
their standard errors.
Results

Growth and Morphology

On all days of the study, SFD piglets were lighter (P<0.05) than the LFD group but were only lighter than NFD piglets on days 0, 4 and 7 (Figure 1a). The weight ranges for the groups were SFD, 0.8-1.33kg; NFD, 1.24-1.75kg; LFD, 1.35-2.11kg. The SFD piglets were also colder on day 0 of postnatal age, with a colonic temperature approximately 1°C lower than that of LFD piglets (SFD, 37.5°C ± 0.35; NFD, 38.4°C ± 0.2; LFD, 38.4°C ± 0.19; P<0.05). Morphometric measurements indicated that SFD piglets had reduced CRL on days 0 and 4 (Figure 1b), girth on days 0, 4 and 14 (data not shown) and head circumferences on all days (data not shown). TOBEC measurements demonstrated that SFD piglets had lower fat free (i.e. lean) mass up to 7 days of age (Figure 1c). Relative lung weight was found to be significantly higher in the SFD piglets on day 7 only (Figure 1d).

UCP2 and 3 mRNA expression in adipose tissue, skeletal muscle and lung

UCP2 mRNA was reduced (P<0.05) in adipose tissue sampled from SFD piglets on day 7 (Figure 2a) compared to LFD piglets only. This trend was not sustained to day 14 of postnatal age when UCP2 was down-regulated in all groups. UCP3 expression followed a similar pattern to UCP2, with mRNA expression highest on day 7 in NFD piglets and lowest in the SFD piglets (Figure 2b). By day 14 of postnatal age, there was no longer a significant effect of birth weight. Neither UCP3 mRNA expression in skeletal muscle (e.g. day 7; SFD 277.3±74.9; NFD 320.8±35.8; LFD 374.0±34.9 UCP3 percentage of reference) or UCP2 mRNA expression in the lung (e.g. day 7; SFD 44.1±19.3; NFD 26.5±7.6; LFD 12.5±2.5 UCP2 percentage of reference) were significantly affected by size at birth. UCP3
was more than 3 fold higher in skeletal muscle when compared to adipose tissue (e.g. day 7; NFD adipose tissue 69.5±12.8; NFD muscle 320.8±35.8 (n=5); day 14 NFD adipose tissue 62.0±23.4; NFD muscle 292.6±23.4 (n=6) UCP3 percentage of reference) (assessed using the internal control sample) a finding previously reported in rodents (46).

_Plasma hormones and metabolites_

There were no significant differences between groups with respect to any of the hormones and metabolites measured (Table 1). However, a number of relationships were observed between UCPs and plasma hormones known to be involved in growth and metabolism i.e. leptin, IGF-1 and insulin (Table 2). Most notably, UCP3 mRNA expression in adipose tissue was found to be differentially associated with leptin between groups.
Discussion

We have demonstrated for the first time a pronounced association between size at birth and expression of UCPs in adipose tissue of neonatal pigs. These effects are not “global”, as differential responses were observed between tissues in responses to birth weight and there was no effect on skeletal muscle or lung.

Genetic linkage studies have implicated UCP2 and 3 in the development of NIDDM (9) and polymorphisms which impair the activity of the UCP3 gene are linked to altered insulin activity and diabetes (48). The SFD piglets in the present study exhibited lower UCP2 and 3 on day 7 in adipose tissue. Given that resting insulin concentrations are similar among birth weight groups this observation suggests that if insulin is acting via UCP2/3, the activity of insulin may be impaired due to the reduced expression of UCPs. A common polymorphism in UCP2 leads to increased UCP2 mRNA in human adipose tissue; the presence of this variant is associated with a reduced risk of obesity. The presence of this polymorphism in the pig and its allelic frequency in a low birth weight cohort is an intriguing possibility.

A postulated role of UCP3 is as an exporter of FFAs from the mitochondrial matrix (40). This suggests that the reduced UCP3 in adipose tissue may predispose the SFD piglets to impaired FFA metabolism. A build up of FFAs within the mitochondrial matrix can lead to cell damage as FFAs are prone to lipid peroxidation. Damage of this type could lead to functional and structural damage of lipid-metabolising enzymes of the mitochondria. This is the proposed mechanism responsible for obesity-induced diabetes secondary to lipid accumulation in non-fat tissues, as seen in obese Zucker rats (45). Although these changes were no longer
apparent at day 14 of postnatal age, the potential cellular damage may have already occurred, leaving the individual at risk of NIDDM. In fact, recent work has demonstrated that piglets of low birth weight are glucose-intolerant at 12 months of age (31). However, the mechanism causing this is currently unknown. The present study investigated only subcutaneous adipose tissue. However, it is possible that UCPs in other fat depots may be affected by size at birth, for example omental tissue. It should be noted however, that piglets are born with very little adipose tissue. Even by 2 weeks of age there is little omental or other fat depots present.

Clearly, these results would be strengthened with the inclusion of UCP2 protein data. However, only one antibody which has been fully validated as specific for UCP2 has been produced worldwide – and not on a large scale (28). We have previously published porcine UCP2 protein data using this antibody (25). However, the latest batch does not cross-react well with porcine UCP2 therefore we have been unable to include protein information.

In the mouse, UCP3 is not expressed until suckling is initiated, suggesting that UCP3 is not expressed until the ingestion of a fat-rich meal (10). Fasted newborn mice treated with Intralipid, but not glucose, exhibit a marked induction of UCP3 expression, demonstrating that UCP3 expression is induced by circulating fatty acids (10). The lower UCP3 expression observed in adipose tissue of SFD piglets may be due to these piglets having a diet with less fat than larger litter mates. It is well documented that large piglets have preferential access to anterior mammary glands which produce more milk of higher quality than posterior glands (20).
When subjected to *toxoplasma gondii*, UCP2 knockout mice had increased survival rates (2) suggesting that a lower UCP2 abundance is preferential for survival. The increased survival in knockout mice was linked to an increased bactericidal activity of macrophages via ROS production. Our results demonstrated no effect of size at birth on UCP2 mRNA expression in the lung. A high UCP2 level in the lung might have predisposed piglets to reduced resistance to bacterial infection. However, our results suggest that the lung is protected against alterations in UCP2 expression, maintaining full immune protection for the young piglet.

Piglets with a low birth weight remained lighter than their normal and large litter mates and did not show any signs of “catch-up” growth during the first 2 weeks of life. In this respect, SFD piglets are more likely to be hypothermic, which increases morbidity and mortality (14) and impairs growth. Although the variation of birth weight within litters in the present study was only 1.7 fold (range: 0.8-2.1kg), this subtle reduction was sufficient to produce significant changes in UCPs. Studies have demonstrated that with advancing age there is a reduction in skeletal muscle mass, associated with increased risk of insulin resistance and NIDDM (43). As SFD piglets begin life with a reduced muscle mass this may predispose them to a more rapid onset of insulin resistance.

Birth weight did not influence any of the measured plasma metabolites or hormones i.e. FFA, glucose, T₃, although a number of relationships between these and UCP3 mRNA expression were observed. However, due to the low “n” values caution must be taken when interpreting the results. Of particular interest was the positive association between leptin and adipose tissue UCP3 mRNA expression in NFD.
piglets, an opposite result to the SFD and LFD groups. The hormone leptin signals satiety to the brain (21), which in turn promotes storage of lipids or activation of UCPs to oxidise excess lipid (22). A negative association between leptin and UCP3 suggests loss of the normal activation of UCP3 in LFD and SFD piglets, thus potentially leading to greater fat deposition. A similar differential association was observed between insulin and muscle UCP3 mRNA expression. An association between adipose tissue UCP3 mRNA expression and IGF-1 was observed only in the NFD group. IGF-1 can enhance UCP3 mRNA abundance at the transcriptional level (16) thereby contributing to IGF mediated protection from ROS, oxidative stress and apoptosis. Potential dysregulation in this feedback loop could present LFD and SFD piglets with an increased risk of cellular damage from oxidative stress.

In conclusion, we have shown for the first time an association between birth weight and UCP expression in the pig. The altered expression of UCP2 and 3 in adipose tissue may have deleterious effects on piglets with low birth weight. The implications of these results could potentially increase with age and contribute to later metabolic disease.
Acknowledgments

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Figure Titles

**Figure 1** Mean body weight (kg) (a), mean crown to rump length (CRL) (b), mean total body electrical conductivity (TOBEC) (c) and mean lung weight as a percentage of body weight (d) in SFD (□), NFD (■) and LFD piglets (■) over the first 14 days of postnatal age. Values are means with their standard errors. Mean values at the same postnatal age with unlike superscripts letters were significantly different (P<0.05). Mean values within the same group between different postnatal ages with an asterisk were significantly different (P<0.05).

**Figure 2** Mean uncoupling protein 2 mRNA expression in adipose tissue on day 7 and 14 of postnatal age (a) and mean uncoupling protein 3 mRNA expression in adipose tissue on day 7 and 14 of postnatal age (b) in SFD (□), NFD (■) and LFD piglets (■). Values are means with their standard errors. Mean values at the same postnatal age with unlike superscripts letters were significantly different (P<0.05). Representative image of day 7 adipose tissue UCP2/3 and 18S expression is shown.
Table 1

Hormone and metabolite values for small (S), normal (N) and large for dates (LFD) piglets ate 4, 7 and 14 days of age. Values are means with sem. IGF-1, insulin-like growth factor 1; NEFA, non-esterified fatty acids; TAG, triacylglycerol; T₃, triiodothyronine; T₄, thyroxine

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Table 2

Influence of size at birth on the relationships between insulin-like growth factor 1 (IGF-1) and insulin with adipose tissue and skeletal muscle UCP3 mRNA on day 14 of age.

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<th>X axis</th>
<th>R²</th>
<th>n</th>
<th>P value</th>
<th>Equation, y=</th>
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<td>Adipose tissue</td>
<td>Leptin</td>
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<td>5</td>
<td>0.05</td>
<td>-0.005x+2.9</td>
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<td>Leptin</td>
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<td>5</td>
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<td>Adipose tissue</td>
<td>Leptin</td>
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<td>6</td>
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<td>Adipose tissue</td>
<td>IGF-1</td>
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