Influence of size at birth on the endocrine profiles and expression of uncoupling proteins in subcutaneous adipose tissue, lung, and muscle of neonatal pigs

Alison Mostyn, Jennie C. Litten, Katharine S. Perkins, Philippa J. Euden, Anne M. Corson, Michael E. Symonds, and Lynne Clarke

1Centre for Reproduction and Early Life, Institute of Clinical Research, University Hospital, Nottingham; and 2Department of Agricultural Sciences, Imperial College London, Wye Campus, Ashford, Kent

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Epidemiological studies have shown that low birth weight is associated not only with 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 ischemic heart disease and noninsulin-dependent diabetes mellitus (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 used 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 threefold difference in body weight among litter mates in normally fed sows, thus providing a natural form of fetal growth retardation without the complication of a different maternal phenotype. Previous investigations have demonstrated that pigs that 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 UCP3 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 UCP3 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 UCP3 between obese and nonobese 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 UCP3 (11, 34, 41). However, further work is required to fully understand the roles and regulation of UCP2 and UCP3.

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 with 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 intrauterine 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 free fatty acid (FFA) accumulation. During situations when FFA delivery exceeds oxidation, for example, 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 cause 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, for example, leptin (35, 36). However, it is not known whether birth weight influences the relationships between these hormones and UCPS in the pig.

Given the postulated roles of UCP2 and UCP3, it is possible that disrupted expression of these proteins during development could lead to 1) altered fatty acid metabolism and therefore ultimately diabetes and obesity and/or 2) 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 hypothesized that piglets with low birth weight will be predisposed to a reduction in UCP2/UCP3 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 noninvasive technique designed to estimate fat-free mass and, thereby, indirectly measure 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) was less than 3%. TOBEC values are computed using the following formula, which takes into account body size: 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 postfeeding. 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 euthanized with an overdose of barbiturate anesthetic (200 mg/kg pentobarbital sodium; Euthatal: RMB Animal Health, UK). The tissues were rapidly dissected, weighed, placed in liquid nitrogen, and stored at −80°C until analyzed. 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) and FFA (CV = 2.8%) (Wako NEFA-C, Alpha Labs) in plasma were determined enzymatically (37). Total plasma triiodothyronine (T3), thyroxine (T4), leptin, and insulin concentrations were assessed by radioimmunoassay (ICN Pharmaceuticals, Basingstoke, UK) and leptin (Linco Research, St. Charles, MO). IGF-1 was measured using an ELISA kit from DRG International (Mountainside, NJ).

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 maximize 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 dNTPs, 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 (Lewes, UK).

The expression of mRNA for UCP2, UCP3, and 18S was determined by using the following set of cDNA primers to the appropriate porcine gene e.g., UCP2: 5’-cttcggctcctcttcgtg-3’ and 5’-cataggtcaccagctca-3’ (Genbank GI4154206); UCP3: 5’-gactgggtgaaagtcgggtaggtg-3’ 5’-caggctagtacgctgccgtct-3’ (Genbank GI4928051). Introns 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 to normalize the samples. 18S was chosen as a “housekeeping” gene, as the alternatives (e.g., 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.
RESULTS

Growth and morphology. On all days of the study, SFD piglets were lighter (P < 0.05) than the LFD group, but SFD were only lighter than NFD piglets on days 0, 4, and 7 (Fig. 1A). The weight ranges for the groups were SFD, 0.8 –1.33 kg; NFD, 1.24 –1.75 kg; and LFD, 1.35 –2.11 kg. The SFD piglets were also colder on day 0 of postnatal age, with a colonic temperature ~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 (Fig. 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 (Fig. 1C). Relative lung weight was found to be significantly higher in the SFD piglets on day 7 only (Fig. 1D).

UCP2 and UCP3 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 (Fig. 2A) compared with LFD piglets only. This trend was not sustained to day 14 of postnatal age when UCP2 was downregulated 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 (Fig. 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, 27.7 ± 4.9; NFD, 32.0 ± 35.8; LFD, 37.4 ± 34.9 UCP3 percentage of reference) nor 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 threefold higher in skeletal muscle compared with adipose tissue (e.g., day 7; NFD adipose tissue, 69.5 ± 12.8; NFD muscle, 320.8 ±
35.8 (n = 5) and 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, such as 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 UCP3 in the development of NIDDM (9), and polymorphisms that 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 UCP3 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 as a result of 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 buildup 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-metabolizing enzymes of the mitochondria. This is the proposed mechanism responsible for obesity-induced diabetes secondary to lipid accumulation in nonfat 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 mo 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 adipose tissue. It should be noted, however, that piglets are born with very little adipose tissue. Even by 2 wk 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 that 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
Influence of size at birth on the relationships between IGF-1 and insulin with adipose tissue demonstrated that with advancing age, there is a reduction in only 1.7 fold (range: 0.8 – 2.1 kg), this subtle reduction was SFD piglets are more likely to be hypothermic, which increases "catchup" growth during the first 2 wk of life. In this respect, normal and large litter mates and did not show any signs of immune protection for the young piglet.

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 metabolism or hormones, that is, FFA, glucose, T3, although a number of relationships between these and UCP3 mRNA expression were observed. However, because of 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 oxidize 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 UCP3 in adipose tissue may have deleterious effects on piglets with low birth weight. The

<table>
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<th>Group</th>
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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>0.05</td>
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<tr>
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<td>Adipose tissue</td>
<td>Leptin</td>
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<td>0.001</td>
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<tr>
<td>LFD</td>
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<td>6</td>
<td>0.05</td>
<td>-150.3x + 487.4</td>
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<tr>
<td>NFD</td>
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<td>IGF-1</td>
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<td>6</td>
<td>0.001</td>
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<tr>
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Table 2. Influence of size at birth on the relationships between IGF-1 and insulin with adipose tissue and skeletal muscle UCP3 mRNA on day 14 of age
implications of these results could potentially increase with age and contribute to later metabolic disease.

GRANTS
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REFERENCES


