Influence of birth weight on gene regulators of lipid metabolism and utilization in subcutaneous adipose tissue and skeletal muscle of neonatal pigs

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Abstract

Epidemiological studies suggest that low-birth weight infants show poor neonatal growth and increased susceptibility to metabolic syndrome, in particular, obesity and diabetes. Adipose tissue development is regulated by many genes, including members of the peroxisome proliferator-activated receptor (PPAR) and the fatty acid-binding protein (FABP) families. The aim of this study was to determine the influence of birth weight on key adipose and skeletal muscle tissue regulating genes. Piglets from 11 litters were ranked according to birth weight and 3 from each litter assigned to small, normal, or large-birth weight groups. Tissue samples were collected on day 7 or 14. Plasma metabolite concentrations and the expression of *PPARG2*, *PPARA*, *FABP3*, and *FABP4* genes were determined in subcutaneous adipose tissue and skeletal muscle. Adipocyte number and area were determined histologically. Expression of *FABP3* and *FABP4* was significantly reduced in small and large, compared with normal, piglets in adipose tissue on day 7 and in skeletal muscle on day 14. On day 7, *PPARA* and *PPARG2* were significantly reduced in adipose tissue from small and large piglets. Adipose tissue from small piglets contained more adipocytes than normal or large piglets. Birth weight had no effect on adipose tissue and skeletal muscle lipid content. Low-birth weight is associated with tissue-specific and time-dependent effects on lipid-regulating genes as well as morphological changes in adipose tissue. It remains to be seen whether these developmental changes alter an individual's susceptibility to metabolic syndrome.

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Introduction

Human epidemiological studies have demonstrated that suboptimal conditions *in utero*, for example resulting in a low-birth weight offspring, are associated with development of metabolic syndrome in later life (Barker 1998). The molecular and pathological features of a number of the components of metabolic syndrome, including obesity and type-2 diabetes, have therefore been explored through a range of both small and large animal studies (Poore & Fowden 2002, Gardner *et al.* 2005, Taylor *et al.* 2005). These studies have demonstrated that suboptimal conditions *in utero*, such as under- or overnutrition, which may lead to altered fetal growth, can 'reprogram' tissue-specific endocrine and physiological regulatory systems (Poore & Fowden 2004a, Gardner *et al.* 2005, Erhuma *et al.* 2007). As a consequence, the offspring are at increased risk of developing obesity, and subsequently, diabetes via dysregulation of adipose tissue distribution, and impaired

lipid metabolism. In the pig, low-birth weight is associated with catch-up growth and then increased fat mass, impaired glucose tolerance, and increased adrenal responsiveness to insulin at 1 year of age (Poore & Fowden 2002, 2004a, 2004b, Marten *et al.* 2009).

These findings support human studies indicating that low-birth weight followed by catch-up growth is associated with increased risk of developing obesity, diabetes, and hypertension in later life (Eriksson *et al.* 2003a, 2003b, Min *et al.* 2007). The molecular mechanisms behind these pathologies, however, remain to be established. There is also evidence to suggest that offspring born to mothers who have experienced over-feeding in pregnancy are at risk from metabolic disease in later life, although these studies are currently limited to feeding pregnant rats abnormally high quantities of lipid during pregnancy (i.e. 5 (corn oil) versus 25% (lard)) and the offspring are not heavier at 7 days of postnatal age (Khan *et al.* 2005, Taylor *et al.* 2005).

Obesity is the most common risk factor for development of metabolic syndrome, which is characterized by insulin resistance leading to increased plasma triglyceride and non-esterified fatty acid (NEFA) concentrations (Frayn 2002, Grundy 2004, Zimmet *et al.* 2005). Although the molecular pathways that link obesity with such a wide spectrum of metabolic and cardiovascular disease are not well defined, recent studies have focussed on the role of adipose tissue in the development of metabolic syndrome (Ferroni *et al.* 2004, Moller & Kaufman 2005) in which fatty acid binding protein (FABP)4 may have a central role (Hotamisligil *et al.* 1996, Scheja *et al.* 1999, Uysal *et al.* 2000, Furuhashi *et al.* 2007, Ordovas 2007).

Adipose tissue differentiation is regulated by a number of transcription factors, with the nuclear receptor peroxisome proliferator-activated receptor (PPAR)G considered to be the master regulator of adipogenesis and is involved in the regulation of fatty acid uptake and storage (Rosen *et al.* 1999). There are two distinct isoforms of PPARG, with PPARG2 expression limited to adipose tissue where it is ten times more active than PPARG1 in ligand-independent transcriptional activation (Werman *et al.* 1997), and its induction occurs very early in pre-adipocyte differentiation (Saladin *et al.* 1999). PPARG2 induction is followed by the expression of multiple genes and proteins including FABP4 that are involved in intracellular fatty acid transportation, cell growth and differentiation, cellular signaling, gene transcription, and modulation of enzyme activity (Heuckeroth *et al.* 1987, Amri *et al.* 1991, Zimmerman & Veerkamp 2002). The FABP with the widest tissue distribution is FABP3 (muscle FABP) that plays a critical role in the uptake and utilization of NEFA, in fuel selection and energy metabolism, and in adult pigs it is genetically linked to intramuscular fat content (Glatz *et al.* 1998, Binas *et al.* 1999, Gerbens *et al.* 2001, Luiken *et al.* 2003). Recent studies have suggested a more wide spread tissue distribution of FABPs (Gardan *et al.* 2007, Li *et al.* 2007) that may be linked to PPAR action (Motojima 2000, Tan *et al.* 2002).

We have previously shown that birth weight is one factor determining gene expression of uncoupling protein (UCP)2 and 3 in adipose tissue of piglets (Mostyn *et al.* 2005). The reduction in UCP expression observed in small piglets may be mediated in part by differential regulation of PPARs and FABPs gene expression, and ultimately may lead to a build up of fatty acids in of adipose tissue and cause lipotoxicity (Aubert *et al.* 1997, Schrauwen *et al.* 2001). The aim of the present study was therefore to investigate the influence of birth weight on expression of FABP3 and 4, PPARG1 and PPARG2 in adipose tissue and skeletal muscle during early neonatal development.

Results

Gene expression

FABP3

The abundance of *FABP3* mRNA in adipose tissue was lower in both small and large compared with normal sized piglets on day 7, but not day 14, of postnatal age (Fig. 1a). In skeletal muscle, when compared with normal-sized piglets, gene expression of *FABP3* was significantly reduced in both small and large offspring on day 14, but not day 7, of postnatal age when compared with normal offspring (Fig. 1b). An unexpected observation was that gene expression of the muscle specific *FABP3* was higher in adipose tissue compared with skeletal muscle. A significant downregulation of *FABP3* with age only occurred in skeletal muscle of small piglets.

FABP4

FABP4 mRNA abundance was lower in the adipose tissue of small and large compared with normal-sized offspring on day 7 only (Fig. 2a). In contrast, gene expression of *FABP4* in skeletal muscle only differed with birth weight at day 14, but not day 7, when its expression

Figure 1 (a) Mean fatty acid-binding protein (*FABP*)3 gene expression in subcutaneous adipose tissue on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. (b) Mean *FABP*3 gene expression in skeletal muscle on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. Values are means with their S.E.M. Significant differences ($P<0.05$) between birth weight groups are represented by different superscripts, significant differences between postnatal ages are represented by an asterisk.

Figure 2 (a) Mean fatty acid-binding protein (*FABP4*) gene expression in subcutaneous adipose tissue on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. (b) Mean *FABP4* gene expression in skeletal muscle on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. Values are means with their S.E.M. Significant differences ($P<0.05$) between birth weight groups are represented by different superscripts, significant differences between postnatal ages are represented by an asterisk.

in normal-sized offspring was greater compared with small and large piglets (Fig. 2b). There was a significant downregulation of *FABP4* in skeletal muscle of small piglets between days 7 and 14. The expression of *FABP4* mRNA was greater in adipose tissue compared with skeletal muscle.

PPARA and PPARG2

As observed with *FABP4*, gene expression of *PPARA* and *PPARG2* was lower in the adipose tissue of small and large size offspring on day 7, but not day 14, of postnatal life (Figs 3 and 4). There was a significant downregulation of *PPARA* mRNA abundance in adipose tissue of normal piglets between days 7 and 14. *PPARA* and *PPARG2* were below the level of detection in all skeletal muscle samples.

Tissue lipid content

There were no differences in adipose or skeletal muscle tissue lipid concentrations between small, normal, or large piglets on day 7 or 14 (e.g. adipose tissue day 7 small, 102.5 ± 55.4 ; normal 98.1 ± 21.5 and large 93.9 ± 15.7 mg/g). There was, however, a positive

relationship observed between the skeletal muscle lipid content and *FABP4* and *FABP3* in normal-sized piglets on day 14 (Table 1).

Plasma metabolites

We have previously shown that there were no differences in plasma metabolite concentrations between small, normal, or large piglets on days 7 or 14 (Mostyn *et al.* 2005). There was, however, a significant relationship between plasma NEFA concentrations and *FABP4* gene expression in skeletal muscle on day 14 (Fig. 5). In this regard, small and large piglets exhibited a negative relationship whereas it was positive in normal-sized piglets. No significant relationships were found between the abundance of any gene examined and individual growth rates (fractional or absolute) in any birth weight group.

Histological examination

Adipocyte area was not statistically different between groups (small, 2843 ± 231.1 ; normal, 3061 ± 285.0 ; large, $3362.5 \pm 466.5 \mu\text{m}^2$), but this difference may be biologically important. Adipocyte number was significantly higher in the small compared to the normal and large groups (Fig. 6) on day 7. There were significant relationships between adipocyte number and size and a number of the genes and metabolites of interest (Table 2). A negative relationship was observed in the small piglets only between adipocyte number and adipose tissue *FABP3* expression on day 7. Adipocyte area was negatively related to birth weight and plasma NEFA on day 7 in the normal piglets only.

Discussion

Our study has demonstrated that birth weight has a substantial effect on the primary genes involved in adipocyte development and lipid metabolism in both

Figure 3 Mean peroxisome proliferator-activated receptor (*PPAR*)A gene expression in subcutaneous adipose tissue on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. Values are means with their S.E.M. Significant differences ($P<0.05$) between birth weight groups are represented by different superscripts, significant differences between postnatal ages are represented by an asterisk.

Figure 4 Mean peroxisome proliferator-activated receptor (*PPAR*)*G* gene expression in subcutaneous adipose tissue on day 7 ($n=5$ per group) and 14 ($n=6$ per group) of postnatal age in small, normal, and large piglets. Values are means with their S.E.M. Significant differences ($P<0.05$) between birth weight groups are represented by different superscripts.

adipose tissue and skeletal muscle during the neonatal period. Interestingly, in our porcine model, a lower or higher than normal birth weight was found to affect the gene expression of *FABP3*, *FABP4*, *PPARA*, and *PPARG2*, which are likely to be linked to changes in lipid metabolism, although we have not measured this directly in the current study (Binas *et al.* 1999, Norris *et al.* 2003, Boord *et al.* 2004, Martin *et al.* 2007). Further work should include assessment of whether the changes in mRNA expression are translated to protein.

FABP3 and *FABP4*

FABP3 is traditionally considered to be a 'muscle' *FABP* (and commonly called heart *FABP*) as confirmed by studies in adult pigs (Li *et al.* 2007). Our findings in the neonatal pig suggest, however, that the expression profile is very different in early life and is dependent, in part, on birth weight. More recently, the presence of *FABP3* in subcutaneous adipose tissue and intramuscular adipocytes has been shown in adult pigs, supporting the proposal that *FABP* distribution is not tissue specific (Gardan *et al.* 2007). We have demonstrated that *FABP3* is expressed in adipose tissue at quantities tenfold greater than in skeletal muscle and high abundance is attained soon after birth irrespective of body weight, in keeping with Li *et al.* (2007) who demonstrated that adult levels were achieved by day 7 of postnatal age. *FABP3* in

adipose tissue decreases with age in small piglets only which may represent a developmental difference in these animals.

Decreased gene expression of *FABP3* and 4 in adipose tissue of small and large piglets on day 7 suggests that fatty acid metabolism is reset in these offspring. There were, however, no immediate differences in lipid content of adipose tissue or plasma NEFA or triglycerides between groups. Small piglets did, however, possess more adipocytes compared with their large siblings, although the extent to which this may determine later fat mass remains to be established (Spalding *et al.* 2008). It is possible that lower *FABP3* could be a beneficial adaptation as both gene ablation or partial reduction in *FABP3* protein is protective against the onset of symptoms of type-2 diabetes, at least in mice fed a very high fat diet (Binas *et al.* 1999, Shearer *et al.* 2005). Indeed, reduced gene expression of both *FABP3* and 4 may actually be protective against the development of metabolic syndrome as a number of recent studies suggest *FABP4* is central to its onset (Boord *et al.* 2004, Makowski & Hotamisligil 2004, Maeda *et al.* 2005). A protective effect in this case seems unlikely, given the well-described U- or J-shaped relationship between birth weight and obesity and metabolic syndrome (Martorell *et al.* 2001).

The differential relationships between plasma NEFA and *FABP4* expression in muscle that was dependent on size at birth suggests altered integration between plasma lipids and tissue *FABP4* in both small and large piglets. Coordination of *FABP4* and *PPARG* expression has previously been demonstrated (Tan *et al.* 2002), a relationship necessary to mediate the transcriptional activities of the *PPAR* ligand (for example NEFA) that is carried by the *FABP*. Despite this coordinated relationship, there were no differences in skeletal muscle triglyceride content between groups in the present study.

The unexpectedly high gene expression of *FABP3* in adipose tissue of neonatal pigs may be due to its extensive growth and differentiation as subcutaneous fat mass increases by 10–20-fold after birth (Mitchell *et al.* 2001), requiring a significant influx of lipid. *FABP3* is known to facilitate long-chain fatty acid uptake, oxidation, and esterification in myocytes (Binas & Erol 2007). Currently, there is little information regarding the

Table 1 Influence of postnatal age and relative size of birth on the relationship between fatty acid-binding protein (*FABP*) and peroxisome proliferator-activated receptor (*PPAR*) gene expression in subcutaneous adipose tissue or skeletal muscle with plasma non-esterified fatty acid (NEFA) concentration or triacylglyceride (TAG) content.

Age	Birth weight	Tissue	X-axis	Y-axis	R^2	Equation	n	P value
7	Large	SAT	<i>PPARG2</i>	<i>FABP4</i> SAT	0.848	$y=0.028x+3.18$	5	0.026
7	Small	SAT	<i>PPARG2</i>	NEFA	0.989	$y=0.023x+0.90$	4	0.005
7	Large	SAT	<i>FABP4</i>	TAG	0.791	$y=-0.016x+1.29$	5	0.041
14	Normal	Muscle	<i>FABP3</i>	Muscle TAG content	0.78	$y=0.03x+1.3$	6	0.02
14	Normal	Muscle	<i>FABP4</i>	Muscle TAG content	0.733	$y=0.28x-0.98$	5	0.037
14	Normal	SAT	<i>FABP4</i>	<i>PPARG2</i> SAT	0.764	$y=15.8x-25.7$	6	0.023

Figure 5 Relationship between plasma non-esterified fatty acid (NEFA) concentration and gene expression of *FABP4* in skeletal muscle at 14 days of postnatal age. Small, $R^2=0.97$ ($y=-0.17x+0.7$); normal, $R^2=0.91$ ($y=0.09x-0.41$); large, $R^2=0.16$ ($y=-0.07x-0.63$).

role of FABP3 in adipose tissue, but we hypothesize that NEFA metabolism has a central role during neonatal fat development. In contrast, changes in gene expression of *FABP3* and *FABP4* were not observed until day 14 in skeletal muscle when reduced expression was seen in all offspring that were not within the normal ranges of birth weight. The emergence of differences in *FABP3* and 4 in skeletal muscle at an older age compared to adipose tissue may reflect the fact that carbohydrate, rather than lipid, metabolism in skeletal muscle (and liver) is crucial during the first week of life in the pig due to the lack of lipid reserves at birth (Mersmann 1974, Mitchell *et al.* 2001). In the first 14 days of life, adipose tissue in the pig becomes unilocular with a substantial accumulation of triglycerides (Mersmann 1974), changes requiring co-operation with FABPs as well as PPARG. It is very unlikely that the presence of *FABP4* in skeletal muscle could be due to the accumulation of intramuscular adipocytes at this early stage of development.

PPARG2 and A

PPARG is considered as the master controller of adipogenesis, and a reduction in expression of PPARG2 in adipose tissue of the small and large piglets suggests delayed development. The histological data partly confirm this as the small but not the larger piglets have more adipocytes per visual field on day 7 (Fig. 6),

Figure 6 Mean adipocyte number in subcutaneous adipose tissue on day 7 ($n=5$ per group) of postnatal age in small, normal, and large piglets. Values are means with their S.E.M. Significant differences ($P<0.05$) between birth weight groups are represented by different superscripts.

yet these are of a similar size to normal and large piglets and possess similar triglyceride concentrations. This suggests that triglyceride is not being taken into the adipocytes of the small piglets at the same rate as normal and large piglets. In addition, PPARG2 plays a role in controlling adipose tissue expandability and increasing the capacity for buffering toxic lipids in a mouse knockout model (Medina-Gomez *et al.* 2007). Under conditions of positive energy balance, these mice exhibited severe insulin resistance and other features of metabolic syndrome (Medina-Gomez *et al.* 2007). While extrapolation to the pig is limited, one may speculate that reduced PPARG2 on day 7 may cause an increase in reactive lipid species and a reduced ability to buffer excess lipid. Such an adaptation would be further exacerbated by the reduction in expression of UCP2 and 3 in adipose tissue of these offspring (Mostyn *et al.* 2005). Offspring born to rats fed a low-protein, high-carbohydrate diet in pregnancy have reduced in PPARG in adipose tissue (Burdge *et al.* 2004), which is accompanied with impaired insulin sensitivity. These findings are in accord with glucose intolerance in low-birth weight offspring at 12 months of age (Poore & Fowden 2002). In the adult human, activation of PPARG by members of the thiazolidinedione (or glitazone) drug group sensitizes peripheral tissues to insulin by stimulating glucose and fatty acids uptake into adipocytes and increasing transcription of genes involved in insulin signaling. These functions may be impaired in both small and large offspring and fit in with epidemiological data indicating an increase risk of later metabolic disease in both small (Eriksson *et al.* 2003a, 2003b, Min *et al.* 2007) and large infants (Gillman *et al.* 2003, Leong *et al.* 2003, Danielzik *et al.* 2004).

PPARA has a well-documented role in hepatic lipid metabolism (Stienstra *et al.* 2007), but its role in adipose tissue and relationship to birth weight are less clear. Previous studies investigating the role of fetal programming in the female offspring of mice fed a high unsaturated fat diet during pregnancy have demonstrated increased PPARA protein in liver along with decreased hepatic triglyceride concentrations (Zhang *et al.* 2005). This is perceived as a potentially protective mechanism, although it should be noted that the high-fat diets adopted contain large quantities of saturated animal fats (Khan *et al.* 2005) and thus are not representative of the diet normally consumed by rodents. In the present study, the reduction in PPARA in low- and high-birth weight piglets on day 7 suggests that lipid oxidation is reduced. Despite tissue triglyceride being similar between groups, we have not assessed tissue lipids. PPARA is also a modulator of NEFA uptake into cells and upregulates the expression of key proteins that facilitate NEFA transport, although this is most pronounced in hepatocytes; however, clofibrate (a PPARA agonist) was found to upregulate fatty acid translocase and fatty acid transport protein in white adipose tissue of mice

Table 2 Influence of size at birth on the relationship between subcutaneous adipocyte number or area and fatty acid-binding protein (*FABP*) gene expression or plasma non-esterified fatty acid (NEFA) concentration.

Group	X-axis	Y-axis	R ²	Equation	n	P value
Small	Adipocyte number	<i>FABP3</i>	R ² =0.87	$y = -11.6x + 56.3$	4	0.001
Normal	Adipocyte area	Body weight day 0	R ² =0.74	$y = -0.0003x + 2.3$	5	0.037
Normal	Adipocyte area	NEFA day 7	R ² =0.97	$y = -0.001x + 5.7$	4	0.001

(Motojima *et al.* 1998). A reduction in lipid uptake into subcutaneous adipose tissue would be consistent with the reduced influence of PPARG2 on adipocyte maturation at this stage in the small and large piglets. The lack of *PPARA* mRNA expression in skeletal muscle may be related to the age of the piglets in this study. Evidence for the developmental ontogeny of *PPARA* in skeletal muscle of the pig is sparse; a small trial of two porcine breeds by Sundvold *et al.* demonstrated no expression of *PPARA* in muscle from 5-day-old pigs, with a slight increase up to 5 weeks and no further increase at 22–28 weeks (Sundvold & Lien 2001). Sebert *et al.* (2005) demonstrated an increase in muscle *PPARA* expression in mini-pigs between 10 and 16 months; however, this increase was restricted to the overfed group. The undetectable level of *PPARA* in skeletal muscle may provide the pigs with a greater circulating source of lipid during the neonatal period. Sundvold *et al.* also demonstrated a genotype effect with differential expression of *PPARA* in two porcine breeds. In contrast to the present study, *PPARA* was undetectable in adipose tissue expression; this could be a genotype effect (Duroc and Landrace pigs were used by Sundvold *et al.* (2001)) or due to northern blotting being insufficiently sensitive to detect *PPARA*.

In conclusion, we have shown that both a low- and high-birth weight can impact the development and potential function of both adipose tissue and skeletal muscle during the neonatal period. Further studies are now needed to investigate how long these alterations in gene expression persist and what impact they may have on disease susceptibility particularly when followed by catch-up growth that often occurs in small offspring (Ibanez *et al.* 2008).

Materials and Methods

Animals

Eleven large white sows of similar body weight (day 109; 274 ± 5.2) and parity (2.7 ± 0.3) were entered into the study and were all fed a standard commercial diet through pregnancy. All sows were housed individually in a temperature-controlled barn (24 °C ± 2) and gave birth normally at term. 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 (*n*=11), normal (*n*=11), or large (*n*=11) groups and randomly designated for tissue sampling on either day 7 (small, normal, and large; *n*=5 per group) or day 14 (small, normal, and large; *n*=6 per group) of postnatal age. The birth

weight ranges for the groups were small, 0.8–1.33 kg; normal, 1.24–1.75 kg; and large, 1.35–2.11 kg. The numbers of male and female piglets were evenly distributed among the groups. On days 0, 4, 7, and 14 of postnatal age, piglets were weighed. 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. All piglets from each litter, whether included in the study or not, remained with their mother for the duration of the experiment. On the assigned tissue sampling day, piglets were humanely killed with an overdose of barbiturate anaesthetic (200 mg/kg pentobarbital sodium; Euthatal: RMB Animal Health, Dagenham, UK). At this time, a representative sample of s.c. adipose tissue (the predominant fat store in the neonatal pig) together with skeletal muscle (biceps femoris) was rapidly dissected, 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

Preparation of mRNA

Total RNA was extracted from 50 to 100 mg of each tissue using Tri-reagent (Sigma). Following RNA extraction, 1 µg of each sample was reverse transcribed in a 20 µl reaction containing buffer (250 mM Tris-HCl, 40 mM MgCl₂, 150 mM KCl, and 5 mM dithioerythritol; pH 8.5), 2 mM deoxyribonucleotide triphosphates, 1 × hexanucleotide mix, 10 U RNase inhibitor, and 10 U RT (Roche Diagnostics) in a Touchgene Gradient thermocycler (Techne, Barloworld Scientific Ltd, Stone, UK). The conditions used to generate first-strand cDNA were 72 °C (5 min), 4 °C (2 min), 25 °C (5 min), 25 °C (10 min), 42 °C (60 min), and 72 °C (10 min). To ensure that there was no contaminating genomic DNA control, reactions without RT were included. RNA concentration and quality were verified by gel electrophoresis and spectrophotometrically using a standard conversion factor of 1 absorbance unit at 260 nm/40 mg RNA/ml. RNA concentration was adjusted by dilution with nuclease-free water (Ambion, Warrington, UK) before cDNA synthesis to a standard concentration of 1 µg/ml. All cDNA samples were stored at –20 °C until use.

Standard curve generation

Standards for *FABP3*, *FABP4*, *PPARA*, *PPARG2*, and for the housekeeping gene 18s ribosomal RNA were made from cDNA obtained from a randomly selected normal animal using semiquantitative PCR. The method used oligonucleotide primers to *FABP3*, *FABP4*, *PPARA*, *PPARG2*, and 18s genes

Table 3 Primer sequences and optimal PCR conditions used.

Gene	Primer sequence	Product size (bp)	Annealing temp (°C)	GenBank ID (source)
<i>FABP3</i>	Forward 5'-GCACTACGAGAAAGAGGCATGA-3' Reverse 5'-GCTGAGTCCAGGAGTAGCCAATT-3'	74	60	AJ416019 (<i>Sus scrofa</i>)
<i>FABP4</i>	Forward 5'-GGAAAGTCAAGAGCACCATAACCT-3' Reverse 5'-ATTCCACCACCAACTTATCATCTACTATT-3'	116	60	AJ416020 (<i>Sus scrofa</i>)
<i>PPARA</i>	Forward 5'-CGGAGAACCATTTCGGCTAAAGC-3' Reverse 5'-CGAGAGGCACTTGTGGAAACG-3'	110	60	DQ437887 (<i>Sus scrofa</i>)
<i>PPARG2</i>	Forward 5'-GAGATCACAGAGTATGCCAA Reverse 5'-CTTGGGCTCCATAAAGTCA	180	58	DQ437885 (<i>Sus scrofa</i>)
18s	Forward 5'-GATCGCGCGCGCTTATTCC-3' Reverse 5'-CTCCTGGTGGTGCCTTCC-3'	125	60	AB117609 (<i>Sus scrofa</i>)

FABP, fatty acid-binding protein; PPAR, peroxisome proliferator-activated receptor.

generating specific intron-spanning products (see Table 3). The PCR program comprised an initial denaturation stage (95 °C, 15 min), amplification (stage I, 94 °C (30 s); stage II, annealing (60 °C, 30 s); stage III, 72 °C (1 min), and final extension (72 °C, 7 min; 8 °C 'hold'). The PCR mixture (final volume 20 µl) contained 7 µl nuclease-free water (Ambion), 10 µl thermo-start PCR master mix (50 µl contains 1.25 U Thermo-Start DNA polymerase, 1×thermo-start reaction buffer, 1.5 mM MgCl₂, and 0.2 mM each of denatured adenotriphosphate, denatured 2'-deoxycytidine 5'-triphosphate, denatured 2'-deoxyguanosine 5'-triphosphate, and denatured 2'-deoxythymidine 5'-triphosphate (ABgene, Epsom, UK)), 1 µl forward primer, 1 µl reverse primer, and 1 µl RT (cDNA) product. The annealing temperature and cycle number of all primers were optimized and used in their linear range (Table 3). Agarose gel electrophoresis (2.0–2.5%) and ethidium bromide staining confirmed the presence of both the product and 18s at the expected sizes. All procedures were performed with appropriate negative and positive controls as well as a range of molecular weight markers (MBI Fermentas, York, UK).

The resultant PCR product was extracted (QIAquick gel extraction kit, Qiagen, cat no. 28704), sequenced, and results cross-referenced against the GenBank website to determine specificity of the target gene. After confirmation that the product was specific to the target gene, extracted PCR products were resuspended in nuclease-free water and a 10-fold serial dilution performed. Standards were stored at –20 °C until use in quantitative PCR.

Quantitative (real-time) PCR

Quantitative PCR was used to examine the expression of *FABP3*, *FABP4*, *PPARA*, *PPARG2*, and 18s (see Table 3). PCRs, set up in duplicate, were carried out in 20 µl volumes consisting of 1×SYBR PCR mastermix (with ROX passive reference dye; Qiagen), 500 nM forward primer, 500 nM reverse primer, and 7 µl nuclease-free water. Real-time PCR was performed in a Techne Quantica 14 real-time thermocycler (Techne, Barloworld Scientific Ltd) on all samples at 95 °C (15 min) followed by 45 cycles of 95 °C (15 s), annealing temperature (see Table 3; 25 s), 72 °C (25 s). Three negative control reactions were carried out with each set of samples analyzed: 1) no RNA template but RT and polymerase provided; 2) RNA and polymerase provided but no RT; and 3) RNA and RT provided but no polymerase. mRNA abundance

was worked out as a ratio of concentration to the housekeeping gene, 18s. Absolute values for concentration were determined by the use of the standard curve.

Histological analysis

Frozen adipose tissue blocks from day 7 animals were cut to 5 µm sections using a manual sledge microtome in a cryostat and the sections were stained with hematoxylin and eosin. Slides were viewed at 200 times magnification; three randomly selected visual fields were captured and adipocyte number and area (µm²) were assessed using Volocity software (Improvision, Coventry, UK). The assessor was blinded to study group.

Tissue lipid content

Adipose tissue and skeletal muscle lipid content were assessed using the Folch assay (Folch *et al.* 1957) followed by colorimetric analysis for triglyceride content using a commercially available kit (Randox, Crumlin, Northern Ireland). Briefly, 500 mg of tissue were homogenized in chloroform/methanol (1/1) to a final volume 20× the tissue sample. The homogenate was agitated for 15–20 min at room temperature then filtered to recover the liquid phase. The solvent was washed with 0.2 volumes of 0.9% NaCl solution and vortexed for a few seconds. The resulting mixture was centrifuged at 700 g for 10 min to separate the two phases and the upper phase was discarded. The lower chloroform phase was subjected to triglyceride analysis.

Plasma metabolites

Plasma concentrations of NEFA were assessed using commercially available kits as described previously (Mostyn *et al.* 2005).

Statistical analysis

Results for real-time PCR are expressed as arbitrary units as a ratio of concentration of gene of interest/18s. Tests of normality as determined by the Kolmogorov–Smirnov test revealed that the data were parametric, thus statistically significant differences between all groups were determined by one-way ANOVA with *post hoc* individual paired comparisons used to determine differences between individual groups as appropriate. Correlations were tested using Pearson's test for

parametric data; relationships between the genes measured, plasma metabolites and histological results were investigated. Results from the Pearson analysis were reported by group and sampling age. Statistical analysis was carried out using SPSS version 14 software (SPSS Inc., Chicago, IL, USA).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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