The effect of fetal pig size and stage of gestation on tissue fatty acid metabolism and profile

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Abstract

The fetus requires an adequate supply of fatty acids for optimum growth and development. It has been hypothesized that reduced activity of enzymes of fatty acid metabolism could contribute to inadequate fetal growth. In a porcine model of differential fetal growth we examined heart and liver fatty acid synthase, Δ5-desaturase and Δ6-desaturase gene expression and measured hepatic fatty acid profile to assess long-chain polyunsaturated fatty acid status. On gestation days 45, 65 and 100 sows were killed and tissues extracted from an average-sized fetus and the smallest fetus from each litter. As early as day 45, considerable hepatic Δ5- and Δ6-desaturase was detected, and this expression significantly increased as gestation progressed. In contrast, cardiac desaturase expression remained stable with time. Fatty acid synthase expression was greatest at day 65 in the liver, but was not expressed in the heart. Overall, the smallest fetus did not exhibit reduced tissue Δ5- or Δ6-desaturase expression or compromised polyunsaturated fatty acid status at any stage. In fact, small fetuses expressed more cardiac Δ5-desaturase than their average-sized siblings, possibly in response to a stress to the heart. It is clear from this study that fatty acid metabolism changes markedly as gestation progresses, and reduced fatty acid supply does not cause inadequate growth in this porcine model of fetal development.

Introduction

Fatty acids such as linoleic acid and α-linolenic acid and their longer-chain, less-saturated, derivatives such as arachidonic acid (AA), docosahexaenoic acid and eicosapentaenoic acid play a crucial role during fetal development. An inadequate supply of these long-chain polyunsaturated fatty acids (LCPUFAs) in neonates can result in serious developmental problems including reduced cognitive development, visual impairment and restricted growth (Uauy et al. 2000). These problems are thought to arise from the large variety of crucial biological roles these lipids play, and that may, therefore, be compromised during deficiency. For example, they form structural components of cells, they are vital constituents of the nervous system and brain, they are precursors of potent mediators of immunity and also have direct effects on gene expression (James et al. 2000, Nakamura et al. 2000). Reduced abundance of these fatty acids is associated with various problems; for example, the offspring of rats fed an AA-deficient diet had a reduced rate of growth and psychomotor development (Amusquivar et al. 2000). Fatty acid metabolism can also be compromised in conditions of reduced fetal growth. For example, rat pups that were growth-retarded as a consequence of restricting maternal protein intake had a reduced abundance of longer, less-saturated, fatty acids in their hepatic and muscle tissue (Loizou et al. 2001).

The concentrations of certain LCPUFAs in fetal plasma are higher than in the maternal circulation (Hornstra et al. 1989). One possible mechanism for this phenomenon is endogenous fetal desaturase activity (Chambaz et al. 1985, Salem et al. 1996, Su et al. 2001). The enzymes Δ5-desaturase (D5D) and Δ6-desaturase (D6D) modify fatty acid structure by introducing a double bond at the Δ5 and Δ6 positions of polyunsaturated fatty acids (PUFAs). These enzymes are responsible for the synthesis of highly unsaturated fatty acids, which are essential for normal fetal development.
Δ6 positions, respectively. This reaction limits the rate of de novo LCPUFA synthesis, with reduced desaturase activity causing lower tissue LCPUFA abundance in rats (De Tomas et al. 1980). D5D and D6D activity can be downregulated by glucocorticoids and by the abundance of their LCPUFA products (Marra et al. 1986, Cho et al. 1999a, 1999b). In vivo, hepatic D5D and D6D activity is downregulated in rats fed a protein-deficient diet (Mercuri et al. 1979). The fetus can synthesize saturated fatty acids de novo using fatty acid synthase (FAS), a glucocorticoid-sensitive, lipogenic enzyme that catalyses the synthesis of palmitic acid (C\(_{16:0}\)) (Lu et al. 2001). The products of FAS are important for fetal development, and any alteration in FAS expression may influence fetal growth or body composition. Relatively little is known about the ontogeny of these enzymes, particularly during early gestation.

We have chosen to use a pig model to investigate differential fetal growth for a number of reasons. As in humans, low-birthweight pigs suffer from various perinatal and long-term health problems, with piglet birthweight recognized as the major factor determining survival to, and mass at, weaning (Winters & Stewart 1947). The causes of differential growth have not been fully elucidated but using this approach, mechanisms relevant to human fetal growth have been studied. These have revealed altered fetal cortisol exposure, reduced ovarian development, altered muscle growth and altered amino acid transport mechanisms in the inadequately grown pig (Handel & Stickland 1987, Klemcke & Christenson 1997, Da Silva-Buttus et al. 2003, Finch et al. 2004). Exploiting the variability in fetal size within a litter allows us to compare inadequately grown fetuses with normally grown siblings. This has the advantage of removing the influence of confounding maternal factors, and allows us to investigate the growth critical processes of the fetus independently. Allo- metric analysis of fetal and organ weights found no consistent evidence of asymmetric organ growth in the smallest fetus (S E Thomson, G Zuur, C J Ashworth & H J McArdle, unpublished observations). Thus, this model allows us to examine the smallest fetus that is developmentally comparable, but of inadequate size.

In this study we investigated the ontogeny and effect of fetal size on D5D, D6D and FAS in porcine fetuses. We measured the gene expression of these enzymes in the hearts and livers of the smallest and an average-sized fetus at early, mid- and late gestation. Hepatic tissue fatty acid profile in the phospholipid fraction is sensitive to nutritional manipulation of dietary fatty acids and can be used as an index of fetal LCPUFA supply (Marin et al. 2003). We therefore used the fatty acid profile of hepatic tissue to assess the abundance of LCPUFAs as an index of fetal LCPUFA status.

Materials and Methods

Animals and sampling

On days 45 (n = 9), 65 (n = 9) and 100 (n = 7) of gestation multiparous Large White × Landrace sows were anaesthetized by inhalation of 8% halothane (Halothane BP; Rhone-Poulenc Chemicals, Bristol, UK) and medical oxygen (BOC gases, Manchester, UK). Euthanasia was performed by exsanguination under deep anaesthesia. The gravid uterus was removed aseptically and opened to expose each fetoplacental unit. All day 65 and 100 fetuses were killed by a 1 ml intra-cardiac injection of sodium pentobarbitone (Euthatal, 200 mg/ml; Rhone Merieux, Harlow, Essex, UK) and the smallest and an average-sized fetus identified by palpation. The mass and length of all fetuses were measured and used to identify the smallest (‘small’) and an average sized (‘average’) fetus on day 45. The livers and hearts of the small and average fetuses were removed, weighed and snap-frozen in liquid nitrogen before storage at −80°C. All experimental procedures were approved and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

RNA extraction and analysis by Northern blotting

Total RNA preparation

Total RNA was extracted from the liver and heart of the small and average fetus carried by six randomly selected sows at each stage of gestation. Frozen tissue was transferred directly to TRI reagent (Helena Biosciences, Sunderland, UK) and homogenized using 3× 20-s bursts with an Ultra Turrax homogenizer, model T25. RNA was prepared according to the manufacturer’s instructions. RNA concentration was quantified spectrophotometrically (OD\(_{260}\)).

20 μg total RNA was separated on a 1% agarose gel, transferred to a nylon membrane (Hybond; Amersham International) using an electrophoretic wet-transfer apparatus (model TE 62; Pharmacia Biotech), and cross-linked with a ultraviolet crosslinker (Ultraviolet Products, Upland, CA, USA).

Northern analysis

cDNA probes specific for porcine FAS (Mildner & Clarke 1991), human D6D (Cho et al. 1999b) and D5D (Cho et al. 1999a) were radiolabelled with [α-\(^{32}\)P]dCTP using the Ready-To-Go™ DNA-labelling kit (Amersham International). Membranes were pre-hybridized at 42°C for 30 min in UltraHyb (Ambion, Abingdon, Oxon, UK). Hybridizations were carried out overnight at 42°C and the membranes were washed at 42°C to a stringency of 0.1 x SSC (where 1 x SSC is 0.15 M NaCl/0.015 M sodium citrate) + 0.1% SDS. Radioactivity on the membranes was imaged using a wire proportional counter (Packard Instant Imager; Packard Biosciences, Pangbourn, Berks, UK). The mRNA was quantified as the amount of radioactivity hybridizing to the bands on the Northern
membrane. This was corrected for loading by re-probing the membrane by a similar method for 18S rRNA.

**Fatty acid analysis**

*Total lipid extraction*

Lipids were extracted from liver tissue of the small and average fetus from three randomly selected sows at each stage of gestation. The method used was an adaptation of that described by Slayback and colleagues (1977). Approximately 0.2 g frozen liver was homogenized in 2 ml 0.9% saline (w/v) using an Ultra Turrax homogenizer. The homogenate was transferred to a glass tube and 2 ml acetone was added. The sample was heated to boiling (85°C), 4 ml ethyl acetate added, and the sample reboiled. The 2:1:1 (by vol.) ethyl acetate/acetone/homogenate mixture was incubated at 65°C for 40 min and then centrifuged at 1800 g at 4°C for 15 min. The solvent (top) phase was transferred to glass tube and the solvent evaporated under N2. The lipid residue was stored under N2 in the dark.

*Lipid fractionation*

The extracted lipid was fractionated into the phospho-, glyco- and neutral lipids using solid-phase extraction. Silica-packed solid-phase-extraction columns were prepared by adding a slurry of silica/chloroform to plastic solid-phase-extraction columns and washing with excess chloroform. Samples were dissolved in 1 ml chloroform and loaded onto the columns. The neutral, glyco- and phospholipids were sequentially eluted with 10 ml chloroform, 15 ml acetone/methanol (9:1) and 10 ml methanol, respectively. The solvent from all fractions was evaporated under N2 and the lipid stored in the dark under N2.

*Derivatization of lipid fractions for analysis by gas chromatography*

The derivatization of the lipid fractions to their fatty acid methyl esters (FAMEs) was performed using a 1% sulphuric acid/methanol derivatization (Christie 1990). The sample was dissolved in 500 μl toluene and 1 ml 1% sulphuric acid/methanol added; then, the tube flushed with N2, sealed and incubated at 50°C for 1 h. 2.5 ml 5% saline were added and the sample mixed. A wash stage was performed by the addition of 1 ml isohexane to the mixture followed by brief centrifugation to allow the phases to separate. The upper sample layer was removed and transferred to a separate tube. This isohexane wash procedure was repeated and samples pooled. 1.5 ml 2% potassium hydrogen carbonate were added, the sample mixed and the top isohexane phase was transferred to a glass tube and the solvent evaporated to dryness under N2. The sample was dissolved in 200 μl isohexane/0.02% butylated hydroxytoluene and transferred to gas chromatography vials before analysis.

**Gas chromatography of lipid fractions**

For analysis by gas chromatography 1 μl of sample was injected using a refrigerated autosampler into a DB-23 capillary column (J & W Scientific, Folsom, CA, USA) of a Hewlett Packard HP 5890 gas chromatograph (Hewlett Packard, Manchester, UK). Injections were made in split mode with a 16:1 split. Separated samples were detected using flame ionization and the data plotted and integrated using a Hewlett Packard integrator. Peak identity was determined by comparison of sample chromatograms with that of a standard mixture of 37 fatty acids (Superco 37 component FAME mix).

**Calculation of fatty acid profiles**

The chromatograms of each sample were compared with that of the standard FAME mixture and peaks identified. The standard mixture was analysed and a response factor (RF) for each fatty acid (FA) calculated using the known proportion of that fatty acid in the standard mix, and the peak area detected:

\[
RF = \frac{\text{Peak area (standard)}}{\% \text{ of standard mixture}}
\]

For all samples each peak of fatty acid was subsequently identified and the fatty acid concentration calculated using the following formula:

\[
\text{Corrected FA concentration (Q)} = \frac{\text{Peak area (sample)}}{RF}
\]

The proportion of fatty acid was expressed as a percentage of the total detected in that sample:

\[
\text{FA concentration as } \% \text{ of total} = \frac{Q_{\text{FA}}}{\sum Q_{\text{all FA}}}
\]

**Statistical analysis**

The effect of stage of gestation, size designation (average or small) and any interaction on fetal and organ weights was analysed by two-way ANOVA. These data were not blocked for sow identity in order to statistically assess absolute weight differences. The relationship between litter size and small or mean fetal weights were assessed by Pearson two-tailed correlation. Significant correlations were detected if \( P < 0.05 \).

D5D, D6D and FAS gene expression, corrected for loading using expression of 18S rRNA, were analysed for effect of stage of gestation, fetal size and for any interaction between these factors by two-way ANOVA. Data were blocked for sow to account for the common maternal environment shared by the small and average siblings. If a significant difference was detected between fetal sizes across gestation the difference at individual stages was assessed. Groups were significantly different if their means differed by more than the least significant
difference at the 5% level. The effect of stage of gestation, fetal size and treatment interaction on fatty acid profile was statistically analysed by two-way ANOVA on the percentage (by mass) that each individual fatty acid contributed to the total identified fatty acid within each sample. Data were again blocked for sow to account for the common maternal environment shared by small and average fetuses. In cases where very small peaks were detected but not integrated, statistical analysis was not performed.

Results

Table 1 shows that at each stage studied, the fetus designated small was significantly lighter than the average fetus from that litter. There was a significant interaction between fetal size and stage of gestation due to the increasing difference between sibling weights as gestation progressed. At day 45 of gestation the small fetus was 84 ± 5% of the weight of their average sibling but by day 100 this had decreased to only 49 ± 5%. Table 2 demonstrates that the livers and hearts of the small fetuses were significantly smaller than those of average fetuses at all stages of gestation.

The effect of litter size on fetal weight was examined by correlating litter size with both average fetal weight of the litter and with the weight of the small fetus (Table 3). At day 45 litter size was not correlated with either mean fetal weight or the weight of the small fetus. At day 65 the weight of the small fetus was inversely correlated with the litter size; however, no correlation was detected with mean fetal weight. No significant correlation between litter size and fetal weight was detected at day 100.

Figures 1 and 2 demonstrate that hepatic D5D and D6D expression in the small fetuses were not significantly different from their average sibling. D5D and D6D increased through gestation with respective expression approximately 70% and 50% greater at day 100 of gestation than at day 45.

Figures 3 and 4 demonstrate that D5D or D6D expression in the heart did not change significantly with gestation. D6D expression did not differ between small and average fetuses but over gestation D5D expression was, on average, 15% higher in the small fetuses (P < 0.05).

Figure 5 shows that hepatic FAS gene expression changed significantly over gestation. Expression was lowest at day 45 and greatest at day 65. In contrast with desaturase ontogeny, peak FAS expression occurred earlier in gestation. Heart RNA blots were also probed using the FAS probe and no expression was detected.
The lipid profiles of the neutral and glycolipid fractions revealed relatively small quantities of the longer, more desaturated products of the desaturase enzymes. For example, mean AA abundance was 6.1 and 0.9% in the glyco- and neutral lipid fractions respectively, compared with 25.1% in the phospholipid fraction. Therefore, the fatty acid profile of the phospholipid fraction was used as the marker of fetal LCPUFA status. Table 4 details the abundances of fatty acids in the phospholipid fraction of hepatic tissue. Of the non-polyunsaturated fatty acids the proportion of C\textsubscript{16}:0 increased at day 100 of gestation. The abundance of LCPUFAs was not different between small and average-sized fetuses. AA was higher at day 65 than at days 45 and 100 ($P = 0.05$). This difference was, however, quantitatively small (26.5 versus 24.7 and 24.2% respectively). The increased desaturase expression at day 100 did not result in greater abundance of hepatic LCPUFAs.

**Discussion**

We have investigated how the steady-state gene expression of three key enzymes of fatty acid metabolism change through gestation and with fetal size and have
examined the abundance of both their products and substrates.

At all stages of gestation, the small fetus was significantly lighter than the average sibling. As gestation progressed the weight of the small fetus, relative to the average, decreased significantly, probably due to the cumulative effect of its lower growth trajectory. The negative correlation between litter size and small fetus weight at day 65 suggests that it is particularly sensitive to a litter-size-related factor at that stage. This factor could be reduced nutrient supply, as some data suggest that increased litter size decreases the availability of certain maternal nutrients (Pere 1997). The considerable level of hepatic desaturase expression at day 45 demonstrates that D5D and D6D may play an important role in LCPUFA supply at this early stage. Although it is known that human and rat mid-term fetal liver demonstrate desaturase activity in vitro (Rodriguez et al. 1998), we have demonstrated that after only 45 days of a ∼116 day gestation, the porcine liver expresses D5D and D6D at 59 and 65% of that at day 100, respectively. The factors that regulate the increasing hepatic D5D and D6D as gestation progresses are not yet known. Some in vitro data suggest that increased glucocorticoids can downregulate desaturase activity (Marra et al. 1986). We have reported that cortisol concentrations increase between days 65 and 100 and on day 100 the small fetus has elevated plasma cortisol (Ashworth et al. 2001). Hence, data from this study demonstrate that hepatic desaturase regulation is not primarily mediated by glucocorticoids in this situation. Cardiac desaturase expression remained unchanged through gestation, suggesting that cardiac LCPUFA demand remained stable.

Increased hepatic desaturase expression did not increase the abundance of LCPUFAs in the liver tissue, probably due to the export of synthesized fatty acids into the fetal circulation. In humans there is an especially high fetal demand for LCPUFAs during the final trimester coinciding with increased plasma LCPUFA concentrations (Al et al. 2000). The brain contains a high proportion of LCPUFAs, and grows most rapidly during late gestation, but has considerably lower D5D and D6D expression than the liver. (Swayne et al. 1976, Clandinin et al. 1980, Passingham 1985, Cho et al. 1999a). It is possible that the increased desaturase expression in the liver may support brain development by increasing fetal LCPUFA supply. The high expression of FAS at day 65 may provide the non-essential fatty acid supply required to support the rapid rate of tissue synthesis and cell hyperplasia during the exponential growth undergone by the fetus during mid gestation (Marrable 1971).

The lack of any hepatic or cardiac deficiency in FAS, D5D or D6D gene expression in the smallest fetus suggests that fatty acid supply did not limit its rate of growth at any stage. The adequate supply of LCPUFAs in the small fetuses is confirmed by their unchanged fatty acid status, as assessed by hepatic phospholipid fatty acid profile. Increased D5D expression in the hearts of the small fetuses was an intriguing observation given the positive correlation measured between organ LCPUFA content and fetal growth seen in some other studies (Burdge et al. 2003). It is important to consider, however, that our experimental model compares naturally occurring variations in fetal size, whereas many other studies induce growth retardation by nutritional intervention. Thus, in our model, upregulated cardiac D5D may be an adaptation to an earlier stress. Although the fetus may respond to a stress by organ-specific desaturase regulation this does not ameliorate either the reduction in growth or affect LCPUFA status.

In conclusion, these data provide the first prenatal examination of D5D, D6D and FAS at the gene-expression level and reveal that even during early pregnancy the fetus may have considerable capacity for the synthesis and modification of fatty acids. Furthermore, the use of this longitudinal model of differential fetal growth demonstrates that inadequate fetal growth is not caused by, or associated with, reduced fatty acid supply at any stage.

Table 4 Fatty acid profile of the phospholipid fraction expressed as a percentage of total detected and identified fatty acids. Trace amounts of C18:1 (n – 6), were detected but were below the threshold for quantification. A and S represent average and small fetuses. Data are means± S.E.M. from three samples.

<table>
<thead>
<tr>
<th>Stage of gestation...</th>
<th>Day 45</th>
<th>Day 65</th>
<th>Day 100</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal size...</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fatty acid</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>9.9 ± 0.28</td>
<td>10.0 ± 0.72</td>
<td>8.5 ± 0.05</td>
<td>9.9 ± 0.30</td>
</tr>
<tr>
<td>C18:0</td>
<td>25.3 ± 0.27</td>
<td>24.7 ± 0.88</td>
<td>26.3 ± 0.15</td>
<td>23.7 ± 0.09</td>
</tr>
<tr>
<td>C16:1</td>
<td>3.0 ± 0.28</td>
<td>3.1 ± 0.47</td>
<td>4.0 ± 0.27</td>
<td>4.6 ± 0.59</td>
</tr>
<tr>
<td>C18:2 (n – 9c)</td>
<td>10.1 ± 0.23</td>
<td>10.6 ± 1.06</td>
<td>9.8 ± 0.11</td>
<td>11.2 ± 0.35</td>
</tr>
<tr>
<td>C18:2 (n – 6c)</td>
<td>4.2 ± 0.34</td>
<td>3.9 ± 0.24</td>
<td>2.3 ± 0.73</td>
<td>3.0 ± 0.12</td>
</tr>
<tr>
<td>C20:2</td>
<td>0.9 ± 0.32</td>
<td>1.4 ± 0.52</td>
<td>0.9 ± 0.46</td>
<td>1.0 ± 0.49</td>
</tr>
<tr>
<td>C20:4 (n – 6)</td>
<td>24.8 ± 0.60</td>
<td>24.6 ± 0.86</td>
<td>27.9 ± 0.67</td>
<td>25.0 ± 0.49</td>
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<td>C22:6 (n – 3)</td>
<td>8.9 ± 0.75</td>
<td>10.1 ± 1.25</td>
<td>9.1 ± 0.36</td>
<td>9.10 ± 0.11</td>
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