Placental transport of leucine in a porcine model of low birth weight

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Abstract

Low birth weight is a major factor in neonatal morbidity and mortality in humans and domestic species and is a predictor of physiological disorders in adulthood. This study utilised the naturally occurring variation in pig fetal size within a uterus to test the hypothesis that placental amino acid transport capability is associated with fetal growth. Leucine uptake by trophoblast vesicles prepared from placentas supplying an average-sized fetus and the smallest fetus in the uterus was assessed. On days 45 and 65 of gestation, uptake of leucine by the porcine placenta was predominantly sodium independent and was inhibited by the non-metabolised leucine analogue 2-amino-2-norbornane-carboxylic acid, indicating that uptake occurs via system L. By day 100 the uptake of leucine by placentas supplying average-sized fetuses had changed from being predominantly sodium independent to involving both sodium-dependent (system B0) and -independent (system L) pathways. This change was not seen in placentas supplying the smallest fetus, which continued to display predominantly sodium-independent uptake. In conclusion, these data show gestational- and fetal size-dependent changes in the transport of leucine across the porcine placenta.

Introduction

In both humans and pigs, low birth weight is a major factor in neonatal morbidity and mortality. Furthermore, low birth weight has been linked to the development of endocrine and cardiac disease during adulthood in humans (Godfrey & Barker 2000), while in pigs birth weight is recognised as the major determinant of subsequent survival and total weaning weight of the litter (Winters et al. 1947). Attempts to alleviate the detrimental effects of low birth weight postnatally have achieved only limited success. Rather, data indicating that the in utero environment can programme fetal gene expression and metabolism suggest that strategies to promote post-natal well-being should focus on key stages of pre-natal development.

In order to gain understanding of the factors associated with differential fetal growth, this study compared the fetoplacental unit associated with the smallest pig fetus in the uterus with a normally sized littermate. This permits assessment of local factors associated with fetal size, free from the confounding effects of maternal husbandry, genetic background and nutrition. Such studies are important for both the pig industry, where low birth weight is associated with significant loss, and for the human condition, as the pig is recognised as a valuable model to study low birth weight infants (Cooper 1975). The capacity of the placenta to transport nutrients has a direct effect on fetal growth. We have demonstrated that placentas supplying the smallest fetus in the uterus are disproportionately lighter than those supplying average-sized fetuses in the same litter (Ashworth et al. 2001). These data, combined with the positive relationship between placental blood flow and fetal weight in the pig (Wootton et al. 1977), imply that there is a generalised reduction in the ability of placentas supplying small fetuses to deliver nutrients. In addition, there is increasing evidence in a variety of species, including rat, guinea pig, sheep and human, of an association between placental amino acid transport and fetal growth (reviewed in Sibley et al. 1997). Direct evidence for a role of placental amino acid transport in fetal growth comes from in vivo studies in sheep, where 13C-labelled leucine was infused during growth-retarded pregnancies and a reduction in maternal leucine flux into the placenta was observed (Ross et al. 1996). Further, abnormalities in amino acid transporter
function have been observed in vesicles prepared from the microvillus membrane of the human placental syncytiotrophoblast associated with low birth weight babies (Dicke & Henderson 1988, Mahendran et al. 1993, Glazier et al. 1997, Jansson et al. 1998, Norberg et al. 1998).

This study tested the hypothesis that the capacity of the placenta to transport amino acids differs between placentas supplying inadequately grown and normally grown fetuses. Leucine was selected as an exemplar amino acid for several reasons. First, it is taken up by both Na\(^+\)-dependent and Na\(^+\)-independent mechanisms (Christensen 1985). Secondly, it is an essential amino acid, and may be rate limiting for growth. Thirdly, day 100 fetal plasma leucine concentrations are lower in the smallest fetus of the litter compared with a normal-sized littermate (C J Ashworth and H J McArdle, unpublished observations).

**Materials and Methods**

**Experimental animals**

Eleven Large White × Landrace sows were used in these studies. On days 45 (n = 4), 65 (n = 4) or 100 (n = 3) following natural mating, sows were exsanguinated under deep anaesthesia which was induced and maintained by inhalation of 8% (v/v) halothane in oxygen (Halothane-M&B, Rhone Merieux Ltd, Harlow, Essex, UK) in a semi-closed system. The reproductive tract was removed, and all feto-placental units were excised in an aseptic manner. Fetal and placental weights were recorded. The lightest fetus and a littermate of average weight were identified and their placentas collected. All experimental procedures were approved by the appropriate ethical committee and conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

**Preparation of placental membrane vesicles**

Placental amino acid transporter function was investigated using an adaptation of a method used extensively to study uptake across the apical membrane of several species (McArdle et al. 1984, Dicke & Henderson 1988, Boyd 1991, Mahendran et al. 1993, Glazier et al. 1997, Jansson et al. 1998, Norberg et al. 1998). On homogenisation, the microvilli of the apical membrane form sealed vesicles that accumulate amino acids and other nutrients and reflect the transport characteristics of the placenta in situ.

Placental membrane vesicles were prepared from the freshly obtained fetal aspect of each placenta (consisting of trophoblast, connective tissue and allantoic layers) using an adaptation of previously published methods (McArdle et al. 1984). Placental tissue was placed in 150 mM NaCl, 5 mM Hepes (pH 7.4), chopped finely with scissors, washed twice in ice-cold 150 mM NaCl, 5 mM Hepes (pH 7.4), and then three times in 100 mM CaCl\(_2\), 5 mM Hepes (pH 7.4), followed by two washes in 250 mM sucrose, 20 mM Hepes (pH 7.4). The placental fragments were homogenised (13 000 r.p.m.) (30 s, 4 °C, ×3). The homogenate was centrifuged at 1500 g for 10 min, 15 000 g for 20 min and 105 000 g for 60 min. The pellet was discarded after the first two spins and the final pellet was resuspended in 10% sucrose and stored in aliquots at −70 °C. A sample of placenta was homogenised in 10% sucrose and kept for comparison with the vesicles. The purity of these membrane vesicle preparations was shown by enrichment of staining by the porcine trophoblast specific antibody SN1/38 (Whyte et al. 1984) on Western blots. Alkaline phosphatase is the standard measure of apical membrane enrichment. However, in early gestation it is not expressed in the pig placenta and in late pregnancy it only localises to discrete patches (Skolek-Winisch et al. 1984). Therefore, vesicles were identified immunologically using the antibody SN1/38.

**Immunoblotting**

Placental vesicles were prepared as described above. Whole placental homogenate was prepared by homogenisation of placental tissue (consisting of trophoblast, connective tissue and allantoic layers) in 10% sucrose solution at a 1:5 w/v ratio. Protein concentration was determined by the Bradford assay (BioRad, Hemel Hempstead, Herts, UK) using BSA as a standard. Crude homogenate and placental vesicle preparations were resolved by SDS/PAGE (10% gel) in the presence of 5% v/v beta-mercaptoethanol as a reducing agent, and electroblotted to a nitrocellulose membrane. The monoclonal antibody SN1/38 (a gift from A Whyte, Babraham Institute, Cambridge, UK) was used (1:100). Immunoreactive species were detected with horseradish peroxidase-conjugated anti-mouse antibody (1:5000) using the enhanced chemiluminescence detection system (ECL; Amersham).

**Immunohistochemistry**

Fixed placentas were processed into wax blocks and sections (thickness, 5 μm) were cut for immunohistochemistry. Treatment with 3% hydrogen peroxide (Sigma, UK) in water (25 °C) quenched endogenous peroxidase activity. The samples were pretreated with a non-immune serum block (1.5% normal horse serum) for 20 min, and incubated with the SN1/38 antibody at a 1:400 dilution (made up in 1% BSA) overnight at 4 °C. Samples were again pre-treated with a non-immune serum block (1.5% normal horse serum; Vector Laboratories, Bredon, Peterborough, UK) and incubated for 30 min at 25 °C with 0.005% biotinylated mouse IgG diluted in 1.5% normal horse serum. Sections were labelled with the avidin-biotin-peroxidase detection system (Vector Laboratories). Thereafter, sections were counterstained with haematoxylin, dehydrated, and cleared in xylene. A matching concentration of mouse IgG was used as a negative control. Appropriate controls were prepared, omitting either primary or secondary antibody as necessary.
Vesicle transporter studies

L-Leucine uptake by microvillous membrane vesicles was determined by a modification of a rapid-filtration technique as described previously (McArdle et al. 1984). Briefly, 25 μg vesicle protein were incubated with 4.5 mM L-[4,5-3H] leucine (Amersham Life Science) in a balanced salt solution with or without Na+*. The balanced salt solution consisted of 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 18 mM HEPES (pH 7.4) and 136 mM of either NaCl (Na+-buffer) or choline chloride (choline-buffer). In inhibition studies, unlabelled amino acids (2-amino-2-norbornane-carboxylic acid (BCH; Sigma), leucine or lysine) were added to the incubation buffer at the final concentrations indicated in the figure legends. BCH is a non-metabolised leucine analogue that is transported by system L (the transport system responsible for the sodium-independent transport of large neutral amino acids). The reaction was started by the addition of vesicles and stopped at 60 s by passing an aliquot (80 μl) through nitrocellulose filters (Whatman, Brentford, Middlesex, UK) followed by two washes of 1 ml ice-cold incubation buffer. The filters were dissolved in 5 ml scintillation fluid (Ultima Gold, Packard Instrument Company, Meriden, CT, USA) and radioactivity was measured in a Packard Beta counter. Appropriate blanks and standards were included as required.

Uptake, as opposed to binding, was measured by incubating vesicles in solutions of increasing osmolarity, achieved by the addition of mannitol, for 5 min by which time equilibrium was achieved (data not shown). Vesicle size (μL/mg protein) was calculated from the specific activity of the extra-vesicular medium.

Data analysis and expression of results

All uptake data were expressed as the difference between uptake at 39 °C and at 4 °C. The total and Na+-independent uptakes were defined as the uptake in the presence of sodium and choline respectively. Data were expressed as the uptake rate (fmol/s/mg vesicle protein) or total placental uptake capacity. The total placental uptake capacity (fmol/s/placenta), an approximation of uptake capacity across the whole placenta, was derived from the uptake rate (fmol/s/mg vesicle protein) × placenta protein concentration (mg protein/placenta). No difference in protein concentration was seen between placentas (or the vesicles derived from them) supplying the low weight fetuses in comparison with those supplying the average-sized fetuses. Hence, total placental uptake capacity is a function of amino acid uptake and placental mass. Significance was determined via ANOVA and a post hoc Newman Keuls test, with the exception of the amino acid inhibition data for which significance was determined via ANOVA and Dunnet’s multiple comparison test.

Results

Fetal and placental size

At each gestational age studied, the smallest fetus from each litter was significantly lighter than that of the average-sized fetus to which it was compared (Table 1). On day 100 the smallest fetus was 52 ± 3% the size of its average-sized littermate. Interestingly, the placentas supplying the smallest fetuses were only significantly smaller than their normal counterparts at day 45 (Table 1). The small fetuses also displayed an increased fetal/placental weight ratio at day 45 (P < 0.05) (Table 1).

Vesicle validation

The monoclonal antibody SN1/38 shows specificity for the trophoblast brush border of the pig placenta (Fig. 1A). The purity of the membrane vesicle preparations was shown by enrichment of staining by the porcine trophoblast specific antibody SN1/38 on Western blots (Fig. 1B). The relationship between leucine uptake and the inverse of the osmolarity was linear, with no uptake when the line was extrapolated to zero (infinite osmolarity) (Fig. 2). This indicated that the leucine was transported into a vesicle space rather than binding to the vesicle membrane. From these data the vesicles have an apparent size of 10 μL/mg protein, similar to that described for rat microvillar vesicles, but larger than human (McArdle et al. 1984).

Characterisation of leucine uptake in differential fetal growth

On day 45 of pregnancy, the calculated total uptake of leucine per placenta was decreased in placentas supplying the smallest fetus of each litter (Fig. 3). However on days 65 and 100 of pregnancy this difference in the total uptake capacity was no longer detected (Fig. 3).

To examine further the transport system/s involved in leucine transport by placentas supplying fetuses of differing

<table>
<thead>
<tr>
<th>Day</th>
<th>No.</th>
<th>Fetal weight (g)</th>
<th>Placental weight (g)</th>
<th>Fetal/placental weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>Small</td>
<td>Average</td>
</tr>
<tr>
<td>45</td>
<td>4</td>
<td>19.1 ± 0.9</td>
<td>15.9 ± 1.1*</td>
<td>76.1 ± 10.0</td>
</tr>
<tr>
<td>65</td>
<td>4</td>
<td>215.3 ± 13.0</td>
<td>170.0 ± 22.9*</td>
<td>186.1 ± 47.0</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>839.3 ± 40.2</td>
<td>431 ± 9.5*</td>
<td>206.2 ± 35.5</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with average.
sizes, we determined the sodium dependence of uptake and the inhibition of leucine uptake by other amino acids. At days 45 and 65 of pregnancy, in both the placentas supplying a normal-sized and the smallest fetus of each litter, the uptake of leucine was predominantly via a sodium-independent transport system (Fig. 4). The synthetic amino acid, BCH, inhibited this sodium-independent uptake of leucine (*P*, 0.05) (Fig. 5). In contrast, at day 100 of pregnancy leucine uptake by the placenta supplying the normal-sized fetuses was only 51 ± 12% sodium independent (Fig. 4). Both the sodium-dependent and -independent transport in the placentas supplying the average-sized fetus at day 100, was inhibited by BCH (*P* < 0.05) (Fig. 5). At day 100, in the placentas supplying the average-sized fetus of each litter, leucine but not lysine inhibited the uptake of 3H-leucine in the presence of sodium in a dose-dependent manner (0.1 mM leucine *P* < 0.05, 1 mM leucine *P* < 0.01) (Fig. 6). This change in the sodium dependence of leucine uptake was not observed in the placentas supplying the smallest fetus of each litter, which continued to display predominantly sodium-independent uptake inhibited by BCH (*P* < 0.05) (Figs 4 and 5).

**Discussion**

While there have been many studies of amino acid transport systems in the haemochorial placenta, those present in the epitheliochorial placenta of the pig remain characterised. In this study we identify two of the amino acid transport systems present in the trophoblast brush border of the pig placenta and establish the trophoblast membrane vesicle as an experimental system to investigate amino acid transport in epitheliochorial placenta.

Leucine transport across the trophoblast brush border of the placentas supplying the average sized fetuses, during early to mid gestation (day 45/65), is predominantly via system L, as demonstrated by the sodium-independent nature of uptake and its inhibition by BCH (Christensen et al., 1968). The particular system L...
transporter involved resembles the recently described LAT3 transporter (Babu et al. 2003). LAT3 mediates facilitated diffusion and does not require 4f2 for its function, unlike the obligatory exchangers LAT1 and LAT2 (Yanagida et al. 2001).

During late gestation, leucine uptake by the placenta supplying the average-sized fetus occurs by both sodium-independent and -dependent systems. The inhibition by BCH indicates that the sodium-independent component continues to be via system L. The sodium-dependent component can be identified as system B(0) also by its inhibition by BCH and its inability to be inhibited by lysine (Stevens et al. 1982). System B(0) (ASCT2) is present in late gestational rat placenta and in human placenta (Carbo et al. 1997, Knerr et al. 2003). In humans, ASCT2 is a dual function protein also acting as the receptor for syncytin. The expression of syncytin and ASCT2 are essential for the formation of the syncytiotrophoblast layer (Frendo et al. 2003). However, in the pig placenta, in which a cellular trophoblast is maintained, the physiological...
igermial growth factor (EGF) in amniotic fluid are
associated with IUGR (Varner et al. 1996) and the
expression of ASCT2 (system B0) has been shown to be
stimulated by EGF (Torres-Zamorano et al. 1997). This
may be the mechanism behind the lack of system B0 seen
in the placentas supplying the low weight fetuses.

In conclusion, this study has demonstrated that the
porcine placenta shows similar transport systems for neutral
amino acids as those of other species (Carbo et al. 1997,
Jansson et al. 1998) with the presence of system L, and
later in gestation, system B0 transport across the brush
border trophoblast membrane of the normal porcine pla-
centa. In placentas associated with small fetuses, system L
transport is present throughout pregnancy and system B0
is absent.

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