Reduced placental amino acid transport in response to maternal nutrient restriction in the baboon

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MATERNAL UNDERNUTRITION is a major public health concern world wide. Maternal undernutrition constitutes the most common cause of intrauterine growth restriction (IUGR) in developing countries, causing significant perinatal mortality and morbidity (6) and programming of adult disease (3). Babies who were in utero during the Dutch wartime famine in the winter of 1944-45 in Holland were growth restricted and have an increased incidence of obesity and metabolic and cardiovascular disease in adulthood (49), demonstrating that maternal undernutrition during gestation has profound effects on the health of the offspring. The mechanisms linking reduced maternal nutrition to IUGR and developmental programming of adult disease remain to be elucidated.

Experiments in animal models have demonstrated alterations in placental growth, structure and function in response to reduced maternal nutrient availability, resulting in adverse pregnancy outcomes (9, 18, 35). In Western societies, IUGR has been primarily related to placental insufficiency, or impaired uteroplacental vascular remodeling, while in developing countries maternal undernutrition remains the most common cause of restricted fetal growth. However, both perturbations appear to cause similar changes in placental signaling and function (18, 46, 48, 52). Investigating the effects of maternal undernutrition is, therefore, directly relevant also for restricted placental and fetal growth in association with placental insufficiency. Decreased expression and activity of key placental amino acid transporters has been reported in IUGR in humans (13, 22, 34, 38), as well as in animal models (18, 35). It has therefore, been proposed that changes in the expression and activity of placental nutrient transporters may precede the development of IUGR (18), and that the placenta acts as a nutrient sensor regulating the delivery of nutrients to the fetus in response to changes in the maternal nutrient supply line (21).

The system A amino acid transporter is encoded by three members of the slc38 family, and all three isoforms [sodium-coupled neutral amino acid transporter 1 (SNAT1), SNAT2, and SNAT4] are known to be expressed in the placenta (10). System A functions in a sodium-dependent manner to mediate the uptake of nonessential amino acids into the cell. System A activity establishes a high intracellular concentration of nonessential amino acids, such as glycine, creating a gradient that is used to drive the exchange for extracellular essential amino acids (EAs) via system L. System L activity is, therefore, necessary for the transport of both essential and nonessential amino acids in the placenta. The system L transporter functions as an amino acid exchanger, mediating the uptake of EAs in a sodium-independent manner (55). It is a heterodimer comprising a light chain, either the large amino acid transporter 1 (LAT1) or LAT2, and a heavy chain, 42hc/CD98.

Placental amino acid transport is regulated by maternal hormones, such as insulin and IGF-1 and mechanistic target of rapamycin (mTOR) signaling (17, 23, 26). Maternal undernutrition in humans and animal models results in reduced circu-
lating and tissue levels of insulin and IGF-I (4, 32). mTOR is a serine/threonine kinase that plays a key role in nutrient sensing and is an important regulator of cell growth, proliferation, and metabolism in response to changes in nutrient availability. Reduced placental mTOR signaling has been observed in human and animal IUGR, including the model we employ in this study (27, 46–48).

Placental amino acid transport has not been adequately studied in women subjected to naturally occurring undernutrition, and controlled experimental undernutrition in pregnant women is not possible. The effects of maternal nutrient restriction (MNR) on placental amino acid transporter expression and activity have, therefore, been investigated utilizing rodent and ovine models. However, the placentas of these animals are not directly comparable to the human placenta (8). Studies using nonhuman primate models that are more similar to the human in terms of physiological changes during pregnancy and placenta are likely to be more informative. To this end, we recently reported that the protein expression of glucose (GLUT-1) and amino acid transporter isoforms (taurine transporter TAUT, SNAT2, and LAT1 and 2) was reduced in syncytiotrophoblast microvillous membranes isolated from placentas of MNR baboons at gestational day (GD) 165 compared with controls (27). Furthermore, the phosphorylation of proteins in the mTOR and insulin/IGF-I signaling pathways, including placental S6K, S6 ribosomal protein, protein 4E-binding protein 1, insulin receptor substrate-1, Akt, ERK-1, and glycogen synthase kinase-3 was also reduced (27). However, the impact of MNR in humans or nonhuman primates on placental transport activity is unknown. Herein, we used in vitro and in vivo approaches to test the hypothesis that MNR decreases placental amino acid transporter activity, leading to reduced transplacental transfer of amino acids.

METHODS

Animal care. All animal procedures were approved by the Texas Biomedical Research Institute Institutional Animal Care and Use Committee and conducted in facilities approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. Baboons (Papio species) were maintained in groups of 10–16 females with 1 male in outdoor metal and concrete cages at the Southwest National Primate Research Center, as described in detail previously (51).

System for controlling and recording individual feeding. Animals were weighed and fed using a feeding system that has previously been described in detail (51). Briefly, once a day before feeding, all baboons passed along a chute, over a scale, and into an individual feeding cage. The weight of each animal was obtained as it crossed an electronic scale system (GSE 665; GSE Scale Systems, Livonia, MI) and recorded as the mean of 50 individual measurements over 30 s. Each animal was fed between either 0700 and 0900 or 1100 and 1300. Water was available ad libitum.

Baboons were fed Purina Monkey Diet 5038 (Purina, St. Louis, MO). The basic composition of each biscuit was ≥15% crude protein, ≥5% crude fat, ≤6% crude fiber, 5% ash, ≤3% added minerals, solubilized vitamin C, and other required vitamins. All animals were initially fed 60 biscuits ad libitum in the feeding tray of each individual cage. Baboons were returned to the group cage at the end of the 2-h feeding period, and biscuits that remained in the tray, floor of the cage, and pan beneath the cage were counted. Food consumption, weight, and health status of each animal were recorded daily.

Study design. Female baboons (8–15 yr old) were selected on the basis of reproductive age, body weight, and absence of extra-gestional pathological signs, and mated as described previously (51). Pregnancy was dated according to time of ovulation and changes of sex skin color, and confirmed by ultrasonography at GD 30. From that point forward, animals in the MNR group were fed 70% of the total intake of corresponding controls, calculated per kilogram body weight.

Infusion of EAAs and collection of blood and tissue samples. Conventional cesarean sections using general anesthesia and standard sterile techniques were performed at GD 165 (term 180–184 days), as previously described (27). Animals were tranquilized with ketamine hydrochloride (10 mg/kg), intubated, and anesthetized with isoflurane (starting rate 2% with oxygen: 2 l/min).

To assess transplacental transport of EAAs, baboons (n = 5 controls, n = 4 MNR) were infused with a cocktail of nine EAAs labeled with stable isotopes 13C or 2H, as described previously in pregnant women, with modifications (12). At the time of cesarean section, a bolus infusion of EAAs diluted to 10 ml with isotonic saline solution was infused into a maternal peripheral vein over a 2-min time period. A catheter placed in the maternal femoral artery was used to obtain maternal blood samples, which were collected just before the EAA infusion, and then at ~2-min intervals for up to 16 min after the completion of the infusion. Umbilical vein blood samples were obtained 5 min after the completion of EAA infusion. Heparinized maternal and fetal blood samples were centrifuged at 2,500 g for 15 min, and plasma was collected and stored at −80°C until further analysis. Fetuses and placentas were towel dried and weighed. Villous trophoblast tissue was processed for the isolation of syncytiotrophoblast membranes. Following cesarean section, maternal postoperative analgesia was provided (buprenorphine 0.015 mg·kg⁻¹·day⁻¹ as 2 doses for 3 days).

Transplacental amino acid transport in baboon was compared with previous stable isotope studies conducted in women (n = 5–7) undergoing cesarean section (12).

Isolation of trophoblast plasma microvillous membrane vesicles. The placenta was collected, and pieces (1 cm³) of choricion villous tissues were immediately dissected, washed in saline, and placed in buffer D (250 mM sucrose, 10 mM HEPES-Tris, and 1 mM EDTA, pH 7.4) at 4°C with protease and phosphatase inhibitors. Syncytiotrophoblast plasma microvillous membrane vesicles (MVM) were isolated as described in detail previously (16, 19), with minor modifications (33). Briefly, following initial centrifugation steps, MVM were isolated using Mg²⁺ precipitation, followed by further purification with differential centrifugation steps. Samples were snap frozen in liquid nitrogen and stored at −80°C. Purity of MVM was assessed using a standardized assay for alkaline phosphatase activity compared with homogenates. Microvillous membrane vesicle enrichment of alkaline phosphatase activity was not significantly different between the control and MNR groups (control, 4.2 ± 0.6; MNR 3.16 ± 0.7, n = 7/group, P = 0.26). Protein content of MVM was assessed using the method of Bradford (5).

Activity of amino acid transporters in MVM. System A transporter activity was assessed by measuring the uptake of the amino acid analog methylaminoisobutyric acid (MeAIB) using modification of the protocol of Mahendran et al. (34). System L activity was studied by determining the uptake of L-leucine, as described previously with minor modifications (19, 22). Vesicles were preloaded and incubated overnight in 300 mmol/l mannitol and 10 mmol/l HEPES-Tris, pH 7.4, at 4°C. Vesicles were subsequently pelleted and resuspended in the same buffer at a protein concentration of ~6 mg/mM. MVM were kept on ice until immediately before transport measurements, when samples were warmed to 37°C using a water bath. Vesicles were mixed rapidly with 30 μl of the appropriate incubation buffer (1:2), including [14C]MeAIB (150 μmol/l) or L-[2H]Ileucine (0.375 μmol/l). To determine the time course of MeAIB and leucine uptake by MVM (n = 2–3), uptake of radiolabel was terminated at 8, 12, 20, and 30 s using ice-cold PBS (pH 7.4) followed by rapid filtration. The uptake of MeAIB and leucine was rapid and time dependent, not reaching
equilibrium by 30 s. Uptakes at 20 s were chosen to approximate initial rate (22) (n = 7 in each group). Vesicles were subsequently separated from the substrate medium using rapid filtration over mixed ester filters (0.45 μM pore size, Millipore, Bedford, MA) and washed using 3 × 2 ml PBS. In studies of MeAIB transport, 150 mmol/l NaCl and 150 mmol/l KCl were used in incubation buffers to measure total and sodium-independent uptake, respectively. In leucine transport experiments, nonmediated flux was studied in the presence of 20 mmol/l unlabeled L-leucine. Using BCH [2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid], a specific system L inhibitor, our laboratory has previously reported that leucine uptake in human MVM is mediated almost exclusively by system L (22), providing the justification for using unlabeled leucine to determine system L activity.

Each condition was measured in triplicate for each placenta in all uptake experiments. Filters were dissolved in 2-ml scintillation cocktail and counted. Blanks were subtracted from counts, and uptakes are expressed in terms of picomoles per milligram protein at 20 s. System A activity corresponding to the Na+ -dependent uptake of MeAIB was calculated by subtracting Na+ -independent uptake from total uptakes. Mediated uptake of leucine was calculated by subtracting nonmediated transport from total uptake. Protein content was measured using the Bradford assay (5).

Plasma preparation, derivatization, and analysis of amino acid derivatives. Plasma samples were prepared as described previously (12). Briefly, 0.5 ml of plasma was mixed with norleucine (0.05 ml of 50 μg/ml), which served as an internal standard, followed by acidification with 0.15 ml of 50% acetic acid, and washed with AG-50 hydrogen form (0.05 ml). Samples were centrifuged at 4,500 g for 1 min, supernatant was discarded, and the pellet was washed four times with 1 ml of 70% isopropanol. Amino acids were released by adding 0.2 ml of 5 M ammonium hydroxide, samples were centrifuged, and supernatant was collected. Elution was repeated twice, and fractions were lyophilized.

Amino acid enrichment was carried out as previously described (12) by converting the amino acids to their respective tert-butylidethylsilyl derivatives, by adding 0.05 ml of the silylating agent [1:1 mixture of N-(r-butylidethylsilyl)-N-methyl-trifluoroacetamide with 1% tert-butylidethylsilyl chloride and acetonitrile] at 65°C for 1 h. Analysis of amino acid derivatives was performed using a gas chromatograph mass spectrometer (model 5975; Agilent Technologies, Santa Clara, CA), on an HP-5-ms column (Hewlett-Packard, Palo Alto, CA). The following ions were monitored for the nine EAAAs: 289/288 for valine, 303/302 for leucine and isoleucine, 323/320 for methionine, 408/404 for threonine, 337/336 for phenylalanine, 435/431 for lysine, 441/440 for histidine, and 380/375 for tryptophan (12).

The mole percent excess (MPE) is defined as a quantitative measure of the concentration of a stable isotope as a percentage of all isotopes, over and above its usual occurrence in nature. MPE of each amino acid was calculated in maternal and fetal plasma. The fetal umbilical vein/maternal artery (Fv/M) MPE was calculated as the ratio between fetal plasma amino acid enrichment over maternal circulation in an exponential manner that was similar for the two amino acids (Fig. 4A). This decrease in isotope enrichment in the maternal circulation is representative of all nine amino acids studied.

Fv/M MPE ratios. Fv/M MPE ratios were calculated for the nine amino acids that were infused in pregnant baboons and pregnant women at cesarean section (12, 43). In the baboon at GD 165, leucine and lysine were rapidly cleared from the maternal circulation in an exponential manner that was similar for the two amino acids (Fig. 4A). This decrease in isotope enrichment in the maternal circulation is representative of all nine amino acids studied.

Fv/M MPE ratios for isoleucine, methionine, histidine and lysine were comparable between pregnant women (n = 5–7) at term and control baboons at GD 165 (n = 5) (Fig. 4B). In contrast, the MPE ratios were significantly higher (P < 0.01) for leucine, phenylalanine, threonine, valine, and tryptophan in the baboon compared with the human, which is consistent with the possibility that the baboon placenta has a higher transport capacity for some EAAs. In general, MPE ratios for the branched-chain amino acids leucine and isoleucine were higher than for tryptophan and lysine in both species, indicating a higher placental capacity to transport leucine and isoleucine.

Table 1. Fetal and placental weights

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<tr>
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<th>Control</th>
<th>MNR</th>
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<tr>
<td>n</td>
<td>7</td>
<td>7</td>
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<tr>
<td>Fetal weight, g</td>
<td>837 ± 38.4</td>
<td>678 ± 28.6*</td>
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<tr>
<td>Placental weight, g</td>
<td>225 ± 19.5</td>
<td>179 ± 12.5*</td>
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Values are means ± SE; n, no. of animals. MNR, maternal nutrient restriction. *P < 0.05, control vs. MNR in the baboon at gestational day 165.

RESULTS

Fetal and placental weights. MNR significantly reduced fetal weights of the MNR group by 19% compared with controls at GD 165 (n = 7 in each group, P = 0.01, Table 1). Placental weights were reduced by 20% (n = 7 in each group, P = 0.02, Table 1). Fetal weight was positively correlated to placental weight in the control and MNR groups (control, r = 0.65, P = 0.02; MNR r = 0.75, P = 0.01, n = 7 in each group, Fig. 1).

System A and system L activity in MVM. At GD 165, MVM uptake of MeAIB was reduced by 73% in MNR compared with controls (control, 263 ± 48.5 pmol/mg × 20 s; MNR, 71 ± 13.7 pmol/mg × 20 s, n = 7/group, P = 0.00071, Fig. 2).

Mediated MVM L-leucine uptake, indicative of system L activity, was reduced by 84% in MNR compared with controls (control, 4.6 ± 1.7 pmol/mg × 20 s; MNR, 0.7 ± 0.1 pmol/mg × 20 s, n = 7/group, P = 0.0006, Fig. 3) at GD 165. Out of n = 7 in each group, three fetuses were female and four were male.

Fv/M MPE ratios. Fv/M MPE ratios were calculated for the nine amino acids that were infused in pregnant baboons and pregnant women at cesarean section (12, 43). In the baboon at GD 165, leucine and lysine were rapidly cleared from the maternal circulation in an exponential manner that was similar for the two amino acids (Fig. 4A). This decrease in isotope enrichment in the maternal circulation is representative of all nine amino acids studied.

Fv/M MPE ratios for isoleucine, methionine, histidine and lysine were comparable between pregnant women (n = 5–7) at term and control baboons at GD 165 (n = 5) (Fig. 4B). In contrast, the MPE ratios were significantly higher (P < 0.01) for leucine, phenylalanine, threonine, valine, and tryptophan in the baboon compared with the human, which is consistent with the possibility that the baboon placenta has a higher transport capacity for some EAAs. In general, MPE ratios for the branched-chain amino acids leucine and isoleucine were higher than for tryptophan and lysine in both species, indicating a higher placental capacity to transport leucine and isoleucine.

Fig. 1. Relationship between placental and fetal weights. Fetal weights were positively correlated to placental weights in the control and maternal nutrient restriction (MNR) groups in the baboon at gestational day (GD) 165. Individual Spearman’s rank correlation coefficients were calculated for each slope in control and MNR baboons (control, r² = 0.65; MNR, r² = 0.65, P < 0.05, n = 7 in each group).

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The protein expression of system A and L isoforms in MVM results in the baboon (P protein expression of system A and L isoforms in MVM results in the present study, we show that the downregulation of MVM isolated from control and MNR placentas of baboons at GD 165 are shown. Control and MNR groups were compared using the t-test (n = 7 in each group). Values are means ± SE. **P < 0.05.

DISCUSSION

In this study, we demonstrate that global MNR from GD 30 to GD 165 in the baboon is associated with 1) reduced activity of the placental nutrient transport systems A and L in isolated MVV; and 2) reduced transplacental transfer of EAAs. This is the first report in the nonhuman primate determining placental nutrient transport activity in vitro and in vivo in the same individuals, and our data provide strong validation for previous measurements of amino acid transport in isolated syncytiotrophoblast plasma membranes (11–14). Reduced activity of placental amino acid transporters has been reported in human IUGR due to placental insufficiency in a number of studies and may be correlated with severity of IUGR (13, 18, 22, 34, 35). Downregulation of system A in the rat before the development of IUGR has been demonstrated in response to a low-protein diet, suggesting that reduced placental amino acid transport may directly contribute to, rather than be a consequence of, IUGR (18). However, the rodent placentas differ from that of humans and other primates in terms of structure, function, and physiology (8). The use of this well-established nonhuman primate model, which shares similarity to the human in terms of physiological changes of pregnancy and placentation (8), lends clinical relevance to this study. Furthermore, this type of dietary manipulation and the investigation of changes in placental amino acid transport before term cannot be conducted in pregnant women. However, mothers who experience food insecurity (39) may be exposed to comparable levels of calorie restriction, resulting in a similar degree of fetal growth restriction.

Our laboratory reported previously that the protein expression of SNAT2 as well as LAT1 and 2 isoforms mediating system A and system L transport, respectively, is reduced in MVM isolated from nutrient-restricted baboons at GD 165 (27). In the present study, we show that the downregulation of protein expression of system A and L isoforms in MVM results in a reduced amino acid uptake capacity mediated by systems A and L in vitro. In addition, we demonstrate that the in vivo fetal-to-maternal plasma enrichment ratios for a number of EAAs are lower in MNR compared with control. These data, together with the lower circulating levels of EAAs in the MNR fetus (27), strongly suggest that transplacental transport of amino acids is impaired in MNR. The activity and expression of placental nutrient transporters in the placenta are regulated by the nutrient sensor mTOR, which, in turn, is influenced by growth factor signaling and the levels of nutrients, oxygen, and ATP. Our laboratory has previously demonstrated a decreased activity in placental insulin/IGF-I and mTOR signaling pathways in response to MNR in the baboon (27) and in pregnant rats subjected to protein restriction (48). Collectively, these observations are consistent with the model that downregulation of placental amino acid transporters, mediated by inhibition of growth factor and mTOR signaling pathways, may directly contribute to decreased fetal nutrient availability and restricted fetal growth in response to MNR.

This is the first study to assess transplacental transport of EAAs in vivo in the nonhuman primate. We have shown that the pattern of transplacental amino acid flux is similar in normal term pregnant women and control late gestation baboons, indicating that amino acid transport likely occurs via similar mechanisms in both species. This is further supported by the observation that the human and baboon placenta express the same glucose and amino acid transporter isoforms with comparable subcellular localization (27). Furthermore, the Fv/M MPE ratios for the amino acids leucine, isoleucine, methionine, phenylalanine, threonine, and tryptophan were reduced in MNR compared with control baboons at GD 165. We, therefore, provide direct evidence that the downregulation of system A and system L activity in MVM isolated from the placental villi of MNR baboons leads to decreased placental amino acid transport and, consequently, reduced amino acid concentrations in the fetal circulation (24). In a previous study, fetal amino acid concentrations were determined in baboons at GD 90 (0.5 of gestation) and were found to be similar in control and MNR animals (37), and it was proposed that impaired placental amino acid transport may be a feature of late gestation, when increasing fetal demand cannot be matched by maternal nutrient supply. The results of the present study are consistent with this hypothesis.

Numerous studies in the human and in different animal models have demonstrated that IUGR due to placental insuf-
ficiency is associated with reduced placental amino acid transport and decreased fetal levels of EAAs. For example, using stable isotopes, it has been reported that in vivo transplacental transport of leucine and phenylalanine is decreased in human IUGR, with decreased fetomaternal enrichment ratios of leucine correlating with disease severity (36, 42). In vivo studies of placental amino acid transport in sheep have shown that transplacental flux of leucine (50) and threonine (1) is decreased in a model of IUGR. In vitro studies of amino acid transport in MVM isolated from human IUGR placentas show decreased leucine and lysine uptake (22). Furthermore, placental amino acid transport is impaired in placental insufficiency subsequent to reduction of uteroplacental blood flow in the guinea pig (20). Collectively, these studies suggest that there are common placental responses, which include downregulation of amino acid transport, to placental insufficiency and maternal undernutrition, and that these responses are conserved from rodents to humans.

The maternal plasma amino acid profile influences amino acid delivery to the fetus, as highlighted by maternal amino acid supplementation studies (24, 25, 44, 45, 56). However, although elevating the maternal concentration of EAAs in human IUGR increased the umbilical uptake of some amino acids, no changes in valine, phenylalanine, lysine, histidine, and threonine umbilical uptake was observed, suggesting that competition for the same transporter may be occurring in the placenta (45). In the present study, the Fv/M MPE ratios of valine, histidine, and lysine were unchanged in nutrient-restricted baboons compared with controls following EAA infusion, suggesting that similar alterations in placental amino acid transport mechanisms occur in human and baboon IUGR. Although EAAs such as valine and histidine are transported by other transporters than system L, they may compete for binding to this transporter in the placental barrier. It is possible to speculate that a preference for certain amino acids by the system L transporter may explain the finding that the in vivo transport of some, but not all, EAAs was decreased in MNR, despite decreased transporter protein expression (27) and leucine transport activity in response to MNR. However, the exact mechanisms are likely to be complex and remain to be elucidated.

IUGR is associated with increased incidence of obesity, insulin resistance, reduced lean body mass, diabetes, and ne-rodevelopmental impairment in adulthood (2, 3, 9, 28, 58). Reduced nutrient supply leads to alterations in the intrauterine environment, and fetal adaptations to this environment ensure maintenance of basal metabolic functions at the cost of fetal growth (15, 53). A reduction in the transplacental transport of amino acids could, therefore, impact a wide variety of fetal tissues in utero, resulting in developmental programming of adult disease in IUGR. Insulin is a primary fetal growth factor, which also acts via IGF-I to promote fetal growth (11, 14, 31, 40, 57). A reduction in the transport of these amino acids could, therefore, impact fetal insulin and IGF-I signaling, and indeed moderate nutrient restriction in the baboon causes reduced IGF-I expression in the placenta (30) and fetal frontal cortex (58), as well as the development of insulin resistance in juvenile offspring (9). Methionine plays a role in DNA methylation and folate metabolism (33). Nutrient restriction in the baboon alters fetal kidney and brain methylation patterns at 0.5 and 0.9 of gestation (54), consistent with an epigenetic mechanism for the developmental programming of adult diseases in IUGR (41). An alteration in folate metabolism during pregnancy is associated with anxiety and altered learning patterns in offspring (29), and offspring of MNR baboons exhibit more variations in emotional behavior compared with controls (28).

**Perspectives and Significance**

Maternal supplementation with amino acids has been proposed to be an attractive option to increase fetal growth in IUGR pregnancies. In this study, we report reduced transplacental amino acid transport in vivo and fetal growth restriction following moderate nutrient restriction at 0.9 of gestation in the baboon, a model that shares extensive similarities with human pregnancy. However, it is important to thoroughly understand the mechanisms of transplacental transport of amino acids, as well as the impact of individual amino acids on placental metabolism and fetal growth, before new treatments for IUGR can be developed. This study represents the first step in understanding transplacental amino acid flux in IUGR in a nonhuman primate model.

**GRANTS**

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CASE STUDY


