Maternal Protein Restriction in the Rat Inhibits Placental Insulin, mTOR, and STAT3 Signaling and Down-Regulates Placental Amino Acid Transporters

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The mechanisms underlying reduced fetal growth in response to maternal protein restriction are not well established. Maternal levels of insulin, IGF-I, and leptin are decreased in rats fed a low protein (LP) diet. Because these hormones stimulate placental amino acid transporters *in vitro*, we hypothesized that maternal protein restriction inhibits placental leptin, insulin/IGF-I, and mammalian target of rapamycin signaling and down-regulates the expression and activity of placental amino acid transporters. Pregnant rats were fed either an isocaloric low protein (LP, 4% protein) or control diet (18% protein) and studied at gestational day (GD)15, GD19, or GD21 (term 23). At GD19 and GD21, placental expression of phosphorylated eukaryotic initiation factor 4E binding protein 1 (Thr-36/46 or Thr-70) and phosphorylated S6 ribosomal protein (Ser-235/236) was decreased in the LP group. In addition, placental expression of phosphorylated S6 kinase 1 (Thr-389), phosphorylated Akt (Thr-308), and phosphorylated signal transducer and activator of transcription 3 (Tyr-705) was reduced at GD21. In microvillous plasma membranes (MVM) isolated from placentas of LP animals, protein expression of the sodium-coupled neutral amino acid transporter (SNAT)2 and the large neutral amino acid transporters 1 and 2 was reduced at GD19 and GD21. MVM SNAT1 protein expression was reduced at GD21 in LP rats. SNAT4 and 4F2 heavy chain expression in MVM was unaltered. System A and L amino acid transporter activity was decreased in MVM from LP animals at GD19 and GD21. In conclusion, maternal protein restriction inhibits placental insulin, mammalian target of rapamycin signaling, and signal transducer and activator of transcription 3 signaling, which is associated with a down-regulation of placental amino acid transporters. We speculate that maternal endocrine and metabolic control of placental nutrient transport reduces fetal growth in response to protein restriction. **(*Endocrinology* 152: 1119–1129, 2011)**

Intrauterine growth restriction (IUGR) is a common cause of perinatal morbidity and mortality (1) and is associated with increased risk of adult disease, such as diabetes and cardiovascular disease (2–6). The primary determinant of fetal growth is the availability of nutrients, which is directly dependent on the transport functions of the placenta. Human IUGR is associated with decreased activity of placental transporters mediating the transfer of amino acids (7–10), ions (11, 12), and lipids (13), suggesting that down-regulation of placental transporters directly contributes to restricted fetal nutrient availability and growth in this condition. However, the cause-and-effect relationship between changes

Abbreviations: AMPK, AMP-Activated protein kinase; C, control; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; 4F2hc, 4F2 heavy chain; GD, gestational day; IUGR, intrauterine growth restriction; LAT, large neutral amino acid transporter; LP, low protein; MeAIB, ^{14}C-methyl-aminoisobutyric acid; mTOR, mammalian target of rapamycin; MVM, microvillous plasma membranes; PI3K, phosphoinositide kinase-3; S6K1, S6 kinase 1; SNAT, sodium-coupled neutral amino acid transporter; STAT3, signal transducer and activator of transcription 3.

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in placental transport function and altered fetal growth remains to be established and cannot easily be addressed in pregnant women.

Maternal dietary protein restriction is a common approach to induce fetal undernutrition and IUGR in experimental animals ranging from rodents to sheep (14). A large number of animal experimental studies has demonstrated that moderate protein restriction during pregnancy typically reduces fetal growth and programs the fetus to develop hypertension, insulin resistance, obesity, and dyslipidemia later in life (15, 16). However, the mechanisms linking maternal protein restriction to slowing of fetal growth and long-term health risk remains to be established. It is reasonable to assume that the placenta, constituting the maternal-fetal interface, plays a critical role in conveying growth-regulating signals from the maternal compartment to the fetus. Accordingly, placental responses to changes in maternal nutrition, such as protein restriction, have recently received increased attention (17, 18). Protein restriction in the pregnant rat has been shown to decrease the *in vitro* activity of specific placental amino acid transporters (19). In addition, we previously demonstrated that down-regulation of the placental system A transporter activity and expression precedes the occurrence of IUGR in pregnant rats fed a low protein (LP) diet (20), implicating decreased placental nutrient transport as the cause of IUGR in this model. However, the signaling mechanisms underlying reduced placental nutrient transport in response to maternal protein restriction remain largely unknown.

System A is a ubiquitous Na^+-dependent transporter that actively transports small, zwitterionic, neutral amino acids with short, unbranched side chains, such as alanine, serine, and glutamine (21). System A is encoded by three different members of the SLC38 gene family (*Slc38a1*, *Slc38a2*, and *Slc38a4*), giving rise to the three subtypes of this sodium-coupled neutral amino acid transporter (SNAT): SNAT1, SNAT2, and SNAT4 (22), which are all expressed in rat (23) and human placenta (24–26). System L is composed of two subunits, one catalytic light chain [large neutral amino acid transporter (LAT)1 or LAT2] and a heavy chain, 4F2 heavy chain (4F2hc), encoded by the gene *Slc3a2*, which is critical for the trafficking of the light chain to the plasma membrane. Both LAT1 and LAT2, which are encoded by the genes *Slc7a5* and *Slc7a8*, are expressed in the placenta (27, 28). Insulin and IGF-I stimulate placental system A (29, 30) and L activity (31). Similarly, we have reported that placental mammalian target of rapamycin (mTOR) signaling is a positive regulator of system A and L amino acid transporters (31, 32), and leptin has been shown to increase system A amino acid transport in placental villous fragments (29). Because we previously have shown that maternal levels of insulin, IGF-I, and leptin are decreased in rats fed a LP diet (20), it is possible that these endocrine changes contribute to the down-regulation of placental nutrient transport in response to maternal protein restriction.

Many of the cellular effects of insulin and IGF-I are mediated by phosphoinositide kinase-3 (PI3K) signaling, leading to the phosphorylation of Akt at Thr-308 (33). Akt plays a central role in the regulation of cell growth and proliferation by activating the mTOR pathway and glycogen synthase kinase 3 (34–36). The mTOR signaling pathway regulates gene transcription and protein translation in response to nutrient and growth factor availability (37), through phosphorylation of the key translation regulators p70 ribosomal S6 kinase 1 (S6K1), S6 ribosomal protein, and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). In addition, cellular energy status influences the mTOR signaling pathway via AMP-activated protein kinase (AMPK), upstream of mTOR (36). Low energy status activates AMPK (by increasing phosphorylation), which in turn inhibits mTOR. The cellular effects of leptin are mediated by a noncovalently associated tyrosine kinase of the Janus kinase family. Upon leptin binding to the ob-rb receptor, Janus kinase 2 phosphorylates signal transducer and activator of transcription 3 (STAT3) at Tyr-705, which results in STAT3 dimerization and migration to the nucleus where transcription initiation is affected (38). However, Tyr-705-STAT3 phosphorylation can also be induced by other cytokines and hormones (39).

Because 1) insulin, IGF-I, leptin, and mTOR signaling all stimulate placental amino acid transport (29–32) and 2) maternal circulating levels of insulin, IGF-I, and leptin are decreased in late pregnant rats subjected to protein restriction (20), we tested the hypothesis that maternal protein restriction inhibits placental leptin, insulin/IGF-I, and mTOR signaling and down-regulates the expression and activity of placental amino acid transporters in rats with LP diet.

Materials and Methods

Animals and diets

Experiments were approved by the local ethical committee for animal research at Göteborg University. Animals and diets have been described in detail previously (20). Briefly, timed pregnant Sprague Dawley rats were fed either a control (C) diet (18% of energy from protein, 67% from carbohydrates, and 13% from fat) or an isocaloric LP diet (4% of energy from protein, 82% from carbohydrates, and 13% from fat). Diets were introduced at gestational day (GD)2 and maintained until the end of the experiment.

Collection of tissue samples

Dams were euthanized at GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), or GD21 (C, n = 8; LP, n = 4) (term 23) using an overdose of barbiturates. After laparotomy, fetuses and placentas were collected and quickly dried on blotting paper, any remaining fetal membranes were removed, and fetuses and placentas were weighed. Placentas in each litter were pooled and homogenized in buffer D (10 mM Tris-HEPES, 250 mM sucrose, 1.6 μM antipain, 0.7 μM pepstatin, 0.5 μg ml^{-1} aprotinin, and 1 mM EDTA) using a Polytron, frozen in liquid nitrogen, and stored at −80 C until analysis.

Isolation of trophoblast microvillous plasma membranes (MVM)

The protocol for isolating trophoblast MVM from rat placenta was adapted from that used previously in mouse placenta (40). All procedures were performed on ice, and centrifugation steps were carried out at 4 C. Homogenates were centrifuged at 10,000 × *g* for 15 min; pellets were resuspended, homogenized in 1 ml of buffer D, and centrifuged at 10,000 × *g* for 10 min. The resulting supernatants were combined and spun at 125,000 × *g* for 30 min. The pelleted crude membrane fraction was resuspended in 2 ml of buffer D, and 12 mM $MgCl_2$ was added. The suspension was stirred for 20 min on ice. After centrifugation at 2500 × *g* for 10 min, the supernatant was centrifuged at 125,000 × *g* for 30 min. The final pellet was resuspended in buffer D using a Dounce homogenizer to yield the vesicle suspension. Protein concentration was determined using the Bradford assay (41), and MVM purity was assessed by MVM/homogenate enrichments of alkaline phosphatase activity (42). Alkaline phosphatase enrichments for C and LP MVM vesicles (n = 15 for both) were 14.9 ± 0.6 and 14.1 ± 0.5 (mean ± SEM), respectively. Statistical analysis revealed no significant difference between the two groups ($P = 0.3$, Student's *t* test).

Western blot analysis

Protein expression of total and phosphorylated S6K1 (Thr-389), 4E-BP1 (Thr-37/46 or Thr-70), S6 ribosomal protein (Ser-235/236), Akt (Thr-308), STAT3 (Tyr-705), and AMPKα (Thr-172) was determined in placental homogenates using commercial antibodies (Cell Signaling Technology, Boston, MA). Protein expression of the system A amino acid transporter isoforms (SNAT) 1, 2, and 4 and the system L amino acid transporter isoforms LAT1, LAT2, and 4F2hc was analyzed in placental MVM preparations. The justification for determining protein expression of transporters in MVM rather than in homogenates is that trophoblast nutrient transporters mediate cellular uptake and transfer across the placental barrier only if localized in the syncytiotrophoblast plasma membranes. Thus, data on amino acid transporter protein expression in MVM is much more informative than determination of protein expression in placental homogenates. The SNAT1 antibody was received as a generous gift from Jean Jiang (University of Texas Health Science Center San Antonio). A polyclonal SNAT2 antibody was generated in rabbits (43). Affinity-purified polyclonal anti-SNAT4 antibodies were produced in rabbits using the epitope YGEVEDELLHAYSKV in human SNAT4 (Eurogentec, Seraing, Belgium). Antibodies targeting the LAT1 and LAT2 were produced in rabbits as described previously (44). The 4F2hc antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-β actin was from Sigma-Aldrich (St. Louis, MO).

Western blot analysis was performed as previously described (31). In brief, 20 μg of total protein were loaded onto a SDS-PAGE gel (7% for S6K1, Akt, STAT3, AMPKα, SNAT 1/2/4, and 4F2hc; 12% for 4E-BP1; and 4–12% for LAT1 and LAT2), and electrophoresis was performed at a constant 100 V for 2 h. Proteins were transferred onto nitrocellulose membranes overnight at a constant 30 V. After the transfer, the membranes were blocked in 5% milk in Tris-buffered saline (wt/vol) plus 0.1% Tween 20 (vol/vol) for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4 C. Subsequently, membranes were incubated with the appropriate secondary peroxidase-labeled antibodies for 1 h. After washing, bands were visualized using enhanced chemiluminescence detection reagents (GE Healthcare, Chalfont St. Giles, UK). Blots were stripped using β-mercaptoethanol as described previously (45) and reprobed for β-actin as a loading control. Analysis of the blots was performed by densitometry using an AlphaImager (Alpha Innotech Corp., San Leandro, CA). For each protein target and gestational age, the mean density of the C sample bands was assigned an arbitrary value of 1. Subsequently, all individual C and LP density values at a particular gestational age were expressed relative to this mean.

MVM amino acid transporter activity measurements

The activity of system A and L amino acid transporters was determined in MVM using rapid filtration techniques slightly modified from procedures previously described for human MVM (46) and mouse MVM (47). MVM vesicles were preloaded by incubation in 300 mM mannitol and 10 mM HEPES-Tris (pH 7.4) overnight at 4 C. Subsequently, MVM vesicles were pelleted and resuspended in a small volume of the same buffer (final protein concentration, ~5–10 mg/ml). Membrane vesicles were kept on ice until immediately before transport measurements when samples were warmed to 37 C. At time zero, 30 μl of vesicles were rapidly mixed (1:2) with the appropriate incubation buffer containing ^{14}C-methyl-aminoisobutyric acid (MeAIB) (150 μM) or ^{3}H-L-leucine (0.375 μM). Uptake of radiolabeled substrate was terminated by addition of 2 ml of ice-cold PBS after 15 sec. Subsequently, vesicles were rapidly separated from the substrate medium by filtration on mixed ester filters (0.45-μm pore size; Millipore Corp., Bedford, MA) and washed with 3× 2 ml of PBS. In all uptake experiments, each condition was studied in triplicate for each membrane vesicle preparation. Filters were dissolved in 2 ml liquid scintillation fluid and counted. Appropriate blanks were subtracted from counts and uptakes expressed as pmol/mg protein. Na^+-dependent uptake of MeAIB (corresponding to system A activity) was calculated by subtracting Na^+-independent from total uptakes. For leucine, mediated uptake was calculated by subtracting non-mediated transport, as determined in the presence of 20 mM unlabeled leucine, from total uptake.

Data presentation and statistics

Data are presented as means ± SEM. Statistical significance of differences between C and LP diet groups was assessed using Student's *t* test. $P < 0.05$ was considered significant.

Results

Maternal weight gain, fetal, and placental weights

As reported in detail elsewhere (20), maternal weight gain was significantly lower in the LP group ($P < 0.05$) compared with the C group at GD18, GD19, and GD21. In the LP group, fetal (−21%; $P < 0.05$) and placental weights (−13%; $P < 0.05$) were significantly reduced at GD21 compared with controls. Fetal/placental weight ratios were similar between the C and LP groups at GD15 (C: 0.86 ± 0.04, n = 8; LP: 0.84 ± 0.04, n = 8), GD19 (C: 3.0 ± 0.1, n = 8; LP: 3.2 ± 0.5, n = 8), and at GD21 (C: 7.0 ± 0.3, n = 8; LP: 6.2 ± 0.5, n = 4). There was no difference in litter size between controls and LP groups at any gestational age studied (20). Food intake was found to be 11% ($P < 0.05$) higher in the LP group (22.9 ± 0.63 g/d^{-1}) compared with C (20.6 ± 0.39 g/d^{-1}).

Maternal protein restriction decreases placental Akt phosphorylation

Phosphorylation of Akt Thr-308, a readout of insulin/IGF-I signaling, was not affected by the LP diet at GD15 or GD19 compared with C diet. However, phosphorylation of Akt Thr-308 was decreased by 50% ($P < 0.02$, n = 4) in LP placentas compared with C (n = 8) at GD 21 (Fig. 1, A and C). There was no significant difference in the total Akt protein expression between C and LP group placentas at GD15, GD19, or GD21 (Fig. 1, B and C).

Inhibition of placental mTOR signaling in response to a maternal LP diet

Mammalian TOR is a key signaling pathway integrating growth factor and nutrient signaling to control cellular processes involved in cell growth, including protein synthesis (48) and amino acid transport (49). These effects are mediated by phosphorylation of 4E-BP1, S6K1, and S6 ribosomal protein. In the present study, we determined the expression of phosphorylated S6K1, 4E-BP1, and S6 ribosomal protein as a functional readout for mTOR activity in placental homogenates of C and LP group at GD15, GD19, and GD21.

Phosphorylated S6K1 (Thr-389)

No significant differences were found in the expression of phosphorylated S6K1 (Thr-389) between placental homogenates obtained from C (n = 8) and LP (n = 8) groups at GD15 ($P = 0.8$) or GD19 ($P = 0.2$) (Fig. 2A). However, by GD21, the expression of phosphorylated S6K1 was down-regulated by 61% ($P < 0.002$) in the LP (n = 4) placentas compared with C (n = 8). There was no significant difference in the total S6K1 expression level between C and LP group placentas at any gestational age tested (Fig. 2, B and C).

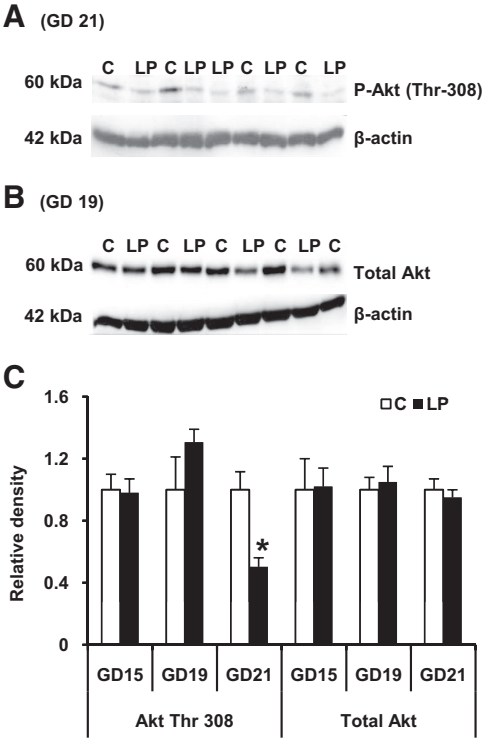

FIG. 1. Placental Akt phosphorylation in rats fed a C or LP diet. Representative Western blottings showing P-Akt Thr-308 (A) and total Akt (B) expression in homogenates of rat placenta at GD21 and GD19, respectively. C, Histogram summarizes the Western blotting data from GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), and GD21 (C, n = 8; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

Phosphorylated 4E-BP1

The 4E-BP1 undergoes phosphorylation at multiple sites, and we studied three of them, Thr-37/46 with one antibody and Thr-70 with another. Figure 3A shows representative Western blottings using antibodies directed against 4E-BP1 phosphorylated at Thr-37/46 or at Thr-70. Phosphorylation of 4E-BP1 is hierarchical, in that phosphorylation of Thr-37/46 is required for further phosphorylation at Thr-70. Densitometry analysis showed no significant difference in the expression of phosphorylated 4E-BP1 (Thr-37/46 or Thr-70) between C and LP placentas at GD 15. However, at GD 19, the expression of phosphorylated 4E-BP1 (Thr-37/46) was reduced by 42% ($P < 0.0001$, n = 8) and phosphorylated 4E-BP1 (Thr-70) expression was decreased by 43% ($P < 0.008$, n = 8) in the LP group compared with C group (n = 8). Similarly, at GD 21, the level of phosphorylated 4E-BP1 Thr-37/46 was decreased by 41% ($P < 0.001$, n = 4) and phosphorylated 4E-BP1 (Thr-70) expression was decreased by 42% ($P < 0.0004$, n = 4) in the LP group compared with C group (n = 7). However, there were no significant differences

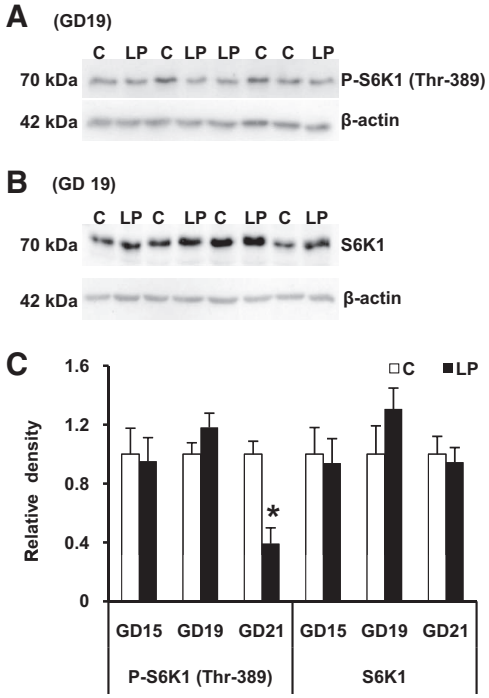

FIG. 2. Placental S6K1 phosphorylation in rats fed a C or LP diet. Representative Western blottings of P-S6K1 (Thr-389) (A) and total S6K1 (B) in homogenates of rat placenta at GD19. C, Data from GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), and GD21 (C, n = 8; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

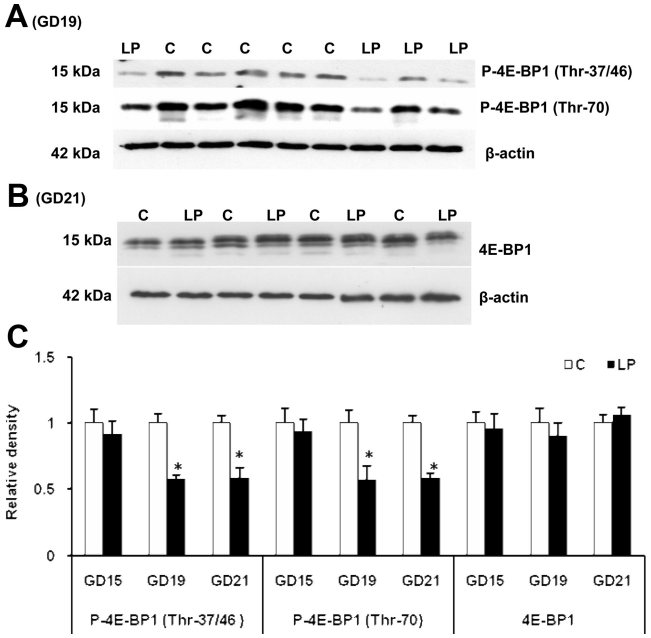

FIG. 3. Placental 4E-BP1 phosphorylation in rats fed a C or LP diet. Representative Western blottings of 4E-BP1 (Thr-37/46) or Thr-70 (A) and total 4E-BP1 (B) in homogenates of rat placenta at GD19 and GD21, respectively. C, Histogram summarizes the Western blotting data from GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), and GD21 (C, n = 7; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

between the LP and C groups at GD15, GD19, or GD21 when analyzing total placental 4E-BP1 (Fig. 3, B and C).

Phosphorylated S6 ribosomal protein (Ser-235/236)

The expression of phosphorylated ribosomal protein S6 (Ser-235/236), a component of the 40S ribosome and a physiologically relevant S6K1 substrate, was decreased by 42% [$P < 0.0001$; n = 8 (C), n = 8 (LP)] and 31% [$P < 0.04$; n = 7 (C), n = 4 (LP) [in LP group placentas compared with C at GD19 and GD21 (Fig. 4, A and C), respectively. Figure 4, B and C, shows that there was no significant difference in the total S6 ribosomal protein expression level between C and LP placentas at GD15, GD19, or GD21.

Maternal LP diet does not alter placental AMPK phosphorylation

Activation of AMPK is critically dependent on phosphorylation of AMPKα at Thr-172, which was measured in placental homogenates obtained from dams on C or LP diet. No change in the protein expression of phosphorylated AMPK or total AMPK was observed in placentas of LP animals compared with controls at GD15, GD19, or GD21 (data not shown).

Maternal protein restriction inhibits placental STAT3 signaling

We have previously reported decreased circulating leptin levels in dams fed a LP diet (20), and STAT3 is a key mediator of intracellular signaling downstream of the leptin receptor (39). STAT3 dimerizes and enters the nucleus after activation by phosphorylation at Tyr-705, which constitutes the critical phosphorylation site determining STAT3 activity. At GD15 and GD19, phosphorylated STAT3 expression was not significantly different in placentas obtained from LP group animals compared with control. In contrast, the protein expression of phosphorylated STAT3 was decreased by 69% in placental homogenates from LP dams ($P < 0.04$) compared with C at GD21 (Fig. 5, A and C). Total STAT3 expression was not significantly different among the C and LP groups at any of the time points tested (Fig. 5, B and C).

Down-regulation of system A and system L transporter isoform protein expression in response to maternal LP diet

System A amino acid transport is mediated by three isoforms, SNAT1, SNAT2, and SNAT4, which all are expressed in the rat placenta. Using Western blot analysis, two distinct bands at approximately 52 and 58 kDa were

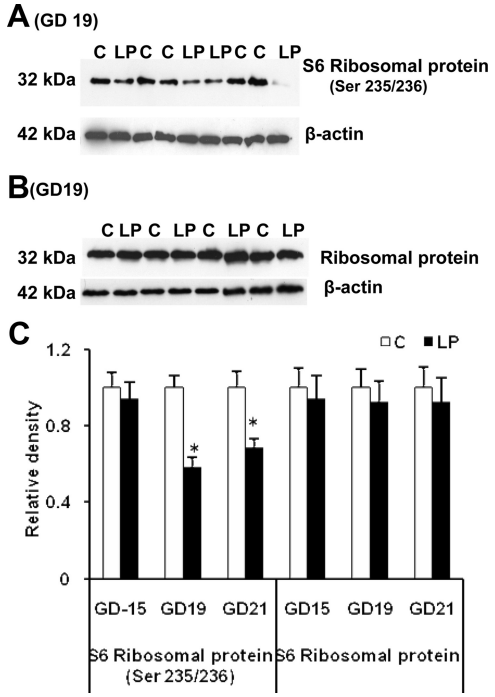

FIG. 4. Placental S6 ribosomal protein phosphorylation in rats fed a C or LP diet. Western blottings of phosphorylated S6 ribosomal protein (Ser-235/236) (A) and total S6 ribosomal protein (B) in homogenates of rat placenta at GD19. C, The expression levels of phosphorylated S6 ribosomal protein (Ser-235/236) and total ribosomal protein from GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), and GD21 (C, n = 7; LP, n = 4) are shown in the histogram. After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

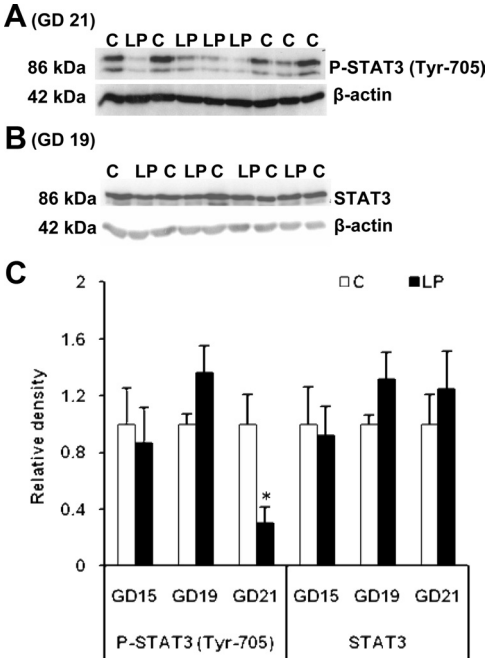

FIG. 5. Placental STAT3 phosphorylation in rats fed a C or LP diet. Representative Western blottings shows (A) phosphorylated STAT3 (Tyr-705) and (B) total STAT3 expression in placental homogenates of rat placenta at GD21 and GD19, respectively. C, The histogram summarizes the Western blotting data from GD15 (C, n = 8; LP, n = 8), GD19 (C, n = 8; LP, n = 8), and GD21 (C, n = 8; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

observed for SNAT1 in MVM isolated from rat placenta (Fig 6A). Protein expression of SNAT1 (analyzing 52 and 58 bands together) in MVM was reduced in LP placentas compared with C at GD21; however, no changes could be observed in SNAT1 expression levels at GD15 and GD19 (Fig. 6, A and D). SNAT2 protein expression was significantly decreased in placental MVM of LP rats at GD19 and GD21 (Fig. 6, B and D). In contrast, MVM protein expression of SNAT2 at GD15 was unaffected by the LP diet. SNAT4 expression in MVM was not significantly different between the C and LP groups at any of the time points tested (Fig. 6, C and D).

System L amino acid transport is dependent on a heavy chain 4F2hc forming a heterodimer with LAT1 or LAT2 in the plasma membrane. MVM LAT1 and LAT2 expression were comparable between LP and C rats at GD15. Feeding a LP diet significantly reduced the LAT1 protein expression in placental MVM at GD19 and GD21 (Fig. 7, A and B). Two distinct LAT2 bands at approximately 30 and 50 kDa, corresponding to two splice variants of the gene (50), were observed in MVM. Both LAT2 proteins (30 and 50 kDa) were down-regulated in MVM of LP placentas at GD19 and GD21 (Fig. 7, A and B). The expression of 4F2hc in MVM was comparable between the C and LP groups at all of the time points tested (Fig. 7, A and B).

Decreased activity of system A and L amino acid transporters in MVM isolated from LP placentas

The Na^+-dependent uptake of ^{14}C-MeAIB (corresponding to system A activity) and the mediated uptake of ^{3}H-leucine (corresponding to system L activity) was linear up to 30 sec (data not shown). Based on these time-course experiments, an incubation time of 15 sec was chosen in subsequent studies. In the C group, the placental capacity to transport MeAIB and leucine increased markedly in late pregnancy (Fig. 8, A and B); however, this gestational increase was not evident in the LP group. As a result, MVM system A activity was decreased by 56 and 59% in the LP group compared with C at GD19 and GD21, respectively (Fig. 8A). Similarly, system L activity was markedly decreased in the LP group compared with C at GD19 (−59%) and GD21 (−68%) (Fig. 8B).

Discussion

The most significant finding in this study is that placental insulin/IGF-I, mTOR, and STAT3 signaling pathways,

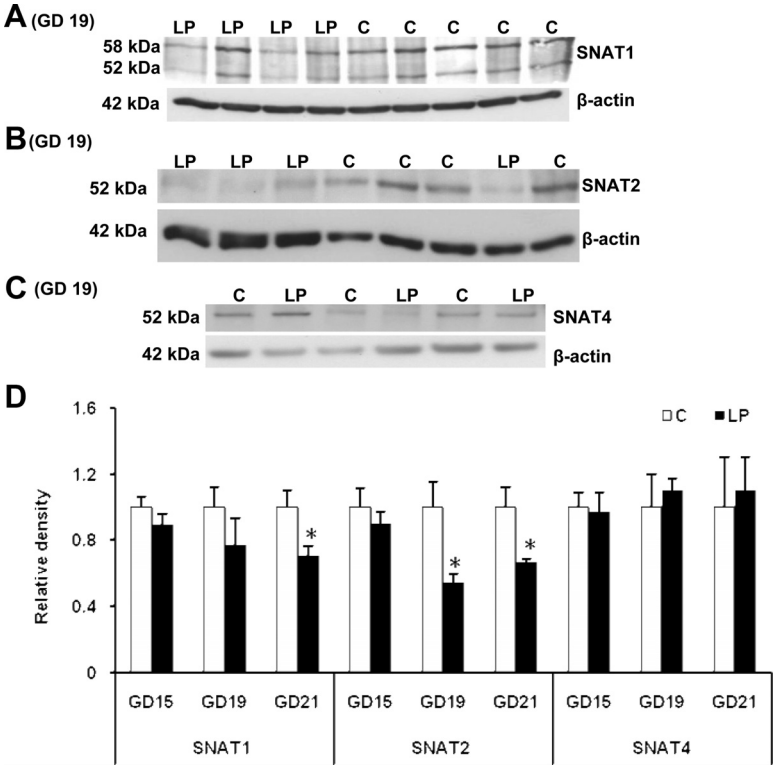

FIG. 6. Protein expression of system A amino acid transporter isoforms in MVM isolated from C and LP placentas. Representative Western blottings are shown for (A) SNAT1, (B) SNAT2, and (C) SNAT4 in MVM isolated from rat placenta at GD19. D, The histogram summarizes the Western blotting data from GD15 (C, n = 5; LP, n = 4 LP), GD19 (C, n = 5; LP, n = 4), and GD21 (C, n = 5; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

known to be positive regulators of placental amino acid transporters, are inhibited in response to maternal protein restriction in the rat. Furthermore, we show that feeding the dam a LP diet results in a down-regulation of the system L amino acid transporter, which is involved in mediating the transfer of many essential amino acids, such as leucine, across the placenta. This data extends previous observations of a decreased placental system A-mediated amino acid transport in this model. Importantly, we demonstrate that many of these changes appear before fetal growth is significantly reduced, suggesting a "cause-and-effect" relationship between inhibition of placental insulin/IGF-I, mTOR, and STAT3 signaling, down-regulation of placental amino acid transport, and reduced fetal growth (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

In the present study, placental phosphorylated Akt Thr-308 expression was unaltered on GD19 in response to a LP diet but was decreased on GD21 in parallel to a significant reduction in fetal weight and reduced levels of maternal IGF-I and insulin (20). This finding is consistent with inhibition of placental Akt signaling in human IUGR (51). Interestingly, in Akt1 deficient mice, the growth and development of the placenta is impaired, resulting in IUGR, and it was proposed that fetal growth restriction was a result of placental insufficiency (52). Collectively, this data support an important role for placental Akt signaling in regulating placental function and fetal growth.

Using phosphorylated S6K1, 4E-BP1, and S6 ribosomal protein as functional readouts, we found that placental mTOR signaling was markedly inhibited in LP group rats on GD19 and GD21. Because mTOR signaling is an important regulator of protein synthesis, placental mTOR inhibition in response to maternal protein restriction may result in decreased protein synthesis in the placenta. Furthermore, because we previously have demonstrated that mTOR signaling is a positive regulator of placental amino acid transporters (32, 49), inhibition of mTOR may play an important role in down-regulating placental amino acid transport *in vivo* (20) and *in vitro* (this study and Ref. 19) in LP rats. Indeed, the timing of changes in placental mTOR signaling and amino acid transport, both occurring by GD19, supports a cause-and-effect relationship between mTOR inhibition and decreased placental amino acid transport. Importantly, mTOR signaling and placental nutrient transport were down-regulated (GD19) before significant fetal growth restriction at GD21 (20). These findings suggests that IUGR develops in this model due to down-regulation of key placental signaling pathways and amino acid transporters. Furthermore, this data are in agreement with the concept of the placenta as a nutrient sensor that regulates fetal growth according to the nutrient availability in the maternal compartment by altering placental nutrient transporter activity (53, 54).

There are a multitude of upstream regulators of the mTOR signaling pathway, including growth factor signaling, amino acid availability, cellular energy, and oxygen levels (55–58). The marked decrease in the expression of phosphorylated Akt at GD21, perhaps due to decreased circulating levels of maternal insulin and IGF-I (20), is likely to contribute to the inhibited mTOR activity at this stage of gestation. However, because mTOR signaling was inhibited already at GD19, when Akt phosphorylation remained unaltered in response to maternal protein restriction, additional upstream regulators of mTOR signaling must be involved. AMPK, a well-known inhibitor

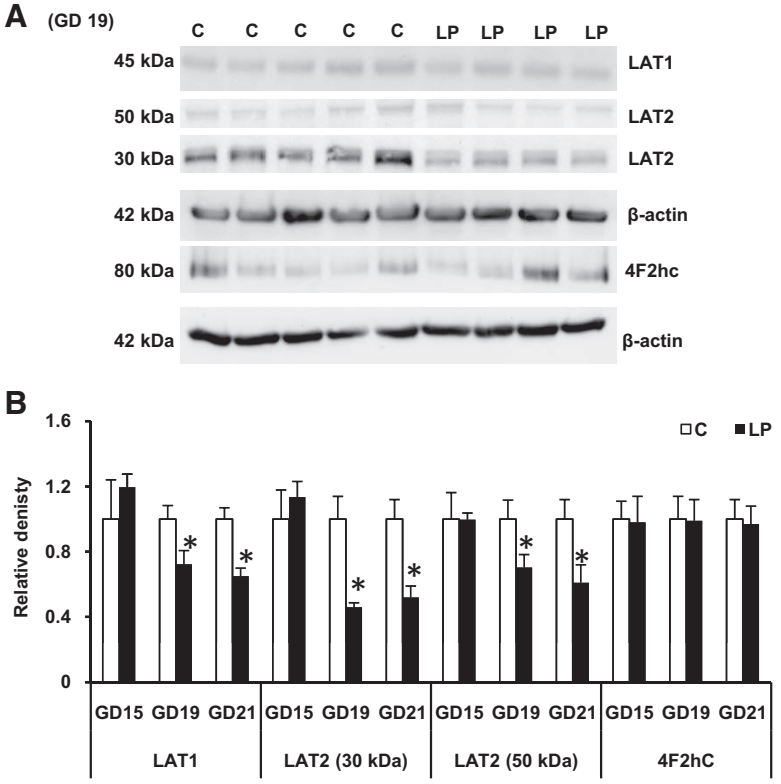

FIG. 7. Protein expression of system L amino acid transporter isoforms in MVM isolated from C and LP placentas. A, Representative Western blottings are shown for LAT1, LAT2, and 4F2hc in MVM isolated from rat placenta at GD19. B, The histogram summarizes the Western blotting data from GD15 (C, n = 5; LP, n = 4), GD19 (C, n = 5; LP, n = 4), and GD21 (C, n = 5; LP, n = 4). After normalization to β-actin, the mean density of C samples was assigned an arbitrary value of 1 at each gestational age. Subsequently, individual C and LP density values were expressed relative to this mean. Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

of mTOR, is unlikely to contribute to the decreased mTOR activity, because placental AMPK phosphorylation was unaffected by maternal protein restriction. Although changes in total maternal amino acid concentrations are limited in pregnant rats fed a LP diet, the concentrations of some individual amino acids do change (20). In particular, maternal plasma concentrations of leucine, an essential amino acid well established to be a potent regulator of mTOR activity (59, 60), were significantly reduced. It is therefore possible that lower leucine concentrations could contribute to the placental mTOR inhibition by GD19.

Placental STAT3 phosphorylation was significantly decreased in LP rats on GD21. We have recently demonstrated that IL-6 stimulates system A amino acid transport in cultured trophoblast cells mediated by increased Tyr-705 phosphorylation of STAT3 (26). Furthermore, blocking endogenously produced leptin in placental villous fragment also decreased system A transport activity through a STAT3-dependent mechanism (61). This indicates that STAT3 inhibition, possibly due to low maternal leptin levels (20), may contribute to down-regulation of placental amino acid transporters at GD21 independent of mTOR. However, it cannot be excluded that the inhibition of placental STAT3 signaling is independent of the decreased maternal leptin levels in this model, because other cytokines and hormones also are known to signal through STAT3 (39).

In this study, system A activity in microvillous membrane vesicles isolated from placentas of LP rats was decreased at GD19 and GD21. This observation is in agreement with the *in vitro* study of Malandro *et al.* (19) showing down-regulation of system A at GD20 and the *in vivo* study of Jansson *et al.* (20) demonstrating decreased system A-mediated amino acid transport across the placenta at both GD19 and GD21 in rats fed a LP diet. Placental SNAT isoforms were differentially regulated by maternal protein restriction. Although SNAT4 protein expression in MVM was unaffected at all three gestational ages, SNAT2 expression in MVM was markedly down-regulated at GD19 and GD21 in LP rats. Furthermore, MVM SNAT1 protein expression was decreased in response to a LP diet at GD21 but not earlier in gestation. These findings are in agreement with other studies of SNAT isoform regulation in the placenta, suggesting that SNAT2 is a highly regulated isoform (26, 40, 62). We reported previously that SNAT2 expression in placental homogenate was unaltered at GD19 in LP rats (20). There may be several possible explanations for this apparent discrepancy. First, although the SNAT isoforms are primarily expressed in the labyrinth trophoblast of the rat placenta, some SNAT expression is also found in other cells, such as marginal giant cells and fetal endothelium (23). Therefore, our previous determination of SNAT protein and mRNA expression in placental homogenates (20) may not closely represent the expression in labyrinth trophoblast. Second, posttranslational regulation of SNAT isoforms, such as effects on transporter trafficking to the plasma membrane, will go undetected in protein expression measurements using homogenates.

Malandro *et al.* (19), using the same rat model as we did in the current study, demonstrated that the fetal serum concentrations of 10 amino acids were lower in the LP group, six of these were essential amino acids transported by system L (Ile, Leu, Val, Met, Thr, and Tyr). Consistent with these data, we report for the first time a decreased placental system L amino acid transport activity in maternal protein restriction. Collectively, these studies sug-

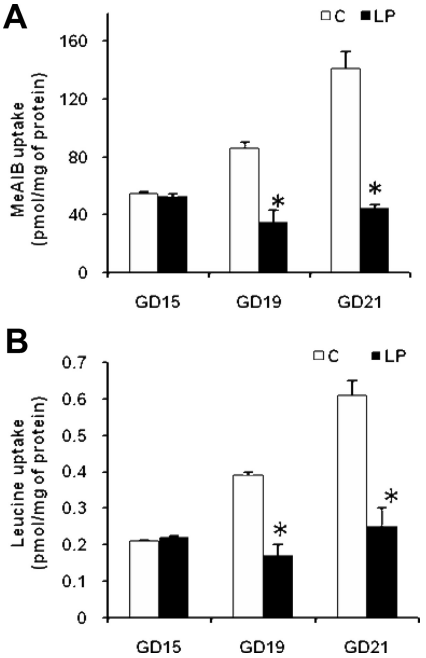

FIG. 8. Activity of system A (A) and system L (B) amino acid transporters in MVM isolated from C and LP placentas. Transporter activities were determined using isotope-labeled substrates and rapid filtration techniques in MVM from GD15 (C, n = 6; LP, n = 5), GD19 (C, n = 5; LP, n = 5), and GD21 (C, n = 5; LP, n = 4). Values are given as means ± SEM; *, $P < 0.05$ *vs.* control; unpaired Student's *t* test.

gest that maternal protein restriction down-regulates placental transfer of essential amino acids, resulting in lower circulating levels of amino acids in the fetus, which may directly limit fetal growth. In addition, because the system L transporter is the primary route for cellular leucine uptake (63), decreased system L activity will contribute to the marked mTOR inhibition observed at GD19 and GD21. A novel finding in the present study is that LAT1 and LAT2 protein expression in placental MVM was reduced at GD19 and GD21 in response to LP diet. To the best of our knowledge, this is the first demonstration of the expression of LAT1 and LAT2 isoforms at the protein level in the rodent placenta. The factors regulating LAT1 and LAT2 expressions in the placenta are largely unknown. However, we have reported that mTOR signaling is a positive regulator of system L activity in human trophoblast cells (31, 32), providing one possible mechanism underlying the decrease in MVM LAT isoform expression in LP rats.

We have reported that maternal leptin, insulin, and IGF-I are significantly decreased in the LP group in late gestation (20). Previous observations in the guinea pig demonstrate that maternal infusion of IGF-I from early pregnancy increases placental uptake and transfer of nutrients, in part by increasing placental transporter gene expression, enhancing placental and fetal growth by midpregnancy (64). Furthermore, IGF-I has been shown to stimulate system A amino acid transport in cultured human trophoblasts (30, 65) and insulin increases amino acid transport in trophoblasts mediated by system A (29, 30) and L (31). In addition, leptin has been reported to stimulate system A amino acid transport in placental fragments (29). It has been shown in skeletal muscle and adipose tissue that translocation of preformed SNAT proteins to the cell membrane can be stimulated by insulin via the PI3K pathway (66, 67). Leptin and insulin activate many of the same pathways, such as MAPK, STAT1 and STAT3, and PI3K. Collectively, these observations suggest that the inhibited placental Akt and STAT3 signaling at GD21 is due to the decreased plasma levels of insulin, IGF-I, and leptin at this stage of gestation and contributes to the down-regulation of placental system A and L amino acid transport on GD21.

Coan *et al.* (68) recently reported that mouse placentas adapt their phenotype both morphologically (relative loss of junctional zone) and functionally (increased the expression of glucose and system A amino acid transporter genes) in response to a 20% global maternal calorie restriction. These data and other observations in mice (69, 70) have provided the basis for the proposal that there are important signals originating in the fetus that initiate compensatory changes in placental structure and function, in particular nutrient transporters, that help maintain fetal growth in situations of restricted nutrient supply. However in our rat model of decreased fetal nutrient availability and IUGR, we found no evidence of compensatory changes in placental signaling or amino acid transport. These distinct responses to maternal undernutrition in the two studies could be due to species differences or related to differences in the severity or type of undernutrition paradigm used. Our data suggest that there are no strong fetal demand signals activated in response to maternal protein restriction in the rat or, alternatively, that fetal demand signals target placental functions not assessed in this study. We speculate that maternal endocrine and metabolic control of placental nutrient transport mediates reduced fetal growth in response to protein restriction and that these maternal signals may be more important for determining placental amino acid transport than regulatory cues originating in the fetus.

Acknowledgments

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