Regulation of placental amino acid transporter activity by mammalian target of rapamycin

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Submitted 24 June 2008; accepted in final form 3 November 2008

Roos S, Kanai Y, Prasad PD, Powell TL, Jansson T. Regulation of placental amino acid transporter activity by mammalian target of rapamycin. Am J Physiol Cell Physiol 296: C142–C150, 2009.—The activity of placental amino acid transporters is decreased in intrauterine growth restriction (IUGR), but the underlying regulatory mechanisms have not been established. Inhibition of the mammalian target of rapamycin (mTOR) signaling pathway has been shown to decrease the activity of the system L amino acid transporter in human placental villous fragments, and placental mTOR activity is decreased in IUGR. In the present study, we used cultured primary trophoblast cells to study mTOR regulation of placental amino acid transporters in more detail and to test the hypothesis that mTOR alters amino acid transport activity by changes in transporter expression. Inhibition of mTOR by rapamycin significantly reduced the activity of system A (−17%), system L (−28%), and taurine (−40%) amino acid transporters. mRNA expression of isoforms of the three amino acid transporter systems in response to mTOR inhibition was measured using quantitative real-time PCR. mRNA expression of L-type amino acid transporter 1 (LAT1; a system L isoform) and taurine transporter was reduced by 13% and 50%, respectively; however, mTOR inhibition did not alter the mRNA expression of system A isoforms (sodium-coupled neutral amino acid transporter-1, -2, and -4), LAT2, or 4F2hc. Rapamycin treatment did not significantly affect the protein expression of any of the transporter isoforms. We conclude that mTOR signaling regulates the activity of key placental amino acid transporters and that this effect is not due to a decrease in total protein expression. These data suggest that mTOR regulates placental amino acid transporters by posttranslational modifications or by affecting transporter translocation to the plasma membrane.

system A; system L; taurine transporter

APPROXIMATELY 15% of all pregnancies result in abnormal fetal growth, either intrauterine growth restriction (IUGR) (1) or fetal overgrowth (2). Babies subjected to altered intrauterine growth are at risk for both short- and long-term complications. IUGR babies have increased perinatal morbidity (8) and are at risk to develop a number of diseases in adulthood, such as cardiovascular disease and type 2 diabetes (4). Fetal overgrowth, resulting in the delivery of a large for gestational age baby, is associated with traumatic birth injuries (11) and the development of metabolic syndrome in childhood (6) and obesity and diabetes in adult life (13, 54). However, the specific mechanisms underlying abnormal fetal growth remain to be fully established.

The most important determinant of fetal growth is the capacity of the placenta to deliver nutrients from the mother to the fetus, which is dependent on the expression and function of nutrient transporters in the placental barrier. Studies in the isolated microvillous membrane (MVM) and basal plasma membrane (BM) of the syncytiotrophoblast, the transporting epithelium of the placenta, have shown that fetal growth abnormalities are associated with alterations of specific placental amino acid transporters. In the fetal overgrowth MVM, system A and system L activities have been reported to be upregulated (30). However, in one study (42), system A activity has been shown to be reduced in MVM from pregnancies where the mothers had insulin-dependent diabetes. The reasons for these discrepancies are unclear but may be related to differences in the two study populations, and placental weight was increased in one study (30), whereas placental weight was unaffected in the other study (42). In contrast, IUGR is characterized by a decreased activity of placental amino acid transporters. For example, the MVM activity of Na⁺-dependent transporter system A, which transports small, neutral, nonessential amino acids, is reduced in IUGR (15, 24, 46). The activities of transporters for the essential amino acids leucine (system L) and taurine (taurine transporter [TAUT]) are also reduced in MVM and/or BM isolated from IUGR placentas (31, 50). The downregulation of placental amino acid transporters in IUGR could be the cause of the reduced fetal plasma amino acid concentrations seen in this pregnancy complication (12, 16). As amino acids are the primary stimuli for insulin secretion from the fetal pancreas, there may be a direct link between changes in placental amino acid transporter activity and altered fetal growth. We (28) recently reported in a rodent model of protein deprivation during pregnancy that placental system A activity is downregulated several days before IUGR develops. These data suggest that downregulation of placental system A activity is a cause, rather than a consequence, of IUGR in this model.

To better understand the pathophysiology of abnormal fetal growth, it is critical to identify the factors regulating placental nutrient transporters. System A is subjected to extensive regulation, and, in the placenta, it has been shown to be inhibited by IL-1β (64), hypoxia (48), nitric oxide (NO) formation (37), hyperglycemia (21), and growth hormone (20). Insulin (27,
mTOR STIMULATES PLACENTAL AMINO ACID TRANSPORT

C143

36), IGF-I (35), EGF (5), amino acid deprivation (34), cortisol (33), and leptin (27) all stimulate system A transport. The regulation of placental system L and TAUT is less well established. Placental TAUT activity is regulated by taurine itself (32), calcium (41), PKC (40, 59), NO (37, 59), and cyclosporin A (57). Increases in intracellular Ca²⁺ concentrations, PKC, and low extracellular pH (7, 51, 56) all stimulate system L activity.

The mammalian target of rapamycin (mTOR) signaling pathway integrates various extracellular and intracellular signals. Growth factor- and hormone-induced stimulation of mTOR is the best-characterized upstream regulator of mTOR, and it is mediated by the activation of phosphatidylinositol 3-kinase (PI3K) (66). Nutrients represent another major signal input that activates mTOR; however, the exact mechanism of nutrient regulation remains unclear. It has recently been suggested that both the class III PI3K hVps34 (10, 49) and the Ste20-related kinase MAP4K3 (22) play a role in mTOR-mediated nutrient sensing. The best-described function of mTOR is its regulation of translation, which occurs by phosphorylation of the key translation regulators p70 ribosomal S6 kinase (S6K1) and eukaryotic initiation factor 4E-binding protein-1 (4E-BP1). There is some evidence indicating that mTOR regulates the expression of nutrient transporters in nonplacental tissues. In FL5.12 cells, growth factors have been shown to not only increase transporter gene transcription but also promote transporter surface expression (17). Treatment of human BJAB B-lymphoma and murine CTLL-2 T lymphocytes with rapamycin leads to the downregulation of the expression of genes participating in nutrient transport (53). Liu et al. (44) have shown that the expression of l-type amino acid transporter (LAT1), an isoform of the l-amino acid transporter light chain, in vascular smooth muscle cells is stimulated by PDGF in a mTOR-dependent manner. We (58) recently reported that rapamycin, a highly specific mTOR inhibitor, reduced system L activity, but not the activities of system A transporters or TAUT, in primary villous fragments from the human placenta. In these experiments, transport measurements were carried out after 4 h of rapamycin incubation, a time period where the regulation of transport mediated by effects on transcription and translation may not be observable. Considering that the most-established and widely reported cellular effects of mTOR signaling are the regulation of protein translation, we tested the hypothesis that mTOR alters amino acid transport activity by changes in transporter protein and mRNA expression. To this effect, mTOR signaling was inhibited for 24 h in cultured primary human trophoblast cells and, subsequently, the activity, mRNA expression, and protein expression of three key placental amino acid transporters were measured.

METHODS

Tissue Collection

Placental tissue was collected with informed consent from the Sahlgrenska University Hospital, and the protocol was approved by the Committee for Research Ethics of the University of Gothenburg. Placentas were obtained at term from women with uncomplicated pregnancies who gave birth to babies with normal birth weight, which were delivered by cesarean section in all cases except one.

Trophoblast Cell Culture

Cytophrophoblast cells were isolated from human placentas and cultured as previously described (38, 45). Cells were plated in either plastic 6-well plates at a density of ~1.5 × 10⁶ cells/well (for amino acid uptake experiments) or 25-cm² flasks at a density of ~10 × 10⁶ cells/flask (for RNA extraction and Western blot analysis) and cultured for 90 h. Between 66 and 90 h of culture, cells were incubated with 100 nM rapamycin (LC Laboratories, Woburn, MA), a specific mTOR inhibitor, or vehicle (0.02% DMSO). After 90 h in culture, cells were either used in amino acid transporter activity assays or collected for Western blot analysis or mRNA extraction.

Assessment of Biochemical Differentiation and Viability

To confirm that our cells were undergoing biochemical differentiation, and to assess their viability with time in culture, the release of human chorionic gonadotropin (hCG) by trophoblast cells into the culture medium after 18, 42, 66, and 90 h was measured using a commercial ELISA kit, which detects the β-subunit of hCG (DRG Instruments). To verify the trophoblast nature of isolated cells, an anti-cytokeratin-7 (clone OVTL 12/30) antibody (antibody 9098, Abcam, Cambridge, UK) was used. Vimentin was used as a marker for the identification of contaminating mesenchyme-derived cells (antibody 20346, Abcam).

Lactate dehydrogenase (LDH) release into the medium was assessed using a LDH-based in vitro toxicity assay (Sigma-Aldrich). LDH release measured after cells had been subjected to sonication was used as a positive control.

Antibodies directed against caspase-3 (R&D Systems, Minneapolis, MN) and cleaved poly(ADP-ribose) polymerase (PARP; Affinity BioReagents, Golden, CA) were used to determine the presence of apoptosis in cultured cells.

Measurement of Amino Acid Transporter Activity

Amino acid transporter activities were measured according to a method developed for primary villous fragments (27, 58, 59), which we modified for use in cultured trophoblast cells. Briefly, system A [Na⁺-coupled neutral amino acid transporter (SNAT)] and TAUT activities were measured as Na⁺-dependent [¹⁴C]methylaminoisobutyric acid (MeAIB) or [¹³H]taurine uptake, respectively, and system L amino acid transporter (LAT) activity was determined as the 2-aminoo-2-norbornanecarboxylic acid (BCH)-inhibitable uptake of [¹³H]leucine. After 24 h of incubation with rapamycin (100 nM) or vehicle (0.02% DMSO), cells were washed twice with 3 ml of Tyrode solution at 37°C with or without Na⁺ and then incubated for variable times for up to 10 min in 1.5 ml of Tyrode solution (with or without Na⁺ and 1 mM BCH) containing [¹⁴C]MeAIB and [¹³H]leucine or [¹³H]taurine in final concentrations of 10 μM, 50 nM, and 25 nM, respectively. Each condition was studied in triplicate. Uptake was terminated by washing three times with 4 ml of ice-cold Tyrode solution without Na⁺. Cells were lysed in distilled H₂O for 1 h and then denatured in 0.3 M NaOH for 2 h or overnight. The water containing the tracers released from the cells was mixed with scintillation fluid and counted in a β-counter. After denaturation, the protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL) or the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Transporter-mediated amino acid transport was calculated by subtracting the uptake in the buffer, representing the nanomediated uptake, from the uptake in the buffer, representing the total uptake. Uptakes were calculated as picomoles of amino acid uptake per milligram of total protein per 8 min. Time courses of taurine, leucine, and MeAIB uptake in cytotrophoblast cells were performed. Control cells were incubated with vehicle only to obtain amino acid uptake under control conditions. Control amino acid uptakes were arbitrarily set to a value of 1 for the purpose of comparison.
Western Blot Analysis

Cells were harvested in buffer D (250 mM sucrose, 10 mM HEPES-Tris, 0.7 μM pepstatin A, 1.6 μM antipain, and 80 μM aprotinin; pH 7.4 at 4°C) with freshly added phosphatase inhibitor cocktails 1 and 2 (1:100, Sigma-Aldrich) using a cell scraper. Cells were then homogenized by passing the lysate several times through a 20-gauge needle fitted to a syringe. Protein concentrations were determined using the method of Bradford (Bio-Rad). To ensure equal loading of samples, all membranes were reprobed with monoclonal anti-β-actin antibody (Sigma-Aldrich).

S6K1 and 4E-BP1. To confirm that rapamycin does inhibit the mTOR signaling pathway in cultured primary human trophoblast cells, equal amounts of protein [20 μg for phospho-S6K1 (Thr389) and phospho-4E-BP1 (Thr70)] were separated on NuPAGE Novex (Invitrogen, Lidingo, Sweden) precast 4–12% (phospho-S6K1) or 10% (phospho-4E-BP1) Bis-Tris gels. All antibodies were purchased from Cell Signaling Technology (Danvers, MA), and antibody incubations were carried out according to the protocol provided by the manufacturer except for the phospho-4E-BP1 (Thr70) antibody, which was diluted 1:2,000 to 0.04 μg/ml. Duplicate gels were probed with antibodies to total 4E-BP1 and S6K1.

Snat2 and Snat4. For SNAT2 Western blots, samples were prepared in Laemmli buffer and heated for 5 min at 95°C. For SNAT4 immunobLOTS, a 3 × DTT sample buffer (8 M urea, 170 mM SDS, 0.04 units of bromphenol blue, and 450 mM DTT in 50 mM Tris-HCl; pH 6.8) was used, and samples were heated for 3 min at 100°C before being loaded. Protein (20 μg) was separated on precast 4–12% Bis-Tris gel (Invitrogen) and transferred onto a nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in PBS-0.1% Tween (PBST) and then washed in PBST (3 × 5 min). The SNAT2 antibody (43) was diluted 1:4,000 to 0.25 μg/ml and incubated overnight at 4°C. The SNAT4 antibody was raised against the amino acid sequence YGEVEDELHAYSKV of human SNAT4 (Eurogentec, Seraing, Belgium) and was used at a dilution of 1:4,000 (0.5 μg/ml).

Table 1. Primers used in PCR amplification

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<th>Gene Name</th>
<th>Oligonucleotide Sequence</th>
<th>GenBank Accession Number</th>
<th>Reference</th>
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SNAT, Na+-coupled neutral amino acid transporter; SDHA, subunit A of succinate dehydrogenase complex; LAT, 1-type amino acid transporter; TBP, TATA box-binding protein.

mRNA Isolation and Quantitative Real-Time PCR

The relative mRNA expression of amino acid transporter was analyzed by quantitative real-time PCR using a LightCycler (Roche). Total RNA was extracted from trophoblast cells, and first-strand cDNA synthesis was then performed as previously described (21). Oligonucleotide primers for subunit A of succinate dehydrogenase complex (SDHA) and TATA box-binding protein (TBP) were designed using the Primer3 program (version 0.4.0, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). SNAT1, SNAT2, SNAT4, SDHA, LAT1, LAT2, 4F2hc, and TBP primers were synthesized by Cybergene (Huddinge, Sweden), and primer sequences are shown in Table 1. For detection of LAT2 (NM_000343) mRNA, the Hs_SLC6A6 Quantitect Primer Assay from Qiagen (Sola, Sweden) was used. For Cybergene-synthesized primers, real-time PCRs were performed in 20 μl mixtures containing 2 μl cDNA (diluted 1:4), 2 mM MgCl2 (LAT1, SNAT2, SNAT4, and SDHA) or 3 mM MgCl2 (LAT2, SNAT1, 4F2hc, and TBP), 0.5 μM of each primer, and 2 μl of LightCycler FastStart DNA Master SYBR Green I (Roche) and carried out according to the manufacturer’s instructions. For the detection of TAU, real-time PCR was performed in 20 μl using the Quantifast SYBR Green PCR Kit (Qiagen) following the manufacturer’s instructions. The mitochondrial protein SDHA and TBP served as internal controls (47). All samples were assayed in duplicate, and water was used as a negative template control.
control. A standard curve for each gene product was generated using a dilution series of cDNA (1:2–1:32). The amplification transcripts were quantified using the relative standard curve and normalized against SDHA and TBP by calculating a normalization factor by averaging SDHA and TBP using the geometric mean. The mean mRNA expression value of control cells for each gene was assigned an arbitrary value of 1 for comparison purposes, and the mean mRNA expression of rapamycin-treated cells was expressed relative to control cells.

Data Presentation and Statistics

The number of experiments (n) represents the number of placentas studied. When amino acid uptake was studied, three separate wells were studied for each condition, and data were averaged to represent that placenta. Values are means ± SE. To evaluate differences between groups, either the nonparametric Kruskal-Wallis test followed by the Mann-Whitney U-test (hCG measurements) or the Wilcoxon signed-ranks test was used (amino acid uptake experiments and Western blot analysis). Spearman’s correlation coefficient (r) was used to determine whether the time courses of amino acid uptake were linear.

RESULTS

Cell Characterization

After 66 h in culture, there was a marked increase in hCG production by trophoblast cells, and the levels remained high until at least 90 h (Fig. 1A). There were no differences in hCG production after cells had been incubated with rapamycin for 24 h (5,229 ± 1,719 mIU·mg protein⁻¹·h⁻¹) compared with control cells incubated with vehicle (4,047 ± 989 mIU·mg protein⁻¹·h⁻¹, n = 8, P = 0.12). After 90 h in culture, total cell lysates were analyzed by Western blot analysis for the presence of cytokeratin 7, a trophoblast-specific cytokeratin, and vimentin, a mesenchyme cell marker. Cultured cells were cytokeratin positive, confirming that cells were trophoblasts, and vimentin-negative, suggesting no contamination with cells of mesenchymal origin (Fig. 1B).

Cell Viability

LDH release. LDH release from cells incubated with media containing rapamycin [0.032 ± 0.011 optical density units (OD) at 490 nm, n = 3] was not different from LDH release from cells grown in control media (0.026 ± 0.011 OD at 490 nm, n = 3). LDH release in cells subjected to sonication was almost 10 times greater than cells grown in rapamycin-containing media (0.26 ± 0.02 OD at 490 nm, n = 3).

Expression of apoptotic markers. Both control and rapamycin-treated trophoblast cells were PARP negative (Fig. 1C), demonstrating that apoptotic activity in our cells is low and that rapamycin does not increase apoptosis. This conclusion was strongly supported by data showing that there were no differences in the expression of active caspase-3 between control and rapamycin-treated cells (Fig. 1C), which is in line with a report (68) demonstrating that trophoblast cells do express active caspasas.

Amino Acid Transporter Activity

Na⁺-dependent uptakes of [³H]taurine (25 nM) and [¹⁴C]MeAIB (10 μM) as well as the BCH-inhibitable uptake of [³H]leucine (50 nM) were all linear up to at least 10 min [system-β, r = 0.84, P < 0.01, n = 9 (Fig. 2A); system A, r = 0.95, P < 0.01, n = 9 (Fig. 2B); and system L, r = 0.89, P < 0.01, n = 16 (Fig. 2C)]. Based on these time course experiments, an incubation time of 8 min was chosen in subsequent experiments of the effect of mTOR inhibition on trophoblast amino acid uptake.

After 66 h in culture, trophoblast cells isolated from the human placenta were incubated with 100 nM rapamycin or vehicle (0.02% DMSO) for 24 h, and the uptakes of [³H]leucine, [¹⁴C]MeAIB, and [³H]taurine into cells were then measured. As shown in Fig. 3, rapamycin inhibited TAUT uptake by 40% (n = 7, P < 0.05), system A uptake by 17% (n = 8, P < 0.05), and system L uptake by 28% (n = 8, P < 0.05).

Fig. 1. Trophoblast cell characterization. A: production of human chorionic gonadotropin (hCG), which is associated with trophoblast differentiation, in isolated trophoblasts after 18 h (n = 8), 42 h (n = 6), 66 h (n = 7), and 90 h (n = 8) in culture. Secretion of hCG was significantly higher in trophoblasts cultured for 66 and 90 h compared with cells cultured for 18 h. Values are means ± SE. *P = 0.001 vs. 18 h by the Kruskal-Wallis test followed by the Mann-Whitney U-test. B: representative Western blot of the trophoblast-specific marker cytokeratin 7 (CK-7, top) and vimentin (bottom), a marker of mesenchymal cells. CK-7 showed positive staining in both vehicle-treated (lanes 1, 3, 5, and 7) and rapamycin-treated (lanes 2, 4, 6, and 8) cells. Lane 9 is a rat heart homogenate, which served as a negative control. The vimentin antibody only showed positive staining in the human placental homogenate sample (lane 1). Trophoblast cells (lanes 2–8) were vimentin negative. C: protein expression of caspase-3 (top) and cleaved poly(ADP-ribose) polymerase (PARP; bottom). Both vehicle-treated (lanes 1, 3, 5, and 7) and rapamycin-treated (lanes 2, 4, 6, and 8) cells were PARP negative. The anti-caspase antibody showed positive staining in both cells incubated with rapamycin and vehicle. Lane 9 is a cell lysate of Jurkat cells treated with staurosporine, which was used as a positive control.
Protein Expression

To confirm that rapamycin inhibits mTOR activity in isolated trophoblasts, the phosphorylation states of S6K1 and 4E-BP1, two downstream targets of mTOR, were measured in cells. Rapamycin completely abolished the phosphorylation of S6K1 at Thr389 (Fig. 4A), a site believed to be directly phosphorylated by mTORC1 and that is essential for enzyme activity. 4E-BP1 undergoes phosphorylation at multiple sites, and we studied three of them: Thr37/46 with one antibody and Thr70 with another antibody. Phosphorylation of 4E-BP1 is hierarchical; phosphorylation of Thr37/46 is required for further phosphorylation at Thr70. Both phosphorylation at Thr37/46 and phosphorylation at Thr70 were significantly inhibited by rapamycin treatment (−21% and −37%, respectively, n = 8, P < 0.05; Fig. 4B). No changes were observed in total S6K1 and total 4E-BP1 expression in rapamycin-treated cells compared with control cells (Fig. 4, A and B, respectively).

The TAUT antibody detected a band at ~70 kDa. Two distinct bands at ~48 and 55 kDa were observed in cells when the SNAT2 antibody was used; both bands were analyzed, and the sum of the two bands was used as the densitometry value for that sample. SNAT4 was detected at ~62 kDa. 4F2hc and LAT2 proteins were identified at 80 and 40 kDa, respectively. A MVM sample from the human placenta was used as a positive control (Fig. 5). The specificity of the SNAT2, SNAT4, and LAT2 bands was verified by incubating the primary antibodies with their respective blocking peptides (data not shown). Trophoblast expression of these amino acid transporter proteins was unaffected by rapamycin treatment (Fig. 5).

mRNA Expression of Amino Acid Transporters

As shown in Fig. 6, the quantity of SNAT1, SNAT2, SNAT4, 4F2hc, and LAT2 mRNA relative to SDHA and TBP mRNA for each sample was unchanged in rapamycin-treated cells (n = 6) compared with vehicle-treated cells (n = 6). mRNA expression of LAT1 and TAUT was downregulated (~13% and ~50%, respectively) in cells incubated with rapamycin for 24 h (n = 6, P < 0.05 by the Wilcoxon signed-ranks test; Fig. 6).

DISCUSSION

The activity of system A transporter (15, 24, 46), system L transporter (31), and TAUT (50) are reduced in the IUGR placenta, and we (58) and others (67) have recently reported that placental mTOR signaling activity is markedly downregulated in IUGR. Here, we show for the first time that mTOR inhibition decreases the activity of system L, system A, and taurine amino acid transporters in primary trophoblast cells. Despite decreased mRNA expression of TAUT and LAT1 in rapamycin-treated cells, protein expression of the transporters was not significantly changed after mTOR inhibition. These findings demonstrate that a change in mRNA expression is not necessarily paralleled by a similar change in protein expression. We propose that mTOR regulates placental amino acid transporters by posttranslational modifications or by affecting transporter translocation to the plasma membrane. Collectively, these data are consistent with the possibility that pla-

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Fig. 2. Time course of amino acid uptake into trophoblast cells. Taurine uptake (A), methylaminoisobutyric acid (MeAIB) uptake (B), and leucine uptake (C) was measured as the Na⁺-dependent (taurine and MeAIB) uptake or 2-amino-2-norbornanecarboxylic acid-inhibitable (system L) uptake of [³H]taurine (25 nM), [¹⁴C]MeAIB (10 µM), and [³H]leucine (50 nM), respectively. Uptakes were linear until at least 10 min (system β: r = 0.84, P < 0.01, n = 9; system L: r = 0.89, P < 0.01, n = 16; and system A: r = 0.95, P < 0.01, n = 9).

Fig. 3. Effect of mammalian target of rapamycin (mTOR) inhibition on trophoblast amino acid uptake. Inhibition of mTOR by 100 nM rapamycin decreased the activity of taurine (TAUT), system A, and system L transporters in cultured human primary trophoblast cells. Values are means ± SE; n = 7 for TAUT, 8 for system A, and 8 for system L. *P < 0.05 vs. control by the Wilcoxon signed-ranks test.
Cultured human trophoblast cells have been used extensively in studies of placental nutrient transporters (5, 23, 35, 36, 48). Our cells were cytokeratin positive and vimentin negative and showed an exponential increase in the secretion of the syncytial biochemical marker hCG, verifying that we had syncytializing trophoblast cells in our culture without significant contamination of cells derived from the mesenchyme. We (58) have previously shown that mTOR protein is present in the syncytiotrophoblast, and, in the present study, we verified that rapamycin efficiently inhibited mTOR signaling in cultured trophoblast cells as evidenced by decreased phosphorylation of 4E-BP1 and abolishment of the expression of phospho-Thr<sup>389</sup> S6K1 in response to rapamycin. This is a specific downregulation, since the expression of total 4E-BP1 and S6K1 was unaffected. Furthermore, rapamycin did not alter the characteristics of the cells, given that there were no differences in cytokeratin 7 expression, hCG production, LDH release, or protein expression of the apoptotic markers PARP and caspase-3 between rapamycin-treated and control cells.

We demonstrated that inhibition of mTOR signaling decreases the activity of system L, system A, and taurine amino acid transporters in cultured primary trophoblast cells.
suggest that the size of the observed changes (17–40% decrease) in the activity of key amino acid transporters are physiologically relevant. For example, TAUT is the only transporter mediating the uptake of taurine across the MVM, and the only known pathway for MVM uptake of essential amino acids such as leucine is system L. Since the transfer across the MVM is likely to be the rate-limiting step for placental amino acid transport (29), a 40% (taurine) and 28% (leucine) decrease in the uptake of these amino acids in vivo is likely to have significant effects on the fetal amino acid supply. Along these lines, the effects of mTOR inhibition on amino acid transport activity in the present study are of similar magnitude as reported for changes in placental transporter activity in human IUGR. For example, we have previously shown that IUGR is associated with a 34% decrease in TAUT activity in the MVM (50) and a 38% and 46% decrease in system L amino acid transporter activity in the BM and MVM, respectively (31).

The finding that mTOR stimulates system L activity is consistent with our previous finding in primary villous fragments from the human placenta (58). Intriguingly, the effect of mTOR inhibition for 24 h on system A and TAUT activity in cultured trophoblast cells differs from the response to 4 h of inhibition with rapamycin in primary villous fragments in which system A and TAUT activities were unaffected (58). The mechanisms underlying these different findings are currently unknown but may be related to distinct differences in the two experimental systems. Alternatively, the much longer rapamycin incubation time used in the cultured trophoblast cells may explain this discrepancy. It has previously been shown in L6 myotubes that short-term rapamycin incubation (3 h) does not affect system A activity (55), and prolonged exposure to rapamycin has been shown to inhibit mTORC2 function (60), which was previously thought to be rapamycin insensitive. Thus, it is possible that the incubation of our primary trophoblast cells for 24 h in rapamycin also inhibited mTORC2, which may downregulate system A and TAUT activity.

In our experiments of the role of mTOR signaling in the regulation of nutrient transporters, we have used transporter activity as the primary outcome measurement, and the experiments have been undertaken in primary human cells, which we believe contribute to the physiological significance of our findings. In contrast, most previous studies on mTOR regulation of nutrient transporters have focused on the effects on transporter expression in cell lines. For example, in lymphoma cells, rapamycin selectively downregulated the expression of five genes involved in amino acid transport (53). LAT1 mRNA has been shown to be increased in PDGF-treated vascular smooth muscle cells, and this induction was dependent on mTOR (44). In a murine T cell line, cell surface expression of 4F2hc was inhibited by 24-h rapamycin incubation (19). However, there are a few reports in which the effects of mTOR inhibition on transporter activity have been assessed. System A activity in L6 myotubes has been shown to be upregulated by leucine in a mTOR-dependent manner (55), and rapamycin treatment reduced basal glucose uptake in these cells (63). Yeast cells treated with rapamycin have reduced leucine uptake (65), and primary hepatocytes incubated with rapamycin for 18 h have reduced glucose uptake (9). Coexpression of mTOR and Na+-coupled phosphate transporter SLC34A2 and creatine transporter SLC6A8 in Xenopus oocytes stimulated the activity of these transporters (61, 62). Collectively, these findings in the literature indicate that various nutrient transporters are regulated by mTOR signaling, and we extend these observations by showing that mTOR regulates the activity of key amino acid transporters in human primary trophoblast cells. In addition, the regulation of trophoblast transporter activity by mTOR has not previously been reported, identifying a novel regulatory mechanism for this transporter.

mTOR is a serine/threonine kinase that has been shown to control cell growth through the regulation of translation and transcription. However, the effect of mTOR on system A transporter, system L transporter, and TAUT activity in primary human trophoblast cells does not appear to be mediated by these mechanisms, since we demonstrated that protein expression in whole cell lysates of SNAT2, SNAT4, TAUT, 4F2hc, and LAT2 was unchanged in response to rapamycin. For the SNAT2 and SNAT4 isoforms of the system A transporter, the lack of change in protein expression was in parallel to the unaltered mRNA expression. Similarly, the unchanged protein expression of LAT2 and 4F2hc in response to mTOR inhibition was consistent with the unaltered mRNA expression of these isoforms. However, the mRNA expression of LAT1 was decreased in rapamycin-treated cells. Since LAT1 protein expression was not measured in this study, due to lack of commercial available antibodies, we cannot exclude a contribution of expression changes to the decreased system L transporter activity when mTOR signaling was inhibited. With respect to TAUT, the protein expression was not significantly altered in response to rapamycin despite a marked reduction in TAUT mRNA abundance. The mechanism underlying this discrepancy remains to be established. Nevertheless, these data suggest that mTOR regulates trophoblast nutrient transporter activity by posttranslational modifications and/or by affecting transporter translocation to the plasma membrane. We propose that mTOR regulates amino acid transporters by affecting the trafficking of the transporters to the plasma membrane, which would alter the expression and overall activity of the transporter in the plasma membrane but not whole cell protein expression. This suggestion is supported by emerging evidence in the literature (18, 19, 25). Edinger and coworkers (19) demonstrated that the expression of an activated mutant of Akt is sufficient to support cell surface expression of 4F2hc in growth factor-deprived FL5.12 cells, and rapamycin reversed this effect. They also reported that a kinase-inactive mutant of mTOR alters 4F2hc localization in these cells (18).
In the fat body of *Drosophila*, the surface levels of Slimfast, a cationic amino acid importer, were increased by mTOR activation through clonal overexpression of Rheb (25). The possibility that mTOR signaling affects the membrane trafficking of transporters is further supported by recent understanding that prolonged incubation of cells in rapamycin may also inhibit, in addition to mTORC1, mTORC2 (60). mTORC2 regulates the actin cytoskeleton (26), which may constitute a mechanism linking mTOR to nutrient transporter trafficking, which relies on the cytoskeleton. However, further research is required to test this hypothesis.

GRANTS

This work was supported by Swedish Research Council Grants 10838 and 14555, the Swedish Diabetes Association, the Frimurare-Barnhus Direktionen, the Magnus Bergvall Foundation, the Åhlens Foundation, the Wilhelm and Martina Lundgren Foundation, and the Swedish Society for Medical Research.

REFERENCES

mTOR STIMULATES PLACENTAL AMINO ACID TRANSPORT


