

## Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling

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Submitted 30 April 2009; accepted in final form 3 July 2009

**Roos S, Lagerlöf O, Wennergren M, Powell TL, Jansson T.** Regulation of amino acid transporters by glucose and growth factors in cultured primary human trophoblast cells is mediated by mTOR signaling. *Am J Physiol Cell Physiol* 297: C723–C731, 2009. First published July 8, 2009; doi:10.1152/ajpcell.00191.2009.—Inhibition of mammalian target of rapamycin (mTOR) signaling in cultured human primary trophoblast cells reduces the activity of key placental amino acid transporters. However, the upstream regulators of placental mTOR are unknown. We hypothesized that glucose, insulin, and IGF-I regulate placental amino acid transporters by inducing changes in mTOR signaling. Primary human trophoblast cells were cultured for 24 h with media containing various glucose concentrations, insulin, or IGF-I, with or without the mTOR inhibitor rapamycin, and, subsequently, the activity of system A, system L, and taurine (TAUT) transporters was measured. Glucose deprivation (0.5 mM glucose) did not significantly affect Thr172-AMP-activated protein kinase phosphorylation or REDD1 expression but decreased S6 kinase 1 phosphorylation at Thr389. The activity of system L decreased in a dose-dependent manner in response to decreasing glucose concentrations. This effect was abolished in the presence of rapamycin. Glucose deprivation had two opposing effects on system A activity: 1) an “adaptive” upregulation mediated by an mTOR-independent mechanism and 2) downregulation by an mTOR-dependent mechanism. TAUT activity was increased after incubating cells with glucose-deprived media, and this effect was largely independent of mTOR signaling. Insulin and IGF-I increased system A activity and insulin stimulated system L activity, effects that were abolished by rapamycin. We conclude that the mTOR pathway represents an important intracellular regulatory link between nutrient and growth factor concentrations and amino acid transport in the human placenta.

placental transport; insulin; insulin-like growth factor I; mammalian target of rapamycin

INTRAUTERINE GROWTH RESTRICTION (IUGR) and accelerated fetal growth represent two important clinical conditions that occur in 15% of all pregnancies (1, 2). Aberrant fetal growth is associated with an increased risk of perinatal morbidity (7) as well as metabolic abnormalities in adult life, such as obesity, type 2 diabetes, and cardiovascular disease (6, 12, 46). The most important determinant of fetal growth is nutrient availability, which is highly dependent on placental transport capacity. The mechanisms underlying altered fetal growth remain to be established, but accumulating evidence implicates changes in the activity of specific placental amino acid transporters as a critical factor contributing to abnormal fetal growth (27, 54).

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Experimental evidence supports the hypothesis that changes in placental nutrient transporter activity are a cause of rather than a response to altered fetal growth. For example, in pregnant rats subjected to protein malnutrition, it is likely that downregulation of the placental system A amino acid transporter directly contributes to the development of IUGR (26).

In IUGR, fetuses may be hypoglycemic (15) and have reduced circulating levels of insulin (43) and IGF-I (4, 34). The maternal levels of glucose (15) and IGF-I (40, 41) may also be reduced in this condition. The placenta of the IUGR fetus could therefore be exposed to decreased levels of glucose, hormones, and growth factors. Both insulin and IGF-I stimulate placental system A activity (24, 30, 31). These results suggest that extracellular cues regulate placental nutrient transporters and, as a consequence, fetal nutrient supply, but the cellular mechanisms remain to be fully established.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is regulated by a multitude of intracellular and extracellular signals. For example, mTOR is activated by growth factors and nutrient levels, such as amino acids (59), and inhibited by numerous stress conditions, such as cellular energy depletion (13, 17). Glucose may also regulate mTOR signaling through energy production in the form of ATP (13, 17). The AMP-activated protein kinase (AMPK) is regulated by the AMP-to-ATP ratio, which rises under nutrient deprivation and activates AMPK (10). Activated AMPK can in turn phosphorylate tuberous sclerosis complex 2 (TSC2), leading to mTOR inactivation (23). AMPK is phosphorylated and activated by LKB1 (52), and it has been shown that phosphorylation of LKB1 at Ser428 is essential for AMPK activation by metformin, and the authors speculate that LKB1-Ser428 phosphorylation may be a common pathway required for AMPK activation (60). There might also be additional, AMPK-independent, pathways involved in energy depletion. A recent report has shown that REDD1 (regulated in development and DNA damage responses 1) in mouse embryonic fibroblasts is induced by chronic energy depletion, and this in turn leads to inactivation of mTOR complex 1 (mTORC1) measured as phosphorylation of S6 kinase 1 (S6K1) at Thr389, independent of AMPK (55).

Insulin and IGF-I activate the tyrosine kinase activity of its receptors to phosphorylate the insulin receptor substrate 1, which in turn activates phosphatidylinositol 3-kinase (PI3K) to generate PI(3,4,5)P<sub>3</sub>. Phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) binding to Akt leads to the translocation of Akt to the plasma membrane, where it is phosphorylated and activated. The activation of Akt positively modulates mTORC1 func-

tion, by phosphorylating, and inhibiting, TSC2 (reviewed in Ref. 59).

We have previously shown that inhibition of mTOR reduces the activity of placental system L, system A, and the taurine transporter (TAUT) (50). Since the activity of these amino acid transporter systems is downregulated in the placenta in association to IUGR (14, 19, 28, 37, 45) and placental mTOR activity has been reported to be decreased in IUGR (49, 62), it is possible that mTOR signaling plays an important role in regulating placental amino acid transporters *in vivo*. However, the upstream regulators of placental mTOR are unknown. We hypothesized that glucose, insulin, and IGF-I regulate placental amino acid transporter activity by inducing changes in mTOR signaling. To test this hypothesis, human primary trophoblast cells were incubated with media containing various concentrations of glucose, insulin, or IGF-I in the presence or absence of the specific mTOR inhibitor rapamycin. Subsequently, the activity of system L, system A, and the taurine transporter was measured. To investigate whether the AMPK pathway and/or REDD1 is activated in glucose-deprived primary trophoblasts, the protein expression of phosphorylated (P)-Thr172-AMPK $\alpha$ , total AMPK, P-Ser428-LKB1, and REDD1 in control and glucose-deprived cells was also studied.

## METHODS

### *Tissue Collection*

Term placental tissue was collected from healthy women delivering infants of normal birth weight, who were delivered by nonlaboring elective cesarean section ( $n = 14$ ) or vaginally ( $n = 15$ ). Tissue collection was carried out with informed consent and was approved by the Research Ethics Committee at the University of Gothenburg.

### *Trophoblast Cell Culture*

Isolation of trophoblast cells was carried out using the method of Kliman et al. (33) as described previously (36, 50). Briefly, placental tissue was minced and then subjected to trypsin (0.25%, Invitrogen, Carlsbad, CA) and DNase (0.2 mg/ml, Sigma-Aldrich, St. Louis, MO) digestion. The cytotrophoblast cells were then separated out by a discontinuous Percoll gradient and diluted to  $3 \times 10^6$  cells/ml in cell culture media made up of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose and Ham's F-12 Nutrient Mixture containing 10 mM glucose plus 10% fetal bovine serum (FBS), 50  $\mu$ g/ml gentamicin, 60  $\mu$ g/ml benzylpenicillin, and 100  $\mu$ g/ml streptomycin. The cells were then further diluted and plated in either 3 ml cell culture media in 6-well plates (approximately  $1.5 \times 10^6$  cells/well) or in 5 ml media in 25-cm<sup>2</sup> flasks at a density of  $10 \times 10^6$  cells/flask. The day after isolation, cells were washed twice with Dulbecco's phosphate-buffered saline (PBS) warmed to 37°C. Cells were cultured until 90 h and the media were changed daily. A sample of culture media from each day was collected at the same time of day and assessed for human chorionic gonadotropin (hCG) content by a commercial ELISA kit (DRG Instruments, Germany). After 90 h in culture, total cell lysates were analyzed by Western blot for the expression of the trophoblast marker cytokeratin 7 (clone OVTL 12/30, ab9098, Abcam, Cambridge, UK) and the mesenchyme marker vimentin (ab20346, Abcam). Lactate dehydrogenase (LDH) release into the medium was assessed using an LDH-based assay (Sigma-Aldrich). To determine the presence of apoptosis in cultured trophoblast cells, the expression of active caspase-3 (R&D Systems, Minneapolis, MN) and cleaved poly(ADP-ribose) polymerase (PARP; Affinity BioReagents, Golden, CO) was investigated in cells after 18, 42, 66, and 90 h in culture, as well as in cells subjected to 24-h glucose

deprivation between 66 and 90 h in culture. Staurosporine-treated Jurkat cells were used as a positive control for these antibodies.

To study the effect of glucose deprivation, human primary trophoblast cells were cultured until syncytialization at 66 h and then incubated with media containing various glucose concentrations (0.5 mM, 4.5 mM, or 16 mM, which corresponds to the concentration in standard media) and with or without the specific mTOR inhibitor rapamycin (100 nM, Sigma-Aldrich and LC Laboratories, Woburn, MA) for 24 h. All glucose-reduced media contained 10% FBS, 50  $\mu$ g/ml gentamicin, 60  $\mu$ g/ml benzylpenicillin, and 100  $\mu$ g/ml streptomycin and were prepared by mixing glucose-free DMEM with glucose-free or regular F-12 Nutrient Mixture (Ham's). Normal maternal venous glucose concentrations during pregnancy are 4–4.5 mM and have been reported to be significantly lower in IUGR (15). Umbilical venous glucose concentrations in appropriate weight for gestational age (AGA) fetuses are  $\sim$ 1 mM lower than maternal, and have also been shown to be reduced in IUGR (15).

To study the impact of growth factor signaling on amino acid transporter activity, trophoblast cells were cultured in regular cell culture media (containing 16 mM glucose) and then incubated with 60 ng/ml insulin or 300 ng/ml IGF-I (Sigma-Aldrich) from 66 to 90 h in culture, with or without the mTOR inhibitor rapamycin (100 nM). The insulin concentration used in these experiments corresponds to the lowest concentration previously shown to stimulate system A in cultured primary human trophoblast cells (31) and is higher than typical postprandial insulin concentrations in pregnant women, which have been reported to be around 10 ng/ml (48). The IGF-I concentration used (300 ng/ml) constitutes physiological concentrations of IGF-I in third trimester maternal serum (25). Before addition of media to the cells at 66 h, cells were washed once with Dulbecco's PBS warmed to 37°C. At 90 h, cells were used either for transport activity assays or for Western blot analysis.

### *Measurement of Amino Acid Transporter Activity*

The activity of system A, the taurine transporter, and system L was assessed by measuring the Na<sup>+</sup>-dependent uptake of [<sup>14</sup>C]methylaminoisobutyric acid (MeAIB) and [<sup>3</sup>H]taurine and the 2-amino-2-norbornane-carboxylic acid (BCH)-inhibitable uptake of [<sup>3</sup>H]leucine after 24 h in glucose-deprived media or in media supplemented with insulin or IGF-I. Methods to measure amino acid transporter activity have been described in detail previously (50). Briefly, cells were washed twice with 3 ml 37°C Tyrode salt solution with or without sodium and then incubated for 8 min with 1.5 ml Tyrode solution (with or without Na<sup>+</sup> and 1 mM BCH) containing [<sup>14</sup>C]MeAIB and [<sup>3</sup>H]leucine or [<sup>3</sup>H]taurine. The uptake was terminated by washing the cells three times with ice-cold Tyrode solution without sodium. The cells were lysed 1–2 h in distilled H<sub>2</sub>O and denatured with 0.3 M NaOH for 2 h or overnight. The water containing the tracers was counted in a  $\beta$ -counter, and protein content was determined after denaturation by the method of Bradford using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). There was no difference in amino acid transport activity in trophoblast cells isolated from placentas delivered by cesarean section as compared with placentas delivered vaginally (data not shown). To facilitate comparisons, the mean of the control uptake data (i.e., at 16 mM glucose) was assigned an arbitrary value of 1, and the amino acid uptake of cells incubated with low (0.5 mM) or medium (4.5 mM) glucose media is expressed relative to the mean value of the control data.

### *Western Blot Analysis*

Cells were washed twice with ice-cold PBS, and then an ice-cold cell lysis buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1% Triton X-100, and 1 mM dithiothreitol, pH 7.2, with freshly added phosphatase inhibitor cocktails 1 and 2 and a protease inhibitor cocktail, all diluted 1:100 (Sigma-Aldrich), was added. Cells were scraped and collected in a 1.5-ml tube, homogenized by passing



(median = 0.50, IQ range = 0.19), although there was a tendency of a decrease in active caspase-3 expression between 18 and 42 h in culture ( $n = 4$ ,  $P = 0.06$ ). Expression of active caspase-3 was not different in cells incubated with low glucose media for 24 h (median = 0.96, IQ range = 0.29,  $n = 7$ ) when compared with cells incubated in control media for 24 h (median = 1.20, IQ range = 0.34). These data are in line with a previous report demonstrating expression of active caspases in trophoblast cells (63).

#### Amino Acid Transporter Activity

**Effect of decreasing glucose concentrations.** Control values (means  $\pm$  SE) for the activity of system A ( $96 \pm 13$  pmol $\cdot$ mg protein $^{-1}\cdot$ 8 min $^{-1}$ ), system L ( $50 \pm 5$  pmol $\cdot$ mg protein $^{-1}\cdot$ 8 min $^{-1}$ ), and TAUT ( $2.64 \pm 0.32$  pmol $\cdot$ mg protein $^{-1}\cdot$ 8 min $^{-1}$ ) were similar to those reported previously (50). The activity of system L decreased in a dose-dependent manner in response to decreasing glucose concentrations when compared with cells cultured with 16 mM glucose ( $P = 0.012$ ,  $n = 11$ , Fig. 2A), the standard concentration of glucose in cell culture media. Incubation with rapamycin, a highly specific mTOR inhibitor, decreased system L activity ( $P = 0.03$ ) in cells incubated with 16 mM glucose. Additionally, in the presence of rapamycin, decreasing glucose concentrations did not affect system L activity ( $P = 0.18$ , Fig. 2A).

The activity of system A was unaffected by the incubation for 24 h with decreasing glucose concentrations when compared with system A activity in cells cultured with 16 mM glucose ( $P = 0.529$ ,  $n = 11$ , Fig. 2B). However, in the presence of rapamycin, system A activity increased in a dose-dependent manner when compared with cells cultured with 16 mM glucose ( $P = 0.001$ ). Under standard conditions, the addition of rapamycin decreased baseline system A activity ( $P = 0.04$ ). The data suggest that two different mechanisms may regulate system A activity, one that is independent of mTOR and one that is mTOR mediated.

TAUT-mediated transport increased significantly in response to low glucose both in the presence and in the absence of rapamycin (Fig. 2C). Furthermore, the size of the increase was similar with and without rapamycin. These data suggest that the stimulation of TAUT activity by hypoglycemia is largely independent of mTOR signaling.

**Effect of insulin and IGF-I incubation.** Insulin and IGF-I stimulated system A activity ( $P = 0.042$ ,  $n = 6$ , Fig. 3A, open bars), in line with previous reports in the literature (24, 30, 31). However, in the presence of rapamycin, insulin (I + R) and IGF-I (IGF-I + R) did not increase system A activity significantly as compared with cells incubated with rapamycin only (R,  $P = 0.6$ , Fig. 3A, gray bars). Insulin induced a significant stimulation of system L activity ( $P = 0.015$ ,  $n = 9$ , Fig. 3B, open bars). With 16 mM glucose standard media, rapamycin decreased baseline system L activity ( $P = 0.008$ ). The combination of rapamycin and insulin blocked the insulin-stimulated increase in system L activity ( $P = 0.767$ , Fig. 3B, gray bars).

#### Intracellular Signaling Pathways

**mTOR signaling.** The phospho-S6K1 antibody detects the two isoforms of the S6K1 protein, p70 S6 kinase when phosphorylated at Thr389 and p85 S6 kinase when phosphorylated

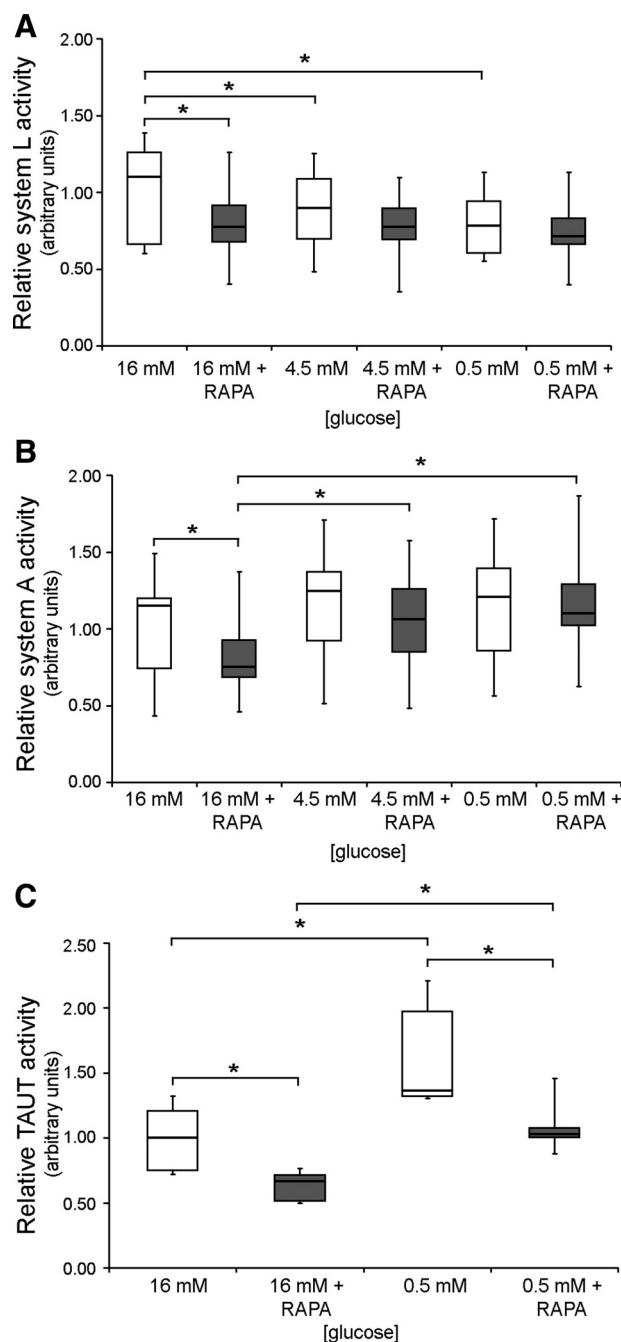


Fig. 2. Effect of decreasing glucose concentrations on amino acid uptake in trophoblast cells. Primary trophoblast cells were cultured for 66 h and then incubated with 16, 4.5, or 0.5 mM glucose [with or without 100 nM rapamycin (RAPA), a specific mammalian target of rapamycin (mTOR) inhibitor] for 24 h. At 90 h of culture, amino acid uptake was measured. **A:** the activity of system L decreased in a dose-dependent manner in response to decreasing glucose concentrations when compared with 16 mM, which corresponds to the glucose concentration in standard cell culture media (white bars). With 16 mM glucose, incubation with rapamycin decreased system L activity (gray bars). In addition, in the presence of rapamycin, decreasing glucose concentrations had no effect on leucine uptake ( $n = 11$ ). **B:** system A activity was unaffected by decreasing glucose concentrations when compared with 16 mM (white bars). However, in the presence of rapamycin (gray bars), system A activity increased in a dose-dependent manner. Under standard conditions (16 mM glucose), incubation with rapamycin decreased system A activity ( $n = 11$ ). **C:** taurine transport (TAUT) increased in response to glucose deprivation (white bars). When incubating cells with 16 mM glucose, rapamycin decreased TAUT activity ( $n = 5$ ). In the presence of mTOR inhibition, glucose deprivation increased TAUT activity ( $n = 5$ ). Values are median and interquartile range. \* $P < 0.05$ .

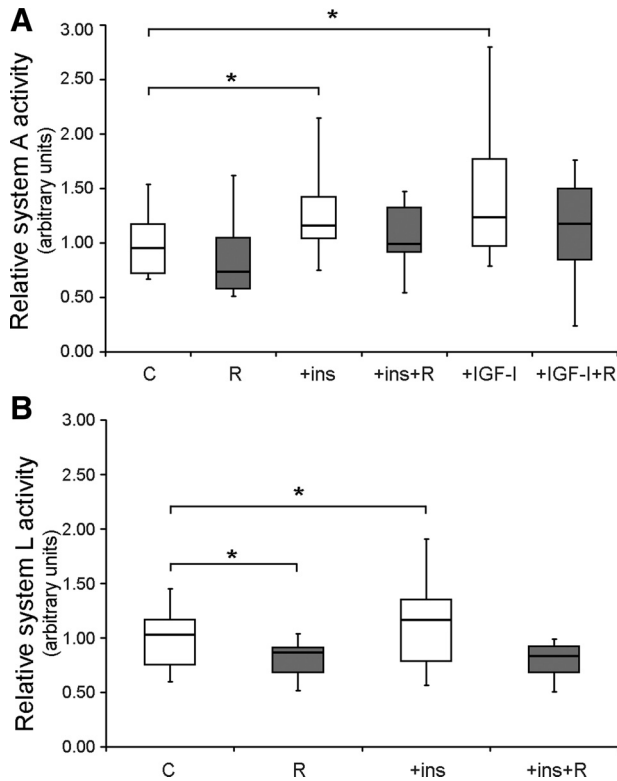


Fig. 3. Effect of insulin and IGF-I on amino acid uptake in trophoblast cells. After 66 h in culture, trophoblast cells were incubated with either 60 ng/ml insulin (ins) or 300 ng/ml IGF-I, with or without the mTOR inhibitor rapamycin (R) for 24 h. *A*: addition of insulin and IGF-I to the cell culture media stimulated the system A transporter (white bars). In the presence of rapamycin, insulin and IGF-I had no effect on system A activity (gray bars,  $n = 6$ ). *C*, control culture conditions. *B*: insulin stimulated the L amino acid transporter (white bars). This effect was abolished when rapamycin was added to the cell culture media (gray bars,  $n = 9$ ). Values are median and interquartile range. \* $P < 0.05$ .

at the analogs site (Thr412). Correspondingly, the antibody directed against total S6K1 detects endogenous levels of both total p70 S6 kinase and p85 S6 kinase. The activity of the mTOR signaling pathway was studied by measuring the protein expression of S6K1 phosphorylated at Thr389, the primary site of mTOR phosphorylation. Phospho-Thr389-S6K1 expression was reduced in cells incubated with 4.5 mM glucose ( $P = 0.053$ ) and in cells incubated with 0.5 mM glucose for 24 h ( $P = 0.009$ ,  $n = 10$ , Fig. 4A) compared with cells incubated with 16 mM glucose. The specificity of this antibody has previously been confirmed by preincubation of the primary antibody with its blocking peptide, which abolished both the 70- and the 85-kDa bands (49). The expression of total S6K1 was unchanged in glucose-deprived cells when compared with cells incubated with standard cell culture media ( $n = 7$ ,  $P = 0.091$ , Fig. 4B). There was no difference in the expression of 4E-BP1 phosphorylated at Thr70 when comparing the three glucose concentrations (data not shown;  $n = 8$ ).

**AMPK pathway.** To investigate whether the AMPK pathway is activated in glucose-deprived primary trophoblasts, the protein expression of P-Thr172-AMPK $\alpha$ , total AMPK, and P-Ser428-LKB1 in control and glucose-deprived cells (16 mM vs. 0.5 mM) was studied. Representative Western blots are shown in Fig. 5. The LKB1-AMPK signaling pathway in

trophoblast cells was unaffected by glucose deprivation [P-LKB1,  $n = 4$ ,  $P = 0.715$  (data not shown); P-AMPK,  $n = 8$ ,  $P = 0.674$ ; AMPK,  $n = 10$ ,  $P = 0.799$ , Fig. 5].

**REDD1.** The protein expression of REDD1 was studied in control (16 mM glucose) and glucose-deprived (0.5 mM glucose) cells to examine the involvement of REDD1 in the inactivation of mTORC1 in response to glucose deprivation. REDD1 expression is shown in Fig. 6 and was not different in cells incubated with glucose-deprived media when compared with cells incubated with control media ( $n = 8$ ,  $P = 0.889$ ).

**ATP levels.** To compare the ATP levels in cells cultured under control conditions with cells cultured under glucose

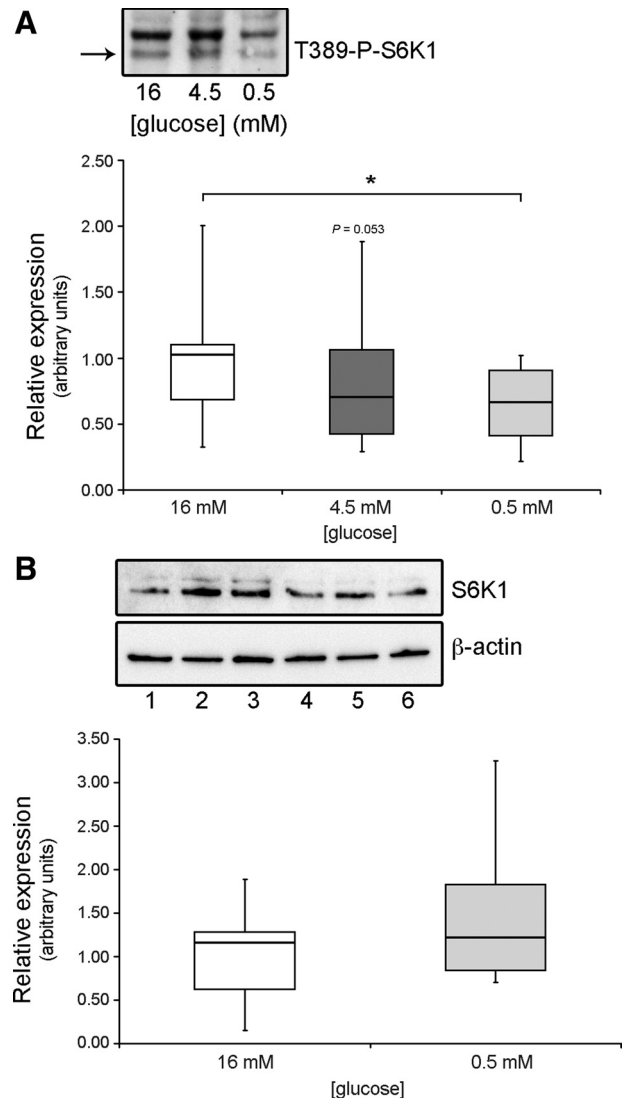


Fig. 4. mTOR pathway signaling in glucose-deprived trophoblast cells. *A*: phosphorylation of S6 kinase 1 (S6K1) at T389 is commonly used to assess the activity of the mTOR signaling pathway. Shown are immunoblots of total cell lysates of trophoblast cells incubated with 16, 4.5, and 0.5 mM glucose. The expression levels of S6K1 phosphorylated at T389 in trophoblast cells are shown in the histogram. Cells exposed to low glucose (0.5 mM) for 24 h had a lower expression of T389-P-S6K1 ( $n = 10$ ) when compared with 16 mM. *B*: the expression of total S6K1 was unaffected by incubating cells with glucose-deprived media (*B*, even-numbered lanes,  $n = 7$ ) when compared with cells incubated with standard media ( $n = 7$ , odd-numbered lanes). Values are median and interquartile range. \* $P < 0.01$ .

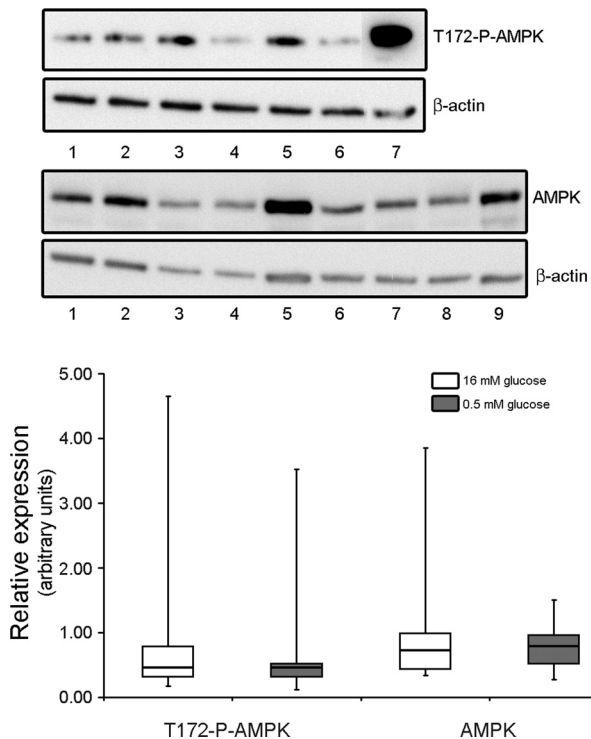


Fig. 5. AMP-activated protein kinase (AMPK) pathway signaling in glucose-deprived trophoblast cells. The expression of phosphorylated (T172-P) and total AMPK is shown. Odd-numbered lanes represent cells incubated with 16 mM glucose media for 24 h and even-numbered lanes represent cells incubated with 0.5 mM glucose media for 24 h. Lane 7 (T172-P-AMPK blot) and lane 9 (total AMPK blot) represent a placental homogenate, used as a positive control. The histogram summarizes the Western blot data, showing that trophoblast expression of total ( $n = 10$ ,  $P = 0.799$ ) and phosphorylated ( $n = 8$ ,  $P = 0.674$ ) AMPK was unaffected by incubation with low glucose media (gray bars). Values are median and interquartile range.

deprivation for 24 h, an ApoSENSOR Cell Viability Assay Kit (BioVision, Mountain View, CA) was used. The levels of ATP in cells were not different between the two culture conditions (C, median = 0.39  $\mu\text{g/ml}$  ATP, IQ range = 0.04; 0.5 mM, median = 0.43  $\mu\text{g/ml}$  ATP, IQ range = 0.17,  $n = 6$ ).

## DISCUSSION

In this study, we show for the first time that glucose deprivation downregulates placental system L activity in an mTOR-dependent manner in cultured primary trophoblasts. Low glucose does not have an effect on system A activity in the absence of rapamycin. However, when mTOR is blocked, decreasing glucose concentrations stimulate system A activity. We further demonstrate that stimulation of system A activity by insulin and IGF-I, a well-known phenomenon, is dependent on mTOR signaling. Furthermore, we show that insulin stimulates system L transport through an mTOR-dependent process. In IUGR, placental system L, system A, and taurine transporters are downregulated (14, 19, 28, 37, 45) and fetal as well as maternal concentrations of glucose and growth factors are reduced (4, 15, 34, 40, 41, 43). Our findings implicate mTOR signaling in the regulation of placental amino acid transporters by glucose and growth factors. We (49) and others (62) have previously shown that placentas from pregnancies complicated by IUGR have reduced mTOR activity, and we

therefore suggest that the downregulation of placental amino acid transport in IUGR may be due to decreased mTOR activity.

In primary trophoblast cells, 24-h glucose deprivation reduced the expression of phospho-Thr389-S6K1, which is frequently used as a cellular readout of mTOR activity (8). The mTOR signaling system has previously been shown to be inhibited in human embryonic kidney (HEK)293T cells and mouse embryonic fibroblasts by short-term incubation with low glucose concentrations (17, 32). We further demonstrate that in the presence of rapamycin, glucose deprivation failed to decrease system L activity in trophoblast cells. Thus, glucose deprivation and rapamycin use a common mechanism to downregulate leucine transport, namely the inhibition of mTOR. Leucine uptake has been shown to be stimulated by glucose deprivation in rat vascular smooth muscle cells (35). This discrepancy could be explained by differences in species and/or cell type.

Under standard culture conditions, we found a significant reduction in placental system A activity after rapamycin incubation, which is in line with our previous studies in cultured trophoblast cells (50). Glucose deprivation did not have an effect on system A activity in the absence of rapamycin, as seen previously in HepG2 hepatoma cells where cells deprived of glucose for 6 and 18 h showed no changes in system A activity (5). However, when mTOR signaling was inhibited, decreasing glucose concentrations stimulated system A activity. We suggest that the lack of apparent effect of glucose deprivation on system A activity in trophoblast cells in the absence of rapamycin is due to two mechanisms regulating system A in opposite directions: 1) inhibition of system A activity mediated by mTOR and 2) an "adaptive" upregulation of system A activity. Adaptive regulation refers to the upregulation of system A activity in response to amino acid deprivation that has been demonstrated in many cell types, including

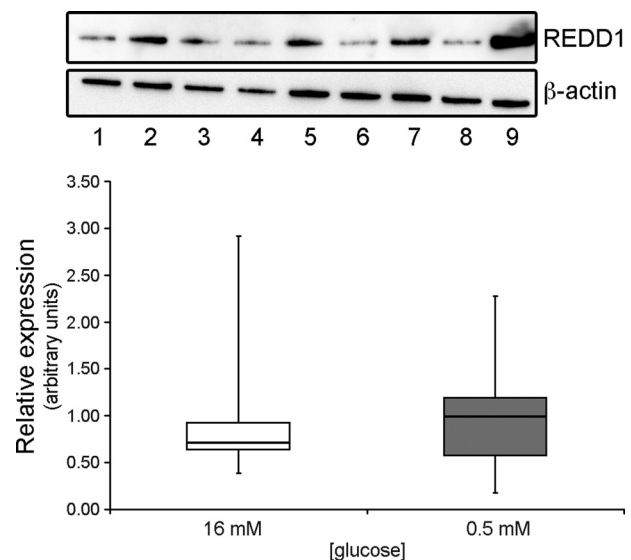


Fig. 6. REDD1 is not activated in glucose-deprived primary trophoblast cells. Expression of REDD1 in cells incubated with low glucose media (even-numbered lanes) and control media (odd-numbered lanes) for 24 h. A Jurkat cell lysate (lane 9) was used as a positive control. The histogram summarizes the Western blot data ( $n = 8$ ,  $P = 0.889$ ). Values are median and interquartile range.

trophoblast cells (29). The basis for this proposal is that, when cells are incubated in rapamycin, the first of these two opposing effects is no longer present and only the second mechanism is observed, i.e., the adaptive upregulation. In BeWo cells, adaptive upregulation of system A may be mediated by translocation of system A amino acid transporter 2 (SNAT2) protein to the plasma membrane and increased SNAT2 mRNA expression (29). To the best of our knowledge, this is the first observation that glucose deprivation may increase system A amino acid activity in trophoblast cells. The underlying mechanisms remain to be established, but they could involve increased SNAT2 expression or SNAT2 translocation to the plasma membrane. It is well established that amino acids are used by the fetus not only as building blocks for proteins but also constitute an important energy substrate. We therefore speculate that upregulation of amino acid uptake in response to low glucose may serve as a physiologically relevant mechanism to ensure the supply of an alternative substrate for energy production.

TAUT activity was stimulated by glucose deprivation, which is consistent with previous findings demonstrating a decreasing TAUT activity in human retinal pigment epithelial cells exposed to hyperglycemia (56). Stimulation of TAUT by low glucose in cultured trophoblast cells does not appear to be controlled by the mTOR pathway, since mTOR inhibition did not block the upregulation caused by incubating trophoblast cells in hypoglycemic medium. Cell swelling, induced by a hyposmotic challenge, leads to an increase in the efflux of taurine from human placental explants (53). In bovine chondrocytes, it has been shown that in addition to increasing taurine efflux, taurine uptake is also stimulated by hyposmolarity (20). It is therefore possible that TAUT uptake in our cells is stimulated by cell swelling following the reduction in medium osmolarity by the removal of glucose. However, the kinetic properties of the volume-activated taurine flux in chondrocytes display behavior typical for a channel or a pore, and in our experiments we are measuring  $\text{Na}^+$ -dependent taurine uptake, suggesting that the observed upregulation of TAUT is not caused by a reduction in media osmolarity. Furthermore, in placental explants, taurine efflux is greatest at osmotic changes above 30% (53), indicating that the changes in media osmolarity by removal of glucose are too small to induce any effect on TAUT activity.

We found that glucose deprivation of primary trophoblast cells inhibits mTOR signaling, indicated by a decrease in phosphorylated S6K1. This is in line with studies demonstrating that incubation with 2-deoxyglucose or glucose deprived media inhibits S6K1 phosphorylation at Thr389 in astrocytes (42), primary cardiac myocytes (38), HEK293 cells (13, 23, 32), and mouse embryonic fibroblasts (17). In cell lines, glucose deprivation has previously been shown to inhibit mTORC1 via the activation of AMPK. Under conditions of energy deprivation that increase the AMP-to-ATP ratio, AMPK becomes active and phosphorylates TSC2, which in turn inhibits mTORC1 (23). Furthermore, evidence was recently provided to suggest that AMPK activation due to glucose deprivation inhibits mTOR signaling mediated by hVps34, independent of TSC2 (9). We therefore tested the hypothesis that AMPK in primary human trophoblast cells is activated in response to low glucose. In contrast to our hypothesis, low glucose did not increase AMPK phosphorylation

significantly, although the relatively high variability in phospho-AMPK expression between samples precludes firm conclusions. However, together with the findings that hypoglycemia did not increase LKB1-phosphorylation, which has been suggested to be essential for AMPK activation (60), or decrease ATP levels, we feel that there is significant support for our conclusions that AMPK is not involved in mediating the effect of low glucose on the mTOR signaling pathway.

The lack of AMPK activation in response to hypoglycemia led us to determine the ATP levels in control cells and in cells subjected to low glucose concentrations. We found that trophoblast cells incubated with glucose-deprived media maintained their ATP-levels at the same values as cells grown in complete media, which is in agreement with the unaltered AMPK activity. Intracellular ATP concentrations may be maintained by intracellular mobilization of glucose from glycogen stores since isolated trophoblast cells contain glycogen (51). ATP could also be produced from other energy substrates such as amino acids or fatty acids. In support of this possibility is the observation that trophoblast cells maintain ATP production in the complete absence of extracellular glucose for at least 8 h (57), suggesting that they indeed can produce ATP from other sources than extracellular glucose. In L6 myotubes, prolonged rapamycin treatment has been shown to increase fatty acid oxidation. The placenta has the capacity to oxidize fatty acids (61) and may switch from glucose metabolism to fatty acid metabolism during glucose deprivation. The capacity of placental mitochondria to generate oxidative energy has been suggested to be low (21), but this has later been questioned and it has been proposed that the mitochondria in the placenta are capable of regular ATP production (22), implicating amino acids as another source of ATP during glucose starvation. Thus, the mechanism linking hypoglycemia to mTOR inhibition in cultured primary human trophoblast cells remains to be established. Our results show that REDD1 is unlikely to be involved. It is possible that in primary trophoblast cells, low glucose levels inhibit mTOR signaling by activation of hVps34 and/or MAP4K3 without involvement of AMPK, in analogy with the signaling event believed to link amino acid deprivation and mTOR signaling (9, 18, 44).

It is well established that system A activity increases in response to insulin in cultured trophoblast cells (31) as well as in primary villous fragments (24). To our knowledge, this is the first time that insulin is shown to activate the system L amino acid transporter. IGF-I has been shown to stimulate system A uptake in cultured trophoblasts (30) as well as in the BeWo cell line (16). In the present study, we extend these studies with the novel finding that insulin and IGF-I stimulate placental amino acid transport through mTOR. Other studies have examined the role of mTOR in the regulation of amino acid transport. Rapamycin did not affect the acute insulin-induced increase in MeAIB uptake in L6 myotubes (39, 47), and incubation of the BeWo cell line with rapamycin had no effect on basal MeAIB transport or on the IGF-I-stimulated system A activity (16). The discrepancies between our study and these reports in the literature may be due to the incubation time during which cells were exposed to rapamycin since less than 1-h incubation time was used in the previous studies. In support of this speculation, we found that mTOR inhibition for 4 h had an inhibitory effect on system L activity in primary human villous fragments but failed to affect system A activity (49).

In the present study, we show that glucose and insulin/IGF-I regulate amino acid transport activity by altering mTOR signaling. We have used primary human trophoblast cells, which we believe to contribute to the physiological significance of our findings. In light of our results, we suggest that mTOR signaling represents an important regulatory pathway for placental nutrient transporters since it is likely that a large number of signals impinge on the placental mTOR signal transduction pathway in vivo. For example, in situations of a compromised nutrient supply to the placenta, it is possible that decreased nutrient concentrations and insulin/IGF signaling may be paralleled by a number of additional stimuli that have been shown to inhibit mTOR signaling, such as energy deprivation (13), hypoxia (3), and low leptin levels (11). Therefore, it is possible that the summation of a number of upstream signals, each only modest in magnitude, is sufficient to explain the downregulation of mTOR and amino acid transporter activity previously demonstrated in the IUGR placenta.

#### GRANTS

This study was supported by grants from the Swedish Research Council (Grant 10838 and 14555), the Swedish Diabetes Association, the Frimurare-Barnhus Direktionen, the Magnus Bergvall Foundation, the Åhlens Foundation, the Wilhelm and Martina Lundgren Foundation, the Anders Otto Swärd Foundation, the Royal Society of Arts and Sciences in Gothenburg, the Herbert and Karin Jacobsson Foundation, and the Royal Swedish Academy of Sciences.

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