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L-Arginine stimulates the mTOR signaling pathway and protein synthesis in porcine trophectoderm cells

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Abstract

Impairment of placental growth is a major factor contributing to intrauterine growth retardation (IUGR) in both human pregnancy and animal production. Results of recent studies indicate that administration of L-arginine (Arg) to gestating pigs or sheep with IUGR fetuses can enhance fetal growth. However, the underlying mechanisms are largely unknown. The present study tested the hypothesis that Arg stimulates the mammalian target of rapamycin (mTOR) signaling pathway and protein synthesis in porcine conceptus trophectoderm (pTr2) cells. The cells were cultured for 4 days in Arg-free Dulbecco's modified Eagle's Ham medium containing 10, 50, 100, 200, 350 or 500 μM Arg. Cell numbers, protein synthesis and degradation, as well as total and phosphorylated levels of mTOR, ribosomal protein S6 kinase 1 (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1), were determined. The pTr2 cells exhibited time (0–6 days)- and Arg concentration (10–350 μM)-dependent increases in proliferation. Addition of 100 and 350 μM Arg to culture medium dose-dependently increased (a) protein synthesis and decreased protein degradation and (b) the abundance of total and phosphorylated mTOR, p70S6K and 4EBP1 proteins. Effects of 350 μM Arg on intracellular protein turnover were only modestly affected when nitric oxide synthesis was inhibited. Collectively, these results indicate a novel and important role for Arg in promoting growth of porcine placental cells largely via a nitric-oxide-independent pathway. Additionally, these findings help to explain beneficial effects of Arg supplementation on improving survival and growth of embryos/fetuses in mammals.

Keywords: Arginine; Conceptus; Placenta; Protein turnover; Fetus

1. Introduction

Intrauterine growth retardation (IUGR), defined as impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy, is a major problem in both human pregnancy [1] and animal production [2]. For example, IUGR infants represent 11% of all newborns in developing countries and also a large number of all newborns in developed nations, e.g., approximately 5% in the United States [1]. In swine, 25% of newborn piglets suffer from IUGR [2], which has a permanent stunting effect on their postnatal growth [3] and efficiency of food utilization [4].

Transfer of nutrients across the placenta is essential for fetal growth and development [5]. Thus, impairment of placental growth is a major factor contributing to IUGR in mammals [6,7]. The placenta is the organ that transports nutrients, respiratory gases and the products of metabolism between the maternal and fetal circulations [8,9]. The blastocyst is composed of two distinct cell layers (trophectoderm cells and the inner cell mass), with the trophectoderm accounting for two thirds of the cells. The trophectoderm is the first epithelium formed in embryonic development, leading predominantly to extraembryonic tissues including the placenta [10]. Interestingly, IUGR is associated with a deficiency of L-arginine (Arg; an amino acid with enormous versatility and importance [11]) in the placenta [6] and fetal fluids [12–14]. Of particular interest, results of recent studies indicate that administration of Arg to gestating pigs [15], sheep [16] and women [17] with IUGR fetuses can enhance fetal growth. However, the underlying mechanisms are largely unknown.

Based on the foregoing, the present study was conducted with the porcine trophectoderm cell line-2 (pTr2) [18] to test the hypothesis that Arg stimulates protein synthesis and growth of placental cells. Because it is now known that certain amino acids activate mammalian target of rapamycin (mTOR), which is the major protein kinase that promotes initiation of polypeptide formation [19], we determined total and phosphorylated (active form) levels of mTOR in the placental cells to identify a molecular mechanism for the effect of Arg on placental protein synthesis. Additionally, because the two

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downstream targets of mTOR are ribosomal protein S6 kinase 1 (p70S6K) and eukaryotic initiation factor 4E-binding protein-1 (4EBP1) [19], the abundance of total and phosphorylated (active form) forms of these two proteins in pTr2 cells was also quantified in the present study.

2. Materials and methods

2.1. Chemicals

We purchased the following chemicals from Sigma-Aldrich (St. Louis, MO, USA): antibiotic–antimycotic solution (P/S), N^G-monomethyl-L-arginine (acetate salt; L-NMMA), D-(+)-α-glucose, Dulbecco's modified Eagle's Ham medium (DMEM)/F-12, fetal bovine serum (FBS, charcoal striped), insulin solution (from bovine pancreas, 10 mg/ml), L-Arg (hydrochloride), L-glutamine, L-leucine, L-phenylalanine and L-proline. Custom-made Arg-free DMEM (Formula #08-5009EF) and 2.5% trypsin solution were obtained from Gibco (Carlsbad, CA, USA). Phosphatase inhibitor cocktail set II was procured from Calbiochem (San Diego, CA, USA), BCA protein assay kit was from Pierce Protein Research (Rockford, IL, USA), and ethylenediaminetetraacetic acid (EDTA, disodium salt) was from Boehringer Mannheim (Indianapolis, IN, USA). L-[Ring-2,4-^{3}H] phenylalanine was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). The sources of other chemicals were previously indicated [20,21].

2.2. Cell culture and preparation

The pTr2 cell line was originally isolated from the elongated porcine blastocysts collected on day 12 of pregnancy and has been previously characterized [18,22] and used for functional studies of porcine trophectoderm. The cells (passages 30–35) were grown in 75-cm^2 flasks containing 15 ml DMEM/F-12 with 5% FBS, 1% P/S and 0.05% insulin. The medium was changed every 2 days. At confluence, the cells were collected using 0.125% trypsin solution (1.25 ml of 2.5% trypsin in 23.75 ml of 0.02% EDTA). After counting the number of cells, they were diluted to 4×10^4 cells/ml in DMEM/F-12 containing 5% FBS, 1% P/S and 0.05% insulin.

2.3. Determination of pTr2 cell growth

Cell growth was determined on the basis of cell numbers on days 0 to 6, as described by Meininger and Wu [23]. Briefly, cells were seeded at 10,000 cells/cm^2 (0.5 ml per well) in 24-well culture plates and cultured overnight (16 h) in complete DMEM/F-12 containing 5% FBS, 1% P/S and 0.05% insulin. The cells were placed for 6 h in 0.5 ml Arg-free DMEM with 5% FBS, 1% P/S and 0.05% insulin to minimize the amount of Arg in the cells. The inclusion of 5% FBS in the Arg-free DMEM provides 10 μM Arg. Concentrations (μM) of amino acids in the Arg-free DMEM, which were based on those in the plasma of gestating swine [15], were as follows: L-cystine, 75; L-glutamine, 500; glycine, 250; L-histidine, 100; L-isoleucine, 150; L-leucine, 250; L-lysine, 200; L-methionine, 75; L-phenylalanine, 100; L-proline, 250; L-serine, 200; L-threonine, 200; L-tryptophan, 75; L-tyrosine, 100; L-valine, 250; L-alanine, 350; L-aspartate, 20; L-asparagine, 50; L-glutamate, 75 and taurine, 100. In experiments to determine effects of Arg on cell growth, pTr2 cells were placed at day 0 in 0.5 ml of DMEM containing 5% FBS, 1% P/S, 0.05% insulin and 10, 50, 100, 200, 350 or 500 μM Arg. The media were changed every 2 days. Eight replicate sets of wells were trypsinized on day 4, and cell numbers were determined with a hemacytometer [24]. Experiments to identify the time course of cell growth in the presence of 10, 100 or 350 μM Arg were conducted as described above except that cell numbers were determined at days 0, 2, 4 and 6. In some experiments, the culture medium contained 400 μM L-NMMA [an inhibitor of nitric oxide (NO) synthase], and the production of nitrite and nitrate (stable products of NO oxidation) was determined by high-performance liquid chromatography as an indicator of NO synthesis [25]. Eight independent experiments were conducted on the basis of statistical power calculation [26].

2.4. Determination of protein turnover in pTr2 cells

The pTr2 cells were seeded at 10,000 cells/cm^2 (2 ml per well) in six-well culture plates with DMEM/F-12 medium containing 5% FBS, 1% P/S and 0.05% insulin. After 16-h culture, the cells were placed for 6 h in 2 ml Arg-free DMEM with 5% FBS, 1% P/S and 0.05% insulin to minimize the amount of Arg in cells. Afterward, the medium was removed and replaced with 2 ml of DMEM containing 5% FBS, 1% P/S, 0.05% insulin and either 10, 100 or 350 μM Arg. There were eight replicate sets of wells per Arg concentration. The culture medium was changed every 2 days.

For determining protein synthesis, culture medium was removed at the end of a 4-day culture period, and the cells were washed once with 2 ml Arg-free DMEM. Then, 2 ml DMEM containing 1 mM L-phenylalanine plus 0.1 μCi L-[ring-2,4-^{3}H] phenylalanine and either 10, 100 or 350 μM Arg was added to each well and cultured for 3 h. At the end of the 3-h culture period, the medium was collected, and cells were rapidly washed three times with 2 ml ice-cold calcium and magnesium-free phosphate-buffered saline (PBS, pH7.4). Protein in the medium was precipitated with 2 ml 10% trichloroacetic acid (TCA) for ^{3}H counting [27]. The cells were scraped from the bottom of the wells after 2 ml of 10% TCA was added to each well, and the whole solution was collected in 15-ml tubes and centrifuged at 3000*g* for 5 min. The supernatant fluid was discarded, and the pellets were washed three times with 5 ml of 2% TCA and dried in air at 37°C. An aliquot (0.5 ml) of 1 M NaOH was added to each cell pellet, and the solution was placed at 37°C for several hours until the pellet was dissolved. Part of the solution (0.4 ml) was transferred to a 20-ml scintillation vial, followed by addition of 15 ml Hionic Fluor Scintillation cocktail (PerkinElmer, MA, USA) for ^{3}H counting after standing overnight at room temperature.

For determining protein degradation, culture medium was removed at the end of a 3-day culture period. The pTr2 cells were then placed in 2 ml DMEM containing 0.1 mM L-phenylalanine plus 0.8 μCi L-[ring-2, 4-^{3}H] phenylalanine and either 10, 100 or 350 μM Arg and cultured for 24 h. After the 24-h culture to label cellular proteins, the cells were washed three times with 2 ml Arg-free DMEM containing 1 mM L-phenylalanine to deplete intracellular free [^{3}H]phenylalanine [27]. Then, 2 ml DMEM containing 5% FBS, 1% P/S and 0.05% insulin and either 10, 100 or 350 μM Arg was added to each well. At the end of a 3-h culture period, cells were rapidly washed three times with 2 ml ice-cold PBS after the medium was collected. The cells were scraped from the wells after addition of 2 ml of 10% TCA. The whole solution was transferred to a 15-ml tube and centrifuged at 3000*g* for 5 min. The supernatant fluid was discarded, and the cell pellet was washed three times with 5 ml of 2% TCA and dried in air at 37°C. An aliquot (0.5 ml) of 1 M NaOH was added to each cell pellet, and the solution was placed at 37°C for several hours until the pellet dissolved. Part of the solution (0.4 ml) was transferred to a 20-ml scintillation vial, followed by addition of 15 ml Hionic Fluor Scintillation cocktail for ^{3}H counting.

For determining the amount of [^{3}H]phenylalanine released from prelabeled proteins into culture medium, the medium was centrifuged at 3000*g* for 2 min to remove dead cells. An aliquot (1 ml) of the supernatant fluid was transferred to a 15-ml tube, followed by addition of 2 ml of 10% TCA. The solution was centrifuged at 3000*g* for 5 min. All the supernatant fluid was transferred to a 20-ml scintillation vial, followed by addition of 15 ml Hionic Fluor Scintillation cocktail for the measurement of ^{3}H radioactivity. The pellet was washed three times with 2% TCA and then dissolved with 1 M NaOH for ^{3}H counting after standing overnight at room temperature. The percentage of protein-bound [^{3}H] phenylalanine released into the culture medium was calculated to indicate protein degradation in pTr 2 cells (namely, [^{3}H]phenylalanine in medium/[^{3}H] phenylalanine in cell protein×100).

Table 1
Antibodies and dilution used for Western blot analyses

Antibody	Catalog number	Dilution
Rabbit monoclonal anti-mTOR (7C10)	2983	1:1000
Rabbit polyclonal anti-phospho-mTOR (Ser2481)	2974	1:1000
Rabbit polyclonal anti-p 70 S6 kinase	9202	1:1000
Mouse monoclonal anti-phospho-P70S6 Kinase (Thr389) (1A5)	9206	1:1000
Rabbit polyclonal anti-4E-BP1	9452	1:1000
Rabbit polyclonal anti-phospho-4E-BP1 (Thr70)	9455	1:1000
Rabbit monoclonal anti-β-actin	4970	1:1000
Horseradish-peroxidase-linked anti-rabbit IgG	7074	1:3000

2.5. Western blot analysis of mTOR-pathway proteins

The pTr2 cells were cultured for 4 days in the presence of either 10, 100 or 350 μM Arg, as described above. Thereafter, the medium was removed, and cells were rapidly

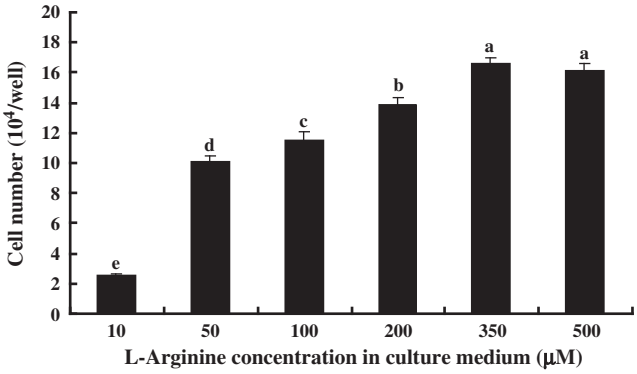

Fig. 1. L-Arginine increased the growth of pTr2 cells. Culture of pTr2 cells in Arg-free DMEM supplemented with either 10, 50, 100, 200, or 350 μM Arg resulted in a dose-dependent increase ($P<.05$) in cell numbers on day 4 of culture. Values are means ± S.E.M., $n=8$. Means sharing different letters (a–e) differ ($P<.05$).

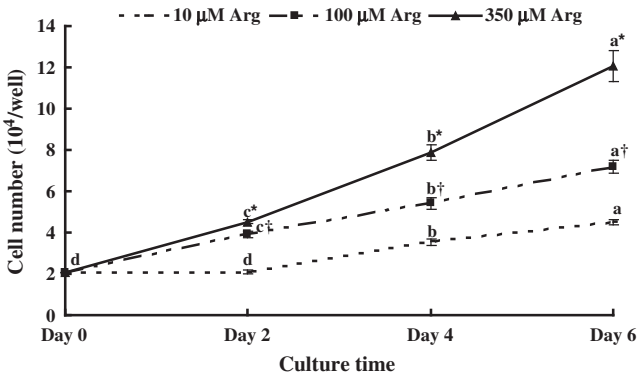

Fig. 2. Time course of pTr2 cell growth at various concentrations of L-arginine. pTr2 cells were cultured in Arg-free DMEM supplemented with either 10, 100 or 350 μM Arg, and cell numbers were determined on days 0, 2, 4 and 6. Values are means ± S.E.M., $n=8$. Within an Arg concentration, means sharing different letters (a–d) differ ($P<.05$) among the four time points. *Cell growth differed ($P<.05$) between the 10- and 350-μM Arg groups at the same time point. †Cell growth differed ($P<.05$) between the 10- and 100-μM Arg groups at the same time point.

rinsed three times with ice-cold PBS. Cells were then lysed for 30 min at 4°C in 0.5 ml of a buffer consisting of 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 30 mM $Na_4P_2O_7$ and 1% protease inhibitor cocktail. The cell lysates were centrifuged (16,000*g* for 15 min at 4°C). Protein concentration in the supernatant fluid was determined using the bicinchoninic acid assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. All samples were adjusted to an equal protein concentration and then diluted with 2× loading buffer [0.63 ml of 0.5 M Tris-HCl (pH 6.8), 0.42 ml of 75% glycerol, 0.125 g sodium dodecyl sulfate (SDS), 0.25 ml β-mercaptoethanol, 0.2 ml of 0.05% bromphenol blue and 1 ml water] to a final volume of 2.5 ml and heated in boiling water for 5 min. After the solution was cooled on ice, it was used for Western blot analysis.

Denatured proteins were separated using SDS–polyacrylamide gel electrophoresis (4%–12% gradient gel) and transferred to nitrocellulose (Bio-Rad, Hercules, CA, USA) overnight at 12 V using the Bio-Rad Transblot apparatus (Hercules, CA, USA). Membranes were blocked in 5% fat-free milk in Tris–Tween-buffered saline (TTBS; 20 mM Tris/150 mM NaCl, pH 7.5, and 0.1% Tween-20) for 3 h and then incubated with the primary antibodies at 4°C overnight with gentle rocking [28]. All antibodies used in these experiments were purchased from Cell Signaling Technology and are listed in Table 1. After washing three times with TTBS, the membranes were incubated at room temperature for 3 h with secondary antibodies. For Western blot analysis of β-actin, the same blots used for mTOR, p70S6K and 4E-BP1 analysis were washed with the Restore Western Blot Stripping Buffer (Pierce, Rockford, IL, USA). The primary antibody and the secondary antibody (horseradish-peroxidase-linked anti-rabbit IgG) were used at dilutions of 1:1000 and 1:3000, respectively. Finally, the membranes were washed with TTBS, followed by development using the Supersignal West Dura Extended Duration Substrate according to the manufacturer's instructions (Pierce, Rockford, IL, USA). The images were detected on Fujifilm LAS-3000 (Tokyo, Japan). Multiple exposures of each Western blot were performed to ensure linearity of chemiluminescence signals. Western blots were quantified by measuring the intensity of correctly sized bands using a ChemiDoc EQ system and Quantity One software (Bio-Rad Laboratories).

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Results were analyzed by one-way analysis of variance and the Student–Newman–Keuls multiple comparison test using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Probability values of $P\leq.05$ indicated significance.

3. Results

3.1. Effects of Arg on pTr2 cell proliferation

Increasing extracellular concentrations of Arg from 10 to 350 μM dose-dependently increased ($P<.05$) the number of pTr2 cells on day 4 of culture (Fig. 1). The effects of Arg on stimulating cell proliferation were observed on days 2, 4, and 6 (Fig. 2). Cell numbers did not differ ($P>.05$) between 350 and 500 μM Arg (Fig. 1).

3.2. Effects of Arg on intracellular protein turnover in pTr2 cells

Increasing extracellular concentrations of Arg from 10 to 350 μM dose-dependently increased ($P<.05$) protein synthesis and decreased ($P<.05$) protein degradation in the pTr2 cells (Table 2). Addition of 400 μM L-NMMA to the culture medium containing 350 μM Arg decreased ($P<.05$) protein synthesis by 13% and increased protein degradation by 21% compared with the 350-μM Arg group (Table 2).

3.3. Effects of Arg on NO synthesis in pTr2 cells

Nitric oxide synthesis was minimal in pTr2 cells cultured in the presence of 10 μM Arg (Table 2). Increasing extracellular concentrations of Arg from 10 to 350 μM dose-dependently increased ($P<.05$) NO synthesis in these cells (Table 2). Addition of 400 μM L-NMMA to the culture medium containing 350 μM Arg reduced ($P<.05$) NO synthesis by 76%, compared with the 350-μM Arg group (Table 2).

3.4. Effects of Arg on mTOR pathway proteins in pTr2 cells

Total and phosphorylated mTOR, S6K1 and 4EBP1 were readily detected in pTr2 cells. Increasing extracellular concentrations of Arg from 10 to 350 μM dose-dependently increased ($P<.05$) the abundance of both total and phosphorylated forms of mTOR (Fig. 3), p70S6K (Fig. 4) and 4EBP1 (Fig. 5).

4. Discussion

L-Arginine has been reported to improve embryonic/fetal survival and growth in several mammalian species, including pigs, rats and sheep [29]. Similarly, intravenous administration of Arg ameliorates fetal growth restriction in women [17]. We recently found that dietary supplementation with Arg increased placental growth in gilts (X.L. Li, F.W. Bazer, G.A. Johnson and G. Wu; unpublished data). To our knowledge, results of the present study are the first to provide biochemical, cellular and molecular data to support the hypothesis that Arg stimulates mTOR signaling and protein synthesis in porcine placental cells, thereby enhancing their growth.

Determining changes in cell number in a culture dish over time is the best available method to assess cell growth [23]. Using this technique, we found that Arg at 50 to 350 μM enhanced pTr-2 cell growth by 4.0- to 6.5-fold compared with 10 μM Arg (Fig. 1).

Table 2
Effects of L-arginine on NO synthesis and protein turnover in pTr2 cells

	Arg concentration in medium (μM)				350 μM Arg+400 μM L-NMMA
	10	50	100	350	
NO synthesis (pmol/h/10^6 cells)	16.2 ± 1.3^e	51.0 ± 2.7^c	78.2 ± 4.4^b	96.0 ± 5.8^a	23.5 ± 1.6^d
Protein synthesis (nmol Phe/mg protein/3 h)	48.4 ± 2.0^d	59.8 ± 3.1^c	70.5 ± 1.5^b	82.6 ± 2.3^a	71.9 ± 1.8^b
Protein degradation (%/3 h)	24.4 ± 2.0^a	18.7 ± 1.2^b	14.1 ± 0.9^c	9.50 ± 0.6^e	11.5 ± 0.7^d

Values are means ± S.E.M., $n=8$. The pTr 2 cells were cultured for 96 h in Arg-free DMEM containing either 10, 100 or 350 μM Arg, or 350 μM Arg plus 400 L-NMMA. At the end of a 96-h culture period, medium was analyzed for nitrite and nitrate as an indicator of NO synthesis, and cells were used for measurement of protein synthesis. Release of ^{3}H-phenylalanine from prelabeled proteins into culture medium was determined to indicate protein degradation in the cells. Means sharing different superscript letters (a–e) differ ($P<.05$).

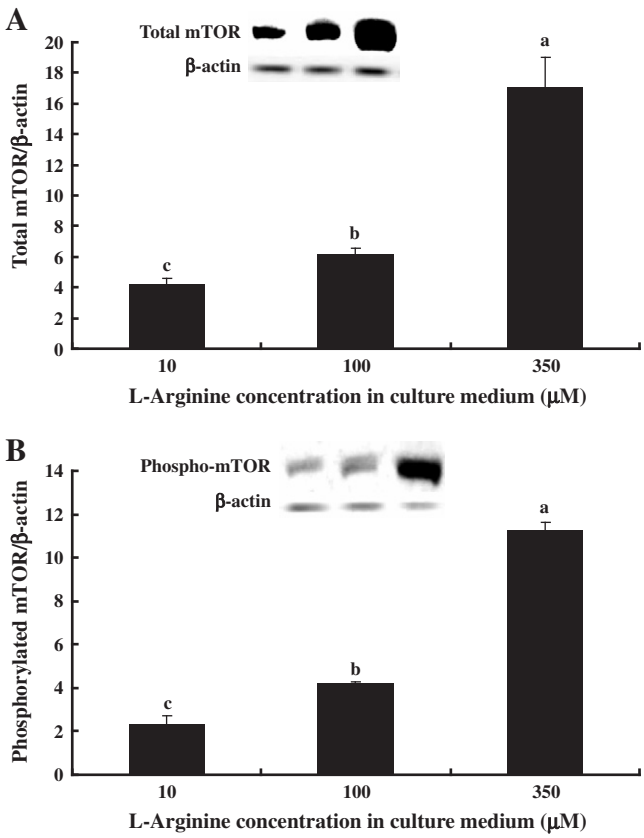

Fig. 3. Abundance of total (A) and phosphorylated (B) mTOR proteins in pTr2 cells cultured for 96 h in the presence of 10 to 350 μM L-arginine. Increasing Arg concentrations in culture medium from 10 to 350 μM enhanced ($P<.05$) the abundance of both total and phosphorylated mTOR proteins in pTr2 cells in a concentration-dependent manner. Data are expressed as means ± S.E.M., $n=8$. Means sharing different letters (a–c) differ ($P<.05$).

However, when extracellular Arg in the medium increased from 350 to 500 μM, there was no further increase in cell growth (Fig. 1). Thus, an optimal concentration of Arg to stimulate pTr2 cell proliferation appears to be 350 μM, which is present in the plasma of gestating gilts receiving dietary supplementation of 0.8% Arg [15]. Accordingly, we conducted all subsequent experiments with 10 to 350 μM Arg.

Cell growth depends on the balance between protein synthesis and degradation [29]. In the present study, we measured the release of protein-bound [^{3}H]phenylalanine into culture medium under the conditions (e.g., the presence of 1 mM unlabeled phenylalanine) of minimizing the incorporation of [^{3}H]phenylalanine into protein [27]. Compared with 10 μM Arg, the addition of 50, 100 or 350 μM Arg to culture medium increased protein synthesis in pTr2 cells by 24%, 46% and 71%, respectively, while decreasing protein degradation in those cells by 23%, 42% and 61%, respectively (Table 2). These results extend the findings recently reported for skeletal muscle of young pigs [30,31], growing–finishing pigs [32], adult rats [33] and enterocytes [34] to explain the Arg-dependent increase in pTr2 cell proliferation (Fig. 2). The effects of physiological levels of Arg in regulating intracellular protein turnover and placental cell growth are interesting and important, as there are marked changes in Arg concentrations in the conceptus during early pregnancy when placental growth is most rapid [1,6]. As for skeletal muscle [32] and enterocytes [35,36], molecular mechanisms for the action of Arg may involve activation of the mTOR protein, which is a master regulator of protein synthesis and possibly autophage-mediated proteolysis [37,38].

Two key downstream targets of mTOR are p70S6K and 4EBP1 [37]. There is growing evidence that Arg activates mTOR and other kinase-mediated signaling pathways in intestinal epithelial cells [36]. For example, Arg enhanced cell migration and activated p70S6K in porcine enterocytes [39]. Additionally, in piglets suffering from severe rotavirus enteritis, the rate of jejunal protein synthesis increased twofold, concomitant with a fourfold increase in jejunal total and phosphorylated forms of p70S6K [36]. Consistent with those reports, we found that when culture medium contained no Arg, the levels of phosphorylated mTOR and p70S6K proteins in pTr2 cells were relatively low, and the cells stopped growing because they could not synthesize Arg from L-glutamine, L-glutamate or L-proline (X.F. Kong and G. Wu, unpublished data). Additionally, our results indicate that Arg supplementation to culture medium can activate the mTOR signaling pathway partly through 4EBP1 and p70S6K phosphorylation in pTr2 cells, as reported for ovine cells [40,41]. For example, 350 μM Arg increased the amounts of total mTOR and phosphorylated mTOR proteins in pTr2 cells by 3.6- and 3.0-fold (Fig. 3), as well as total p70S6K and phosphorylated p70S6K proteins by 6.0- and 1.3-fold (Fig. 4), respectively. Furthermore, Arg enhanced the abundance of total 4EBP1 and phosphorylated 4EBP1 proteins in pTr2 cells in a dose-dependent manner (Fig. 5). In contrast to porcine intestinal epithelial cells (IPEC-J2) [42] or rat intestinal epithelial cells (IEC-6) [43], the fraction of activated p70S6K protein was reduced in arginine-supplemented pTr2 cells compared with the control group of cells. However, it is the total amount of phosphorylated p70S6K that affects protein turnover in cells.

L-Arginine is a physiological substrate for NO synthesis [44]. Nitric oxide, as a major endothelium-derived relaxing factor, plays an important role in regulating placental–fetal blood flows and, thus, the

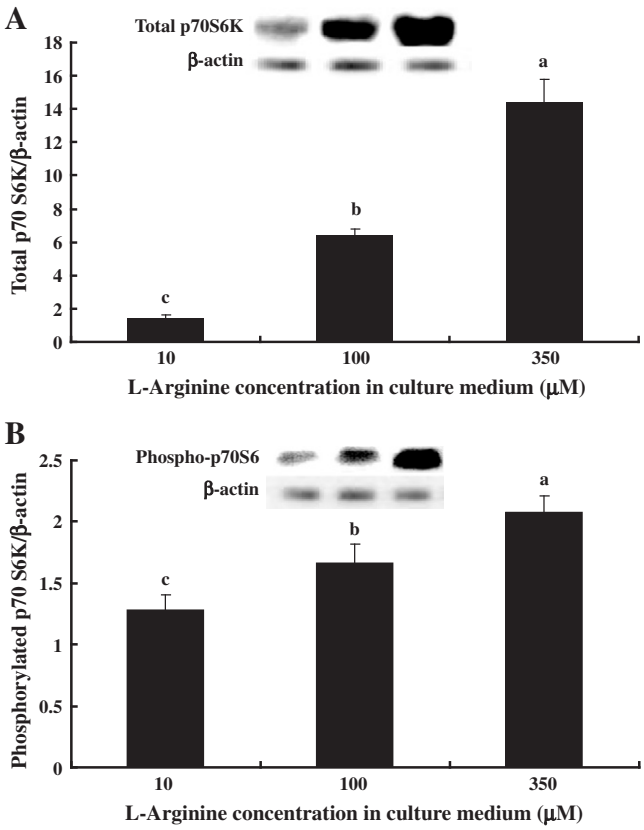

Fig. 4. Abundance of total (A) and phosphorylated (B) S6K1 proteins in pTr2 cells cultured for 96 h in the presence of 10 to 350 μM L-arginine. Increasing Arg concentrations in culture medium from 10 to 350 μM enhanced ($P<.05$) the abundance of both total and phosphorylated S6K1 proteins in pTr2 cells in a concentration-dependent manner. Data are expressed as means ± S.E.M., $n=8$. Means sharing different letters (a–c) differ ($P<.05$).

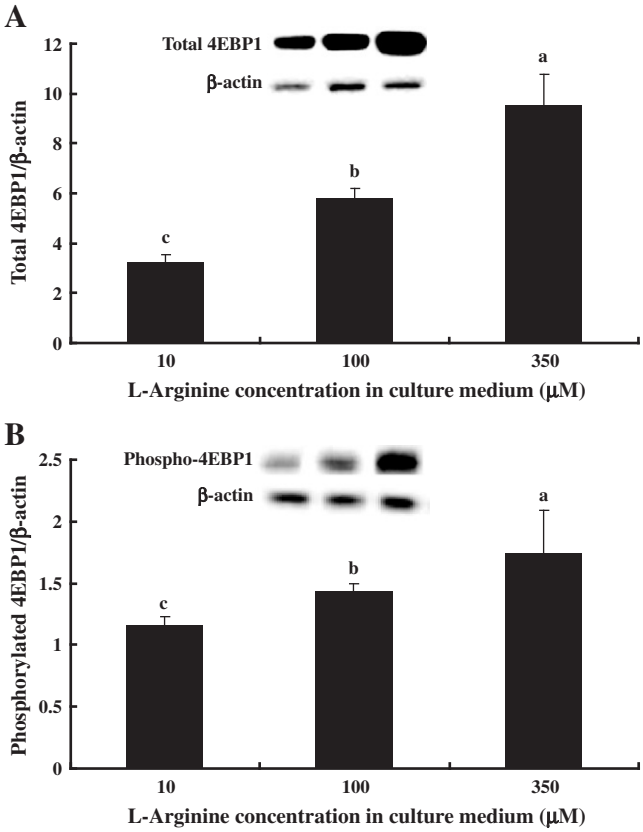

Fig. 5. Abundance of total (A) and phosphorylated (B) 4EBP1 proteins in pTr2 cells cultured for 96 h in the presence of 10 to 350 μM L-arginine. Increasing Arg concentrations in culture medium from 10 to 350 μM enhanced ($P<.05$) the abundance of both total and phosphorylated 4EBP1 proteins in pTr2 cells in a concentration-dependent manner. Data are expressed as means ± S.E.M., $n=8$. Means sharing different letters (a–c) differ ($P<.05$).

transfer of nutrients and O_2 from mother to fetus [45]. Physiological levels of NO may also stimulate DNA and protein synthesis and, therefore, cell proliferation and differentiation [23]. There is also evidence that NO is a key regulator of angiogenesis and embryogenesis [39,46]. Consistent with this notion is the high abundance of Arg in porcine allantoic fluid during early gestation (e.g., 0.17 and 4.1 mM on days 30 and 40, respectively) when placental growth is most rapid [47,48]. Conversely, IUGR in mammals (e.g., pigs and sheep) is associated with reduced concentrations of Arg in the conceptus [6], reduced expression of NO synthase in the placenta and endometrium [48,49], as well as impaired placental and fetal growth [50]. Thus, we previously proposed a hypothesis that inadequate placental synthesis of NO *in vivo* is a major mechanism for IUGR in response to both maternal undernutrition and overnutrition [5,51]. In support of this view, increasing extracellular concentrations of Arg from 10 to 350 μM increased NO synthesis by pTr2 cells in a dose-dependent manner (Table 2). Elevated levels of Arg stimulate NO synthesis in placental cells by enhancing the expression of GTP cyclohydrolase I, which catalyzes the first and rate-controlling step in the de novo synthesis of tetrahydrobiopterin, an essential cofactor for NO synthase [29]. Interestingly, at 350 μM Arg, substantial inhibition of NO only modestly affected protein synthesis or proteolysis in pTr2 cells (Table 2). These results indicate that elevated levels of Arg regulate pTr2 cell growth and protein turnover largely via an NO-independent mechanism, as recently reported for intestinal epithelial cells [42]. It is possible that either Arg itself [52,53] or its other metabolites (e.g., polyamines [54]) activate the mTOR signaling to regulate intracellular protein turnover. Additionally, Arg may modulate the production of other signaling molecules (e.g., carbon monoxide and H_2S [55]) in pTr2 cells. Future studies are required to understand the mode of actions of Arg on pTr2 cells. Nonetheless, an important implication of the present study is that dietary supplementation with Arg may be effective in increasing embryonic and fetal survival and growth in mammals [53,56]. This suggestion is substantiated by findings from recent studies with gestating gilts [15,57,58] and multiparous sows [59], as well as pregnant rats [60] and mice [61].

In summary, addition of 50 or 350 μM Arg to culture medium stimulated growth of pTr2 cells. This effect of Arg involves activation of the mTOR cell signaling pathway that increases protein synthesis and reduces protein degradation largely via an NO-independent mechanism. Results from the *in vitro* studies demonstrate a significant role for Arg in promoting placental cell growth. These findings have important implications for the use of Arg in prevention and treatment of IUGR in mammals, including humans, pigs and sheep.

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