OBJECTIVE: Both phosphorylated (p) mammalian target of rapamycin (mTOR) and protein S6 kinase 1 (p70S6K) are known to regulate protein synthesis and are affected during intrauterine growth restriction (IUGR). We studied the mTOR pathway during hyperthermia (HT)-induced IUGR in sheep.

STUDY DESIGN: Beginning at 40 days gestational age, 4 ewes were exposed to HT for 55 days and 4 were exposed for 80 days to induce IUGR. Western blot analyses were performed for mTOR, p70S6K, 4E-binding protein 1, extracellularly regulated kinase (ERK), and AKT.

RESULTS: HT animals showed: smaller fetuses and placentas near term; reduced placental weight at midgestation; increased p-mTOR, p-ERK, and p-AKT; decreased p70S6K in the near-term cotyledons; decreased p-p70S6K; and increased p-ERK in the caruncles (maternal) near term.

CONCLUSION: Near-term IUGR ovine cotyledons showed up-regulation of p-mTOR, whereas p70S6K was decreased. This suggests that the changes in placental mTOR signaling proteins could be driven by the fetal stress observed near term in this model of IUGR.

Key words: hyperthermia, intrauterine growth restriction, protein S6 kinase 1, mammalian target of rapamycin


The mammalian target of rapamycin (mTOR) protein is a phosphatidylinositol kinase-regulated protein kinase that regulates cell growth in response to nutrients and growth factors.1-3 Downstream effectors in the mTOR pathway include the 70-kDa ribosomal protein S6 kinase 1 (p70S6K) and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1).4,5 Activation-phosphorylation of these proteins by mTOR is known to regulate translation initiation and protein synthesis.3,6 Regulation of mTOR and p70S6K activity is mediated by phosphatidylinositol-3 kinase/AKT signaling.7-9 Under physiologic conditions, activation of p70S6K is also mediated by the activation of the extracellularly regulated kinase (ERK) pathway.10-12 In the human placenta, mTOR has been localized to the syncytiotrophoblast cells, suggesting a role for this protein in nutrient sensing during preg-
The primary hypothesis of this study was that mTOR and its downstream effector proteins would be decreased in the placentae of ovine IUGR pregnancies and that these findings would be true both at term and midgestation. Our specific objectives were to assess the following at midgestation (95 days of gestational age [dGA]) and near term (130 dGA) in the fetal (cotyledon) and maternal (caruncle) components of the placentae: (1) phospho (p)-mTOR protein concentration, (2) activation of p70S6K and 4EBP1, and (3) activation of ERK and AKT.

**Materials and Methods**

**Animal care**

A total of 16 mixed-breed Columbia-Rambouillet ewes with time-dated singleton pregnancies were used for this study, which was approved by the University of Colorado at Denver and Health Sciences Center Animal Care and Use Committee. To induce placental insufficiency and intrauterine growth restriction (PI-IUGR), ewes were exposed to environmental conditions as previously described. Briefly, the environmental conditions consisted of the following: (1) temperature maintained at 40°C for 12 hours during the day and decreased to 35°C at night, and (2) humidity kept between 35–40%.

Eight ewes were placed in the environmental chamber beginning at 40 dGA (term = 147 days) and then separated into 2 groups based on gestational age at necropsy. In the first group, 4 ewes were housed in the environmental chamber for 55 days, and 4 ewes were housed at ambient temperature (20°C) to serve as controls. The IUGR fetuses were diagnosed as having IUGR if their placental weights were reduced compared with the controls and if the umbilical artery Doppler velocimetry studies showed elevated systolic/diastolic ratios. These animals underwent necropsy at 95 dGA (midgestation).

**Arroyo. Placental mTOR and related pathways in IUGR. Am J Obstet Gynecol 2009.**

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FIGURE 2

Increase in cotyledon mTOR and decrease in p70S6K during IUGR

A significant increase of cotyledon mTOR and a significant decrease in p-p70S6K were observed in treated animals near term.

mTOR, mammalian target of rapamycin.
In the second group, 4 ewes were exposed to hyperthermia (HT) conditions for 80 days and were removed to control conditions at approximately 120 dGA, together with 4 ewes kept at ambient temperature as controls. The latter animal group was euthanized at 130 dGA (near term).

All ewes were pair fed and offered water ad libitum. No fetal loss was found in these studies. At the time of necropsy, fetal and placentome weights were recorded. The placentomes were divided into cotyledon (fetal) and caruncle (maternal) components and frozen in liquid nitrogen for Western blot analysis. Whole placentomes were sectioned, fixed in 4% paraformaldehyde, and sent for paraffin embedding for histochemical studies.

**Western blot analysis**

Cotyledon and caruncle tissues were homogenized in protein lysis buffer containing: 10 mM of phenylmethylsulfonyl fluoride, 10 mM of Na₃VO₄, 1% Triton X-100, 150 mM NaCl, 20 mM Tris base, 5 μM of 4-(2-aminoethyl)benzene sulfonyl fluoride, 5 μM of EDTA, 10 nM of E-64, 10 nM of leupeptin, and 10 ng/mL of aprotinin. Tissue lysates containing 50 μg of total protein were separated on 2–14% Bis-TRIS gel and transferred to a nitrocellulose membrane.

Membranes were incubated in rabbit antibodies against phospho-AKT (Thr308), total AKT, phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204), total p44/42 MAPK, phospho-mTOR (Ser2448), total mTOR, phospho-p70 S6 kinase (S6K) (Thr389), total p70S6K (all 1:500 in tris-buffered saline and 0.1% Tween 20 buffer [TBST] with 5% BSA; Cell Signaling Technology, Danvers, MA).

A secondary antirabbit immunoglobulin-horseradish peroxidase antibody (dilution 1:5000) (Cell Signaling Technology) was incubated for 1 hour at room temperature. After rinsing, the membranes were incubated with enhanced chemiluminescence substrate (Amersham, Princeton, NJ) for 5 minutes. The emission of light was detected using x-ray film. Each membrane was stripped of antibodies and reprobed utilizing antibody against mouse beta-actin (dilution 1:4000; MP Biomedicals, Aurora, OH) to confirm loading consistencies in each lane. Presence of these proteins was quantified by densitometry.

**Immunohistochemistry**

Immunohistochemistry (IHC) was performed on paraffin-embedded whole-placentome sections. Slides were dewaxed with 100% xylene. Slide preparation was done using the Mach 2 double-staining reagents from Biocare Medical (Concord, CA). Protocol was followed as suggested by manufacturer. Briefly, slides were incubated with peroxidase for 10 minutes followed by phosphate-buffered saline rinses. Diva was used for antigen retrieval for 20 minutes.

Slides were blocked with Sniper for 15 minutes followed by rinses with Tris-buffered saline (TBS). Slides were incu-
bated for 1 hour with a mouse monoclonal primary antibody against Pan-Cytokeratin (dilution 1:500; Sigma, St. Louis, MO) for trophoblast localization, rabbit anti-mTOR (dilution of 1:500; Cell Signaling Technology) antibody, or a universal immunoglobulin G-negative control (dilution 1:500; Biocare Medical).

Sections were washed in 1×H11003 TBS. Slides were incubated with Mach 2 double-stain polymer for 30 minutes. Color development was done by incubating the slides for 5 minutes with diaminobenzidine (brown color) substrate for the rabbit antibody (mTOR) for 5 minutes and Vulcan fast red (red color) for the mouse antibody (cytokeratin) for 12 minutes. Hematoxylin was used for nuclear counterstaining. Slides were mounted using Permount (Thermo Fisher Scientific, Waltham, MA) mounting media.

**Statistical analysis**

Comparisons of the following endpoints were made between control and IUGR pregnancies: fetal and placental weights, p-ERK, p-AKT, p-mTOR, p-p70S6K, and p-4EBP1. All data were assessed for normality, and treatment effects were determined using Mann-Whitney test, with \( P < .05 \) considered significant.

**RESULTS**

There was no difference in fetal weights between PI-IUGR and control pregnancies at 95 dGA (682 ± 205 g vs 715 ± 11 g; \( P = .79 \)). However, PI-IUGR pregnancies demonstrated significant reductions in fetal weight compared with controls near term (1.8-fold; 1718 ± 433 g vs 2914 ± 201 g; \( P = .008 \)). At 95 dGA, PI-IUGR pregnancies exhibited a statistically significant decrease (2.4-fold; 186 ± 18 g vs 440 ± 50 g; \( P = .003 \)) in placental weight. The difference in placental weights persisted near term (2.0-fold; 169 ± 43 g vs 349 ± 21 g; \( P = .004 \)).

**Cotyledon tissues (fetal side of placenta)**

A characteristic Western blot for near-term cotyledon mTOR, p70S6K, and 4EBP1 is shown in Figure 1. No other Western blot pictures will be shown in interest of space. A significant increase in cotyledon p-mTOR was observed at 130 dGA (1.8-fold). In contrast, cotyledon total mTOR was increased at 95 dGA, whereas p-mTOR was unchanged in treated animals as compared with controls (Figure 2). p-p70S6K and p70S6K were decreased (\( P < .02 \)) in the cotyledon of treated animals near term, whereas p70S6K only was decreased in this tissue at 95 dGA (Figure 2). There was no significant difference observed for 4EBP1 at 95 dGA during PI-IUGR in the sheep, whereas a significant increase in the 4EBP1 (1.5-fold; \( P < .02 \)) protein was seen in the cotyledon near term (Figure 2).

**Caruncle tissues (maternal side of the placenta)**

There was no impact of p-mTOR protein in the PI-IUGR caruncle at either 95 or 130 dGA (Figure 3). Similarly, treatment did not result in differences at either gestational period for the mTOR downstream effector p-4EBP1 protein. However, there was a significant decrease in p-p70S6K in the caruncle tissues near term between treated animals and controls (Figure 3).

**ERK and AKT**

A characteristic Western blot for near-term cotyledon ERK and AKT is shown in Figure 1. When assessing the ERK and AKT proteins in the sheep placenta during IUGR, there was a significant increase for both p-ERK (1.4-fold; \( P < .02 \)) and p-AKT (2.6-fold; \( P < .03 \)) associated with HT treatment in the cotyledon of the sheep placenta near term (Figure 4). In the caruncular tissues, only p-ERK was increased at this gestational point (Figure 5). A decrease in p-AKT was found in the cotyledon at midgestation (Figure 4), whereas no changes were ob-

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**FIGURE 4**

Cotyledon ERK and AKT

ERK and AKT are increased in the cotyledon of IUGR animals near term.

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served for either ERK or AKT proteins in the caruncle of treated animals as compared with controls (Figure 5).

**Immunohistochemistry**

Tissue samples were confirmed to contain trophoblast cells with cytokeratin positive staining. mTOR protein localized to the cytokeratin-positive trophoblast cells (*black arrows*) in the villi of the sheep placentome (Figure 6).

**COMMENT**

In the present study, mTOR and its downstream effectors were not phosphorylated activated under conditions of PI-IUGR at midgestation. At near term, however, we found that the phosphorylation of AKT, mTOR, and ERK proteins was significantly increased, whereas total and phosphorylated p70S6K were decreased in the cotyledon of PI-IUGR animals.

Previous human studies have shown an increase in total mTOR protein during IUGR in the human placenta at term. In the same study, they found a down-regulation of the phosphorylated downstream effector protein p70S6K, whereas 4EBP1 was unchanged. In our results parallel the human IUGR studies, suggesting that a similar mechanism exists for regulating the mTOR pathway proteins in the ovine placenta in this model of IUGR. Previous studies have shown that p70S6K activation is mediated by the activation of AKT and ERK in fibroblast cells; our results showed that although ERK and AKT were up-regulated, activated p70S6K was decreased, suggesting a different mechanism of regulation for this protein in this IUGR model.

mTOR activation is usually associated with the activation of the effector proteins p70S6K and for 4EBP1; this PI-IUGR ovine model showed an increase in phosphorylated mTOR, with a decrease in total and phosphorylated p70S6K and no changes in 4EBP1 near term. Whereas this was an unexpected finding, previous reports have shown changes in mTOR and p70S6K but not 4EBP1 in other cells, suggesting a different mechanism of regulation for the 4EBP1 protein in the mTOR pathway. Furthermore, in vitro studies have demonstrated that the regulation of 4EBP1 occurs via 5 phosphorylation sites that have been identified (Thr 37, Thr 46, Thr 70, Ser 64, and Ser 82). We evaluated only the Thr 37/46 phosphorylation site of the protein, but it may be that other sites are affected differently under conditions of PI-IUGR.

Interestingly, we showed that it is in the period of maximal placental size in the final one-third of pregnancy and near term that mTOR pathway changes occur. This also coincides with the period of maximal slope of growth of the fetus. More specifically, we found that the differences in the regulation of the cotyledon mTOR pathway between mid- and near-term gestation during PI-IUGR could be secondary to protein production required during the peak of fetal growth observed in the latter one-third of pregnancy (130 dGA) in sheep pregnancy.

These results suggest that the increase in mTOR may be a compensatory mechanism attempting to increase protein synthesis and thus fetal growth near term in this model of PI-IUGR. There could be a different factor or mechanism that decreases p70S6K, which could be affecting placental protein synthesis at this point. This is speculative, because mTOR signaling has not been evaluated at midgestation in human IUGR.

When examining the mTOR signaling pathway in the maternal section (caruncle) under conditions of PI-IUGR, we found a significant effect only in total p70S6K expression near term. This is an interesting result, which will require further evaluation. In contrast, mTOR was not changed in this tissue at any other dGA.

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**FIGURE 5**

**Caruncle ERK and AKT**

ERK is increased in the caruncle of IUGR animals near term.

ERK, extracellularly regulated kinase; IUGR, intrauterine growth restriction.

gestational points studied. This is consistent with the IHC experiments, which showed that in this model of HT-induced placental insufficiency and IUGR, mTOR is primarily localized to the cotyledon tissues, suggesting that hyperthermia effects on mTOR pathway endpoints occur primarily in the cotyledon or on the fetal side of the placenta. Although little staining occurred in the caruncular tissues, it is likely that there are still other mechanisms involved in the mTOR regulation in the maternal-oriented, caruncular tissues.

IUGR is a common clinical problem, with several abnormal placental characteristics, which also include an alteration in the expression and activity of placental nutrient transporters that are important for fetal development. Amino acids are known to regulate signal pathways, including the activation of the mTOR pathway. Activation of this pathway regulates cell growth in response to nutrient stimuli. More specifically, this signaling pathway regulates protein synthesis and modulates insulin signals, both of which are important mechanisms that control cell growth.

mTOR has been shown to regulate trophoblast proliferation and differentiation, suggesting an important role for mTOR in trophoblast function within the placenta. Roos et al has further shown mTOR to be expressed in the syncytiotrophoblasts, which are the cells responsible for exchange of nutrients between maternal and fetal blood. This suggests that mTOR is actively involved in the uptake of amino acids for fetal development during pregnancy.

In this study, p-mTOR was increased near term in the cotyledon of treated animals during IUGR, which was associated with an increase in p-AKT and p-ERK. A reduction in phosphorylated and total p70S6K confirms that although mTOR was activated, this was not enough to increase p70S6K for the purpose of increasing protein synthesis in IUGR. Trophoblast cell activation of these proteins in the mTOR pathway regulates protein translation in a synergistic fashion.

Our model is characterized by a smaller placenta with placental insufficiency and a decrease in amino acid uptake. Although placental mTOR expression is increased, this is not sufficient to maintain normal fetal weight in the latter third of gestation in our ovine model of PI-IUGR and may simply represent a compensatory effort by the placenta to increase protein synthesis for fetal benefit. Perhaps this can be explained in part by an increased apoptosis in the cotyledon of HT IUGR animals near term, resulting in decreased amino acid transport because of reduced trophoblast volume, even though mTOR protein is increased. These findings provide further insight into the molecular mechanisms of placental dysfunction at midgestation and near term in our ovine model of IUGR.

**REFERENCES**