# Nutrient Restriction Modulates mTOR Signaling and Ubiquitin-Proteasome System in Skeletal Muscle of Cows and Fetuses

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## Introduction

For generations, animals are selected for enhanced muscle growth. However, the intensive genetic selection for animals with high lean growth efficiency produces meats with inferior quality, such as pale, soft, and exudative (PSE) meat (Fabian et al., 2003; Koohmaraie et al., 2002; Lonergan et al., 2001). The fundamental reason for this discrepancy is that little is known about the molecular mechanisms controlling muscle growth and meat quality.

The mammalian target of rapamycin (mTOR) signaling pathway is critical for sensing nutrient availability and in the nutrient-stimulated muscle protein synthesis (Bodine et al., 2001; Bolster et al., 2003; Sakamoto et al., 2003). The activation of mTOR promotes protein synthesis in muscle (Bodine et al., 2001; Bolster et al., 2003). The ubiquitin-proteasome system is crucially involved in the degradation of cellular proteins (Costelli and Baccino, 2003), which is up-regulated during muscle atrophy (Taillandier et al., 2003).

Maternal undernutrition due to insufficient food supply influences the physiology and development of both mothers and their fetuses (King, 2003). We observed that undernutrition induced skeletal muscle atrophy (Du et al., 2004b). We hypothesize that this muscle atrophy induced by nutrient restriction is due to modulation of protein synthesis and degradation, and mTOR and ubiquitin-proteasome system plays crucial roles in this process. Thus, the objective of the current study was to evaluate the regulation of mTOR signalling and ubiquitin-proteasome system in the skeletal muscle of both cows and fetuses following global nutrient restriction.

## Materials and Methods

### *Animals*

All animal procedures were approved by the University of Wyoming Animal Care and Use Committee. From d-31 to 125 of gestation, 20 Angus × Gelbvieh rotationally crossed cows were separated into two groups which were balanced for body weight so that the average body weight for both groups was around 580 kg. One group of the cows were allotted to a control diet which met the nutrient requirement recommended by NRC (1996). The other half of the cows were allotted to a nutrient restricted diet, which provided 68.1% the net energy requirement for maintenance and 86.7% of metabolizable protein requirements (NRC, 1996) (Du et al., 2004b). Pregnant cows were slaughtered at Meat Laboratory, University of Wyoming on 125 d of gestation. Immediately following slaughter, a sample was taken from the right side of the *longissimus dorsi* muscle at 12th rib of both cows and fetuses. Muscle samples were snap-frozen in liquid nitrogen and then stored at -80 °C until analysis (Du et al., 2004b).

### *Immunoblotting*

Frozen *longissimus* muscle samples (0.1 g) were homogenized in a polytron homogenizer with 5 vol of ice-cold lysis buffer (137 mM NaCl, 50 mM HEPES, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 10% glycerol, 2 mM PMSF, 10 mM sodium pyrophosphate, 2.5 mM EDTA, 10 μg/ml apropinin, 10 μg/ml leupeptin, 2 mM $Na_3VO_4$, 100 mM NaF, pH 7.4). The protein content of lysates was determined by the Bradford method (Bio-rad Laboratories, Hercules, CA). A 5 to 20% gradient gel was used for SDS-PAGE separation of proteins and transferred to nitro-cellulose membrane. Polyclonal anti-mTOR and anti-ribosomal protein S6 antibodies, and phospho-specific antibodies for mTOR ($Ser^{2448}$) and ribosomal protein S6 ($Ser^{235/236}$) were purchased from Cell Signaling Technology Inc. (Beverly, MA 01915) and used as primary antibodies. A horseradish peroxidase-conjugated monkey-anti-mouse antibody was used as a secondary antibody. The density of bands was quantified by using an Imager Scanner II and ImageQuant TL software (Du et al., 2004ab).

For re-probing the membrane, membranes were incubated in a stripping solution (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-Cl pH 6.7) at 50° C for 30 min with shaking. Then, the membranes were thoroughly washed with TBST and ready for immunoblotting as described above.

### *Measurement of AMPK activity*

The AMPK activity was measured as previously reported (Davies et al., 1989). Briefly, muscle homogenate was centrifuged for 5 min at 13,000 × *g* at 4°C. The supernatant was incubated for 10 min at 37°C in 40 mM HEPES, 0.2 mM SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg; Invitrogen), 0.2 mM AMP, 80 mM NaCl, 8% (w/v) glycerol, 0.8 mM EDTA, 0.8 mM DTT, 5 mM MgCl2, and 0.2 mM ATP + 2 μCi [32P]ATPl. An aliquot was removed and spotted on a piece of Whatman P81 filter paper. The filter paper was washed with 1% phosphoric acid and used for scintillation counting.

### *Statistical Analysis*

Data were analyzed as a complete randomized design using GLM (General Linear Model of Statistical Analysis System, SAS, 2000). The differences in the mean values were compared by the Tukey's multiple comparison, and mean values and standard deviation were reported ($P < 0.05$).

## Results and Discussion

The rate of protein translation is tightly regulated by the availability of nutrients. mTOR is the main kinase involved in the sensing of nutritional status in cells and coordinate it with the protein synthesis (Bodine et al., 2001; Bolster et al., 2003; Sakamoto et al., 2003). The mTOR kinase controls translation by phosphorylating 4E-BP1 and S6K (Hara et al., 2002). S6K is activated by phosphorylation and phosphorylates ribosomal protein S6 which drives translation of a small family of abundant transcripts that encode primarily ribosomal proteins and components of the translational apparatus (Jefferies et al., 1997; Schmelzle and Hall, 2000). Thus, activation of mTOR upregulates the translational machinery and promotes protein translation (Schmelzle and Hall, 2000).

The activity of mTOR is controlled by phosphorylation. Phosphorylation at Ser2448 activates mTOR, while phosphorylation at Thr2446 inhibits its activity (Cheng et al., 2004). The phosphorylation of these two sites are mutually exclusive (Cheng et al., 2004). Using an antibody specific to the Ser2448 phosphorylation of mTOR, it was found that there was significant difference in the phosphorylation of mTOR between muscles from control and nutrient restricted cows and fetuses, while no significant difference in the overall content of mTOR was detected (Figure 1), indicating that nutrient restriction down-regulated mTOR signalling. It is largely unclear, however, how the cells sense the availability of nutrients and phosphorylate mTOR kinase. It was reported that Ser2448 phosphorylation is via protein kinase B which can be stimulated by insulin and certain growth factors, while phosphorylation at Thr2446 is accomplished by AMPK, a kinase sensing energy status in cells (Cheng et al., 2004). AMPK is activated by AMP inside cells. Nutrient restriction is expected to increase the content of AMP and thus activate AMPK. To confirm this, the activity of AMPK was analyzed (Figure 2). However, an expected discovery is that the activity of AMPK in the skeletal muscle of nutrient restricted cows was significantly lower ($P \leq 0.05$) that that of control cows (Figure 2). A very possible explanation is due to feedback inhibition. The AMPK in nutrient restricted animals is constantly activated, but this activation results in feedback inhibition. In support of this notion, a point mutation, Arg200 to Gln, in the AMPK γ3 subunit was found in Hampshire pigs, which resulted in a constitutively active AMPK and led to feedback inhibition of AMPK (Milan et al., 2000).

The AMPK activity in pigs carrying this mutation is only one third of the activation of normal pigs (Andersson, 2003; Milan et al., 2000). For the fetal muscle, however, no difference was detected (Table 2).

mTOR can directly phosphorylate S6K and S6K phosphorylates S6 (Bodine et al., 2001; Jefferies et al., 1997). Figure 3 showed the ribosomal protein S6 in the skeletal muscle of both of cows and fetuses. Similar with mTOR, a higher ratio of ribosomal protein S6 was phosphorylated in control cows and their fetuses compared to that of nutrient restricted cows (Figure 3). The hypophosphorylation of ribosomal protein S6 in nutrient restricted animals down-regulates the translational machinery (Jefferies et al., 1997; Schmelzle and Hall, 2000), which should result in the inhibition of protein synthesis in the skeletal muscle of nutrient restricted cows and their fetuses.

Protein degradation in muscle is largely controlled by calpain and ubiquitin-proteasome systems. In our pervious study, the calpastatin content was down-regulated in the skeletal muscle of nutrient restricted cows, while up-regulated in fetuses from nutrient restricted animals (Du et al., 2004b). In this study, no difference in the ubiquitylation of proteins in the fetal muscle of control and nutrient restricted animals was detected (Figure 4). The cow muscle, however, a significant higher amount of ubiquitylated proteins was detected in nutrient restricted samples, showing that nutrient restriction enhanced muscle protein degradation (Figure 4). This result is in agreement with former reports that food deprivation enhances the expression of proteins associated with ubiquitin- proteasome system (Costelli and Baccino, 2003; Jagoe et al., 2002). The ubiquitin dependent proteolysis is up-regulated during muscle atrophy (Costelli and Baccino, 2003). This result is also consistent with our former report that the content of calpastatin was down-regulated in nutrient restricted animals, which resulted in higher calpain activity and thus higher turnover of muscle proteins (Du et al., 2004b).

The accelerated protein degradation and decreased muscle protein synthesis result in muscle atrophy in the skeletal muscle of nutrient restricted cows. The loin area at the 12th rib for the control cow was 72±9 $cm^2$ versus that for nutrient restricted cow was only 60±7 $cm^2$ ($P < 0.05$). No significant difference in body weight for fetuses was observed (Figure 5).

## Implication

This study demonstrated that the mTOR signalling was down-regulated during the nutrient restriction for both cow and fetal muscles, which may be related to the activity of AMPK in the muscle. This down-regulation of mTOR signalling might be associated with the muscle atrophy in nutrient restricted animals. The ubiquitylization of proteins was increased only for cow muscle under nutrient restriction; in combination with our previous report that calpastatin was differentially regulated in cow and fetal muscle during nutrient restriction (Du et al., 2004b), data suggest that the accelerated degradation of proteins is important for the atrophy of adult muscle, while reducing protein synthesis might be the main reason for the growth retardation of fetal muscle due to maternal nutrient restriction. It is interesting to further study how muscle senses the nutrient availability and translates this into the modulation of mTOR signalling and protein degradation. By illustrating these mechanisms, possible strategies may be developed to promote muscle growth.

**Figure 1** mTOR phosphorylation in cow and fetal muscles

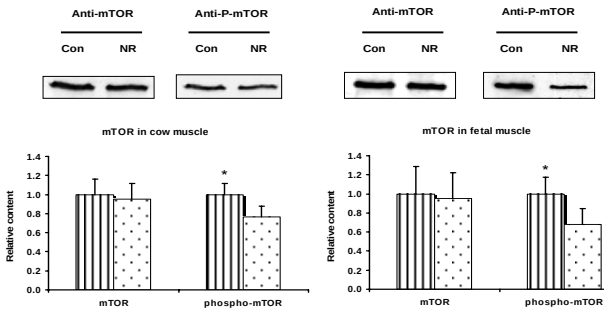


**Figure 2** Activity of AMPK in cow and fetal muscles

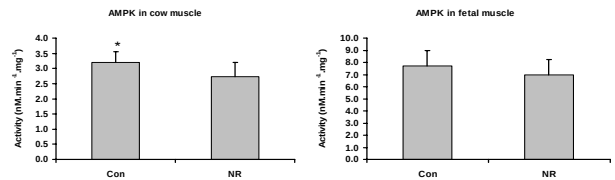


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**Figure 3** Ribosomal protein S6 phosphorylation in cow and fetal muscles

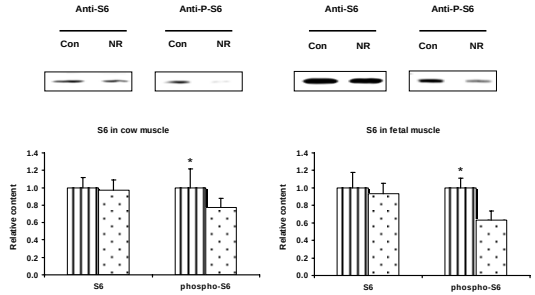
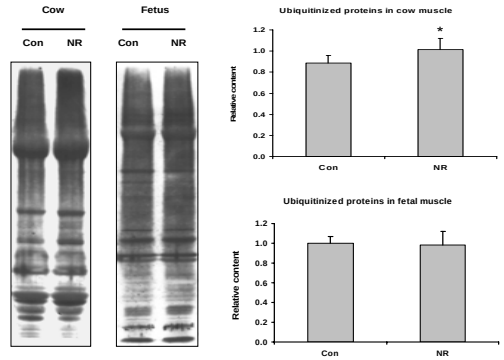



**Figure 4** Content of ubiquitylated proteins in cow and fetal muscles.

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