Short communication: High incubation temperature in bovine mammary epithelial cells reduced the activity of the mTOR signaling pathway

J. D. Kaufman, K. R. Kassube, R. A. Almeida, and A. G. Rius¹

Department of Animal Science, University of Tennessee, Knoxville 37996

ABSTRACT

Hyperthermia alters utilization of AA in protein synthesis and cell-signaling activity in bovine mammary cells. Essential AA and insulin regulate translation of proteins by controlling the activity of mammalian target of rapamycin (mTOR) signaling pathway. The objectives of this study were to evaluate (1) the effects of incubation temperature on the mTOR signaling pathway and transcription of AA transporters in a bovine mammary alveolar cell line (MAC-T) and (2) the combined effects of incubation temperature and insulin on the mTOR signaling pathway in this cell line. Cells were cultured in medium with 10% fetal bovine serum at 37°C and 5% CO₂. In experiment 1, cells were subjected to 37°C (control) or 41.5°C (high incubation temperature; HT) for 12 h. In experiment 2, cells were assigned to 1 of 4 treatments as a 2 × 2 factorial arrangement, including 2 cell culture temperatures (control and HT) and absence or presence of 1.0 µg/mL of insulin. Proteins were harvested and separated by gel electrophoresis. In experiment 1, gene expression of AA transporters (*SLC1A1*, *SLC1A5*, *SLC3A2*, *SLC7A1*, *SLC7A5*, and *SLC36A1*) were evaluated, and changes of ≥2 fold were deemed significantly different. In experiments 1 and 2, immunoblotting was used to identify total and site-specific phosphorylated forms of protein kinase B (Akt1; Ser473), p70 S6 kinase (S6K1; Thr389), ribosomal protein S6 (rpS6; Ser235/236), and eukaryotic elongation factor 2 (eEF2; Thr56). Phosphorylated and total forms of Akt1, S6K1, rpS6, and eEF2 were quantified and expressed as the ratio of phosphorylated to total protein. In experiment 1, HT resulted in a ≥2-fold increase expression of *SLC1A1* and *SLC3A2*. High incubation temperature reduced the phosphorylated to total ratio of Akt1 and rpS6 and increased the phosphorylated to total ratio of eEF2. In experiment 2, we found no temperature by insulin interactions on phosphorylation state of the protein fac-

tors of interest. High incubation temperature reduced the phosphorylated to total ratio of Akt1. The addition of insulin increased the phosphorylated to total ratio of Akt1, S6K1, and rpS6. In summary, HT reduced the activity of the mTOR signaling pathway and increased the expression of AA transporters. High incubation temperature possibly reduced protein translation by reducing the mTOR signaling pathway activity in an effort to adapt to thermal stress. These results may help explain the direct effect of elevated temperature on AA metabolism and protein translation in heat-stressed animals.

Key words: mTOR signaling pathway, insulin, mammary epithelial cell, thermal stress

Short Communication

Heat stress impairs cellular functions and cell growth in rodents, broilers, and livestock species (Bernabucci et al., 2010; Belhadj Slimen et al., 2016; Morales et al., 2016). High incubation temperature elicited a downregulation of genes involved in AA utilization and protein transcription in bovine mammary cells in vitro (Collier et al., 2006). The rate of gene transcription of ribosomal RNA was reduced under high incubation temperature, showing that changes at the transcription level are partially responsible for the reduction in cellular functions in vitro (Jacob, 1995). Furthermore, high incubation temperature decreased expression of genes associated with protein factors in the mammalian target of rapamycin (**mTOR**) pathway, a key regulatory mechanism, in bovine mammary epithelial cells in vitro (Li et al., 2015). The phosphorylation of protein factors in the mTOR signaling pathway activates or inhibits protein translation and the utilization of AA (Hay and Sonenberg, 2004; Wang and Proud, 2006). Thus, the phosphorylated-to-total signaling protein ratios in the mTOR pathway would indicate changes in protein translation and AA utilization in mammary epithelial cells exposed to high incubation temperature. This would allow testing interventions (e.g., nutritional and pharmacological) to reduce negative effects of high temperature on AA utilization and protein synthesis.

Received October 7, 2017.

Accepted March 26, 2018.

¹Corresponding author: arius@utk.edu

Insulin, a potent anabolic signal, and EAA independently control the activity of the mTOR signaling pathway in bovine mammary epithelial cells (Appuhamy et al., 2011). Depending upon the physiological state of the cell, the activity of AA transporters in the cell membrane affects intracellular concentrations of AA and translational effectors associated with the mTOR signaling pathway and protein synthesis (Hundal and Taylor, 2009). The activity of AA transporters on intestinal cells declined in response to heat stress (Morales et al., 2016). Collectively, a reduction in the expression of AA transporters can impair AA availability for protein synthesis and the mTOR signaling pathway (Nicklin et al., 2009). We hypothesized that elevated incubation temperature would reduce expression of AA transporters and thus impair mTOR signaling pathway activity, which potentially could be restored by insulin in bovine mammary epithelial cells. Therefore, the objectives of our study were (1) to evaluate the effect of incubation temperature on the activity of protein factors in the mTOR signaling pathway and expression of AA transporters in bovine mammary cells and (2) to determine the combined effect of incubation temperature and insulin on activity of the mTOR signaling pathway in bovine mammary cells.

Experiments were conducted using immortalized bovine mammary alveolar cells (MAC-T) because studies demonstrated that nutrients and hormones control the activity of the mTOR signaling pathway and protein translation (Appuhamy et al., 2011; Hosseini et al., 2013). However, these cells failed to produce copious amounts of casein (Huynh et al., 1991; Hosseini et al., 2013). Mammary alveolar cells were grown in 15 mL of Dulbecco's Modified Eagle Medium (pH = 7.4) with 10% fetal bovine serum (Corning Inc., Corning, NY), 1.0 µg/mL of bovine insulin (Sigma-Aldrich Co., St. Louis, MO), 1.0 µg/mL of hydrocortisone, 11.5 mL of 1.0 M HEPES (Sigma-Aldrich Co.), 40 mM L-Glu (Sigma-Aldrich Co.), and 1.1 g/500 mL of sodium bicarbonate at 37°C and 5% CO₂, in agreement with previous work conducted in MAC-T cells (Almeida et al., 1996; Appuhamy et al., 2011). Cells were grown and removed at 90% confluence and seeded as a suspension with 1.0 µg/mL of insulin into 6-well plates (Corning Inc.) at 3.5×10^5 cells/well.

Experiment 1 was conducted to determine the effect of incubation temperature on the mTOR signaling pathway and transcription of AA transporters. Each 6-well plate was randomly assigned to 37°C (control; **CTRL**) or 41.5°C (high incubation temperature; **HT**) for 12 h in a 95:5 CO₂ and gas mix balanced incubator. The experiment was repeated 5 times on 5 separate days. Experiment 2 was designed to test the combined effect of temperature and insulin, and it was set up

with a 2×2 factorial arrangement of treatments. Cells were exposed to CTRL and HT with the absence (–) or presence (+) of 1.0 µg/mL insulin (**Ins**) as (1) CTRL/Ins–, (2) CTRL/Ins+, (3) HT/Ins–, and (4) HT/Ins+. The experiment was repeated 5 times on 5 separate days. The concentration of insulin used in the present study promoted protein synthesis and activated the mTOR signaling pathway in MAC-T cells in previous studies (Burgos et al., 2010; Appuhamy et al., 2011). The effect of high incubation temperature reflected core body temperature observed in heat-stressed dairy cows (Kaufman et al., 2017). Moreover, 42.0°C for 8 h promoted the downregulation of genes involved in protein transcription (Collier et al., 2006); therefore, the thermal treatments (CTRL and HT for 12 h) were adequate to achieve the objectives of our study. The temperature of the cell culture incubator was recorded every 5 min using EasyLog EL-USB-2-LCD RH/Temp Data Logger (Lascar Electronics, Erie, PA). Images of cells were collected before and after treatments using optic microscopy (EVOS XL Core Imaging System; Advanced Microscopy Group, Bothell, WA) to assess cell viability and morphology.

Cell lysate aliquots (~15 µg) were subjected to Western blot analysis in experiments 1 and 2 as previously described (Rius et al., 2010) with modifications. Briefly, wells were filled with 200 µL of ice-cold lysis-extraction buffer [1 part 1% protease and phosphatase inhibitor cocktail (Thermo Scientific, Waltham, MA) to 50 parts homogenizing buffer; Rius et al., 2010; Appuhamy et al., 2011] and immediately scraped to collect lysate. Lysates were centrifuged ($12,000 \times g$ at 4°C for 15 min; Allegra 21R, Beckman Coulter, Brea, CA) and supernatants were analyzed for total protein content and incubated at 80°C for 8 min as described previously (Laemmli, 1970).

Proteins were separated electrophoretically using 10 [for protein kinase B (**Akt1**), p70 S6 kinase (**S6K1**), and eukaryotic elongation factor 2 (**eEF2**)] and 12% [for ribosomal protein S6 (**rpS6**)] polyacrylamide gels (SDS-PAGE at 200 V for 45 min; Bio-Rad, Hercules, CA). Three replicates per gel were run, on average, and treatment replications were loaded in adjacent wells within each gel. Proteins were transferred (24 V for 3 h) to polyvinylidene fluoride membranes (Merck Millipore, Billerica, MA). An internal control, α -tubulin, was used to determine consistency of this analysis. Membranes were evaluated for protein transfer, washed, blocked, and incubated overnight in 4°C with primary antibodies (diluted 1:1,000 in blocking buffer) recognizing site-specific phosphorylated and total forms of Akt1 (Ser473), S6K1 (Thr389), rpS6 (Ser235/Ser236), and eEF2 (Thr56). After incubation, membranes were washed and incubated with anti-mouse/rabbit antibod-

ies conjugated with horseradish peroxidase (diluted 1:2,000 in blocking buffer) for 1 h at room temperature. All primary antibodies, except for total S6K1, and secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA); total S6K1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Blots were developed using enhanced chemiluminescence (ECL plus, GE Healthcare, Chicago, IL), digitized using an imaging cabinet (FluorChem 5500; Alpha Innotech, San Leandro, CA), and analyzed by densitometry against the membrane background using NIH ImageJ (Schneider et al., 2012).

In experiment 1, cells were lysed and homogenized for extraction of RNA using RNeasy Mini Kit and including an on-column DNase treatment according to the manufacturer's protocol (Qiagen, Valencia, CA; Bionaz and Loor, 2007); complementary DNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Abundance of mRNA transcripts were quantified using real-time quantitative PCR and select primers (Integrated DNA Technologies, Coralville, IA; Bionaz and Loor, 2011; Supplemental Table S1, <https://doi.org/10.3168/jds.2017-13958>) with associated SYBR Green master mix (iQ Taq Universal, Bio-Rad). Reactions were performed using a CFX96 Real-Time PCR instrument (Bio-Rad) with the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 58°C (annealing and extension). All samples and standards were analyzed in triplicates, and mean values were normalized using reference genes [neudesin neurotrophic factor (*NENF*) and mitochondrial ribosomal protein L39 (*MRPL39*)] as previously described (Bionaz and Loor, 2007). The relative expression and fold change were calculated using $2^{-\Delta\Delta C_t}$ and $2^{-\Delta\Delta C_t}$, respectively (Livak and Schmittgen, 2001).

Individual 6-well plates assigned to each of the treatments were the experimental units. Results were analyzed using the Mixed procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC). Treatment differences were deemed significant at $P \leq 0.05$ and a trend was declared at $0.05 < P \leq 0.10$. All results are reported as least squares means ($\pm$ SEM). Experiment 1 results were analyzed as a completely randomized block design, blocking on independent preparation of cell cultures (i.e., replication):

$$Y_{ij} = \mu + T_i + R_j + e_{ij},$$

where Y_{ij} = the dependent variable, μ = the overall mean, T_i = the fixed effect of the i th temperature treatment ($i = 37$ and 41.5°C), R_j = the random block effect of the j th day that replicated the experiment, and

e_{ij} = the random error. Quantitative PCR data were square root transformed. After analysis, least squares means and standard error of the means were untransformed for interpretation (Manikandan, 2010). False discovery rate was taken into account by adjusting P -values ≤ 0.05 for the differential expression of genes using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Fold change was calculated for CTRL to HT using the mean values for each gene of interest with the threshold of 2.0 to represent significant expression (Li et al., 2005). In experiment 2, Western blotting data were analyzed as a completely randomized block design, blocking on independent preparation of cell cultures:

$$Y_{ijk} = \mu + T_i + I_j + (T \times I)_{ij} + R_k + e_{ijk},$$

where Y_{ijk} = the dependent variable, μ = the overall mean, T_i = the fixed effect of the i th temperature treatment ($i = 37$ and 41.5°C), I_j = the fixed effect of the j th insulin treatment ($j =$ presence or absence of insulin), $(T \times I)_{ij}$ = the fixed effect of the i th temperature and j th insulin treatment interaction, R_k = the random block effect of the k th day that replicated the experiment, and e_{ijk} = the random error. Data were square root transformed, and after analysis, least squares means and standard error of the means were untransformed for interpretation. Phosphorylation states were calculated using the phosphorylated-to-total ratio for each individual factor (Akt1, S6K1, rpS6, and eEF2; Rius et al., 2010).

Cell morphology was not affected by incubation temperature (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-13958>). Cellular confluence of 90% indicated that cells were viable upon completion of treatments (data not shown). In experiment 1, exposure of bovine mammary cells to HT decreased the phosphorylated-to-total ratio of Akt1 (22%; $P = 0.02$) and rpS6 (30%; $P < 0.01$; Table 1) and increased ($P = 0.02$) the phosphorylated-to-total ratio of eEF2 by 22%. Treatments did not affect the phosphorylated-to-total ratio of S6K1 (Supplemental Figure S2; <https://doi.org/10.3168/jds.2017-13958>).

In experiment 1, HT increased ($P < 0.01$) the relative expression of *SLC1A1* by 142% and *SLC3A2* by 91% (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-13958>) and triggered a fold change of 2.8 and 2.0 in relation to the *NENF* reference gene. Compared with CTRL, the HT treatment increased ($P < 0.01$) the relative expression of *SLC1A5* (24%), *SLC7A5* (46%), and *SLC36A1* (54%). The HT treatment increased ($P < 0.01$) the expression of *SLC1A1* by 29% and *SLC3A2* by 44% in relation to the *MRPL39* ref-

Table 1. Effect of temperature [37°C (control) or 41.5°C (high incubation temperature)] on phosphorylated-to-total signaling protein ratios (arbitrary units) in MAC-T cells for experiment 1¹

Signaling protein ²	37°C	41.5°C	SEM	Effect (<i>P</i> -value)
Akt1	0.27 ^a	0.21 ^b	0.06	0.02
S6K1	1.16	1.03	0.11	0.28
rpS6	1.33 ^a	0.93 ^b	0.22	<0.01
eEF2	12.8 ^b	15.6 ^a	4.47	0.02

^{a,b}Means in a row without common superscripts differ ($P \leq 0.05$).

¹Data are LSM $\pm$ SEM.

²Akt1 = protein kinase B; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; eEF2 = eukaryotic elongation factor 2.

erence gene (Supplemental Table S3; <https://doi.org/10.3168/jds.2017-13958>). Compared with CTRL, HT decreased ($P = 0.01$) the relative expression of *SLC7A1* by 23%. Treatments did not affect relative expression of *SLC1A5*, *SLC36A1*, and *SLC7A5* and fold changes above 2.0 were not detected (Figure 1B)

In experiment 2, we found no interaction between temperature of incubation and insulin levels; thus, main effects are reported in Table 2. Cells exposed to HT decreased ($P = 0.05$) the phosphorylated-to-total ratio of Akt1 by 55% (Supplemental Figure S3; <https://doi.org/10.3168/jds.2017-13958>). Compared with Ins⁻, the Ins⁺ treatment increased the phosphorylated-to-total ratio of Akt1 (175%, $P < 0.01$), S6K1 (74%, $P < 0.01$), and rpS6 (56%, $P = 0.01$). High incubation temperature did not affect the phosphorylated-to-total ratio of S6K1, rpS6, and eEF2, and the Ins⁺ treatment did not affect the phosphorylated-to-total ratio of eEF2.

Under the conditions described in the present study, MAC-T cells exposed to 12 h of high incubation temperature (41.5°C) reduced the phosphorylated-to-total ratio in Akt1 and rpS6 but increased that in eEF2 compared with cells exposed to control temperature in experiment 1. In the current study, Akt1, S6K1, rpS6, and eEF2 protein factors were chosen because they are upstream and downstream mediators of the mTOR signaling pathway. Specifically, the Akt factor is an upstream regulator that inhibits the activity of the tuberous sclerosis complex 2 (Choi et al., 2004; Hay and Sonenberg, 2004; Wang and Proud, 2006). Downstream to mTOR protein factor, rpS6 is part of the 40S ribosomal complex required for the initiation of mRNA translation (Hay and Sonenberg, 2004). Eukaryotic elongation factor 2 is influenced by hormones and metabolites but is not a direct substrate of the mTOR factor (Appuhamy et al., 2011); eEF2 is an inversely-related downstream factor of mTOR (Jackson et al., 2010). Dephosphorylation of eEF2 supports the elongation step during protein translation (Kaul et al., 2011). Activation of rpS6 is controlled by phos-

phorylation of S6K1, which contributes to activation of the mTOR signaling pathway (Navé et al., 1999; Chiang and Abraham, 2005). In the current study, the

Figure 1. Fold change ratio of genes of interest [arbitrary unit (AU)] at high incubation temperature (41.5°C) or control (37°C) relative to the reference genes (A) neudesin neurotrophic factor (*NENF*) and (B) mitochondrial ribosomal protein L39 (*MRPL39*). High incubation temperature-to-control fold change ratio was computed on the basis of mean values for each gene of interest with a fold change >2.0 as the cutoff for increased expression.

phosphorylation state of S6K1 was not affected by high incubation temperature. The low phosphorylation state observed in rpS6 during high incubation temperatures may have been mediated by a secondary regulator (e.g., S6K2; Ruvinsky et al., 2005), as previous studies indicated that deletion of S6K1 did not affect mTOR downstream activity (Mieulet et al., 2007). Moreover, the inhibition of the mTOR signaling pathway by Leu deprivation resulted in dephosphorylation of S6K1 but did not affect S6K2 (Pardo and Seckl, 2013). Therefore, it is possible that S6K2 mediated the changes in phosphorylation state in rpS6 and eEF2 in response to high incubation temperature.

Western blot results in experiment 2 indicate that high incubation temperature reduced the phosphorylation state in Akt1; however, the effect of temperature did not affect the phosphorylation state in rpS6 and eEF2. The Western blot procedure is semiquantitative; thus, large variability of phosphorylated and total form of protein factors is reported in the literature (Burgos et al., 2010; Appuhamy et al., 2011). This could explain the lack of statistically significant responses of rpS6 and eEF2 observed in experiment 2. Cellular responses to elevated temperature include inhibition of protein synthesis, defects in protein folding and function, and shifts in the use of energetic metabolites (Collier et al., 2008). In contrast to our results in mammary cells, elevated body temperature increased the phosphorylation state of Akt and stimulated mTOR signaling in skeletal muscle (Konishi et al., 1996; Yoshihara et al., 2013). Although the synthesis of heat shock proteins was not measured in the previous work, it is possible that thermal stress promoted the synthesis of heat shock proteins in skeletal muscle (Collier et al., 2008) because heat shock proteins identify damaged proteins and prevent protein misfolding and aggregation (Chou et al., 2012; Hu et al., 2016). The effect of high in-

cubation temperature on cellular physiology may be tissue-specific, and the same thermal load may induce apoptosis on one cell type and thermal tolerance on another cell type (Collier et al., 2008). Thus, in the present study, cells exposed to high incubation temperature experienced an apparent reduction in the activity of the mTOR signaling pathway that may be associated with greater apoptosis.

High incubation temperature for 12 h increased mRNA expression of AA transporters (*SLC1A1* and *SLC3A2*) by more than 2 fold. The *SLC1A1* gene encodes the excitatory amino acid transporter 3 (**EAAT3**), which transports AA (e.g., Glu, Asp, and Cys) across the cell membrane (Bionaz and Loor, 2011). Expression of *SLC3A2* mRNA promotes the synthesis of the heavy polypeptide chain 4f2hc to guide the neutral AA transporter (**LAT1**) to the cell membrane to transport branched-chain AA (Bionaz and Loor, 2011). Drummond et al. (2010) suggested that the expression of AA transporters increased in response to a lower activity of the mTOR pathway to maintain AA uptake and synthesis of protein. This is because AA (i.e., Glu and Asp) account for the majority of cell mass and determine cell division (Hosios et al., 2016). In our study, a lower activity of the mTOR signaling pathway coupled with increased AA transport activity may be a cellular adjustment to maintain cell mass in rapidly growing MAC-T cells exposed to high incubation temperature.

A high incubation temperature by insulin interaction was not detected in the current study. The lack of this interaction indicates that high incubation temperature and insulin affect the activity of the mTOR signaling pathway by independent mechanisms, despite observations of increased circulating insulin associated with reductions in protein synthesis in livestock exposed to thermal stress (Itoh et al., 1998; Wheelock et al., 2010). The lack of a high temperature-insulin interaction in

Table 2. Main effects of temperature [37°C (control) or 41.5°C (high incubation temperature)] and insulin [absence (–) or presence (+)] on phosphorylated-to-total signaling protein ratios (arbitrary units) in MAC-T cells for experiment 2¹

Signaling protein ²	Temperature		Insulin		SEM	Effect (<i>P</i> -value) ³	
	37°C	41.5°C	–	+		Temp	Ins
Akt1	0.31 ^a	0.14 ^b	0.12 ^z	0.33 ^y	0.12	0.05	<0.01
S6K1	0.72	0.62	0.49 ^z	0.85 ^y	0.18	0.61	<0.01
rpS6	0.77	0.87	0.64 ^z	1.00 ^y	0.30	0.45	0.01
eEF2	6.61	6.18	6.65	6.13	1.26	0.87	0.73

^{a,b}Temperature (Temp) means in a row without common superscripts differ ($P \leq 0.05$).

^{y,z}Insulin (Ins) means in a row without common superscripts differ ($P \leq 0.05$).

¹Data were square root transformed and untransformed LSM $\pm$ SEM are reported.

²Akt1 = protein kinase B; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; eEF2 = eukaryotic elongation factor 2.

³Test statistics are results from square root transformed data.

the mTOR signaling pathway was possibly caused by the absence of other hormones and metabolites in vitro (Collier et al., 2008). The addition of insulin increased the phosphorylated-to-total ratio of Akt1, S6K1, and rpS6. Insulin-induced stimulation of Akt1 inhibits the tuberous sclerosis complex 2 that in turn increases activity of the mTOR signaling pathway (Hay and Sonenberg, 2004; Wang and Proud, 2006) in skeletal muscle cells (Lund et al., 1998), hepatocytes (Khamzina et al., 2005), and mammary cells (Choi et al., 2004; Menzies et al., 2009; Burgos et al., 2010). In the current study, the presence of insulin promoted the mTOR signaling pathway. In agreement with our results, the presence of insulin increased the phosphorylation state of protein factors upstream and downstream of mTOR in MAC-T cells (Appuhamy et al., 2011). Previous research reported that insulin increased mRNA expression of 28 genes directly involved in protein synthesis in primary bovine mammary cells (Menzies et al., 2009). In vitro studies in rodent mammary cells demonstrated that insulin increased AA uptake and protein synthesis (Choi et al., 2004). In our study, compared with temperature, insulin elicited an independent and more consistent effect in the phosphorylated state of mTOR signaling protein factors in MAC-T cells.

In conclusion, high incubation temperature resulted in increased expression of AA transporters and reduced phosphorylation state of upstream and downstream protein factors of the mTOR signaling pathway. Independent of incubation temperature, insulin activated the mTOR signaling pathway. The changes observed in bovine mammary cells in the present study may help explain the direct effect of heat stress on AA utilization, protein translation, and milk protein synthesis.

ACKNOWLEDGMENTS

The authors acknowledge the support provided by the AgInnovation (University of Tennessee) program and the Institute of Agriculture at the University of Tennessee.

REFERENCES

- Almeida, R. A., D. A. Luther, S. J. Kumar, L. F. Calvino, M. S. Bronze, and S. P. Oliver. 1996. Adherence of *Streptococcus uberis* to bovine mammary epithelial cells and to extracellular matrix proteins. *J. Vet. Med. B* 43:385–392. <https://doi.org/10.1111/j.1439-0450.1996.tb00330.x>.
- Appuhamy, J. A. D. R. N., A. L. Bell, W. A. D. Nayananjali, J. Escobar, and M. D. Hanigan. 2011. Essential amino acids regulate both initiation and elongation of mRNA translation independent of insulin in MAC-T cells and bovine mammary tissue slices. *J. Nutr.* 141:1209–1215. <https://doi.org/10.3945/jn.110.136143>.
- Belhadj Slimen, I., T. Najar, A. Ghram, and M. Abdrrabba. 2016. Heat stress effects on livestock: Molecular, cellular and metabolic aspects, a review. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 100:401–412. <https://doi.org/10.1111/jpn.12379>.
- Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* 57:289–300.
- Bernabucci, U., N. Lacetera, L. H. Baumgard, R. P. Rhoads, B. Ronchi, and A. Nardone. 2010. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. *Animal* 4:1167–1183. <https://doi.org/10.1017/S175173111000090X>.
- Bionaz, M., and J. J. Looor. 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. *Physiol. Genomics* 29:312–319. <https://doi.org/10.1152/physiolgenomics.00223.2006>.
- Bionaz, M., and J. J. Looor. 2011. Gene networks driving bovine mammary protein synthesis during the lactation cycle. *Bioinform. Biol. Insights* 5:83–98. <https://doi.org/10.4137/BBI.S7003>.
- Burgos, S. A., M. Dai, and J. P. Cant. 2010. Nutrient availability and lactogenic hormones regulate mammary protein synthesis through the mammalian target of rapamycin signaling pathway. *J. Dairy Sci.* 93:153–161. <https://doi.org/10.3168/jds.2009-2444>.
- Chiang, G. G., and R. T. Abraham. 2005. Phosphorylation of mammalian target of rapamycin (mTOR) at Ser-2448 is mediated by p70S6 kinase. *J. Biol. Chem.* 280:25485–25490. <https://doi.org/10.1074/jbc.M501707200>.
- Choi, K. M., I. Barash, and R. E. Rhoads. 2004. Insulin and prolactin synergistically stimulate β -casein messenger ribonucleic acid translation by cytoplasmic polyadenylation. *Mol. Endocrinol.* 18:1670–1686. <https://doi.org/10.1210/me.2003-0483>.
- Chou, S.-D., T. Prince, J. Gong, and S. K. Calderwood. 2012. mTOR is essential for the proteotoxic stress response, HSF1 activation and heat shock protein synthesis. *PLoS One* 7:e39679. <https://doi.org/10.1371/journal.pone.0039679>.
- Collier, R. J., J. L. Collier, R. P. Rhoads, and L. H. Baumgard. 2008. Invited review: Genes involved in the bovine heat stress response. *J. Dairy Sci.* 91:445–454. <https://doi.org/10.3168/jds.2007-0540>.
- Collier, R. J., C. M. Stiening, B. C. Pollard, M. J. VanBaale, L. H. Baumgard, P. C. Gentry, and P. M. Coussens. 2006. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. *J. Anim. Sci.* 84(Suppl. 13):E1–E13. https://doi.org/10.2527/2006.8413_supplE1x.
- Drummond, M. J., E. L. Glynn, C. S. Fry, K. L. Timmerman, E. Volpi, and B. B. Rasmussen. 2010. An increase in essential amino acid availability upregulates amino acid transporter expression in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* 298:E1011–E1018. <https://doi.org/10.1152/ajpendo.00690.2009>.
- Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes Dev.* 18:1926–1945. <https://doi.org/10.1101/gad.1212704>.
- Hosios, A. M., V. C. Hecht, L. V. Danai, M. O. Johnson, J. C. Rathmell, M. L. Steinhauser, S. R. Manalis, and M. G. Vander Heiden. 2016. Amino acids rather than glucose account for the majority of cell mass in proliferating mammalian cells. *Dev. Cell* 36:540–549. <https://doi.org/10.1016/j.devcel.2016.02.012>.
- Hosseini, A., R. Sharma, M. Bionaz, and J. J. Looor. 2013. Transcriptomics comparisons of MAC-T cells versus mammary tissue during late pregnancy and peak lactation. *J. Adv. Dairy Res.* 1:103. <https://doi.org/10.4172/2329-888X.1000103>.
- Hu, H., Y. Zhang, N. Zheng, J. Cheng, and J. Wang. 2016. The effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells. *Anim. Sci. J.* 87:84–91. <https://doi.org/10.1111/asj.12375>.
- Hundal, H. S., and P. M. Taylor. 2009. Amino acid transceptors: Gate keepers of nutrient exchange and regulators of nutrient signaling. *Am. J. Physiol. Endocrinol. Metab.* 296:E603–E613. <https://doi.org/10.1152/ajpendo.91002.2008>.
- Huynh, H. T., G. Robitaille, and J. D. Turner. 1991. Establishment of bovine mammary epithelial cells (MAC-T): An *in vitro* model for bovine lactation. *Exp. Cell Res.* 197:191–199. [https://doi.org/10.1016/0014-4827\(91\)90422-Q](https://doi.org/10.1016/0014-4827(91)90422-Q).
- Itoh, F., Y. Obara, M. T. Rose, H. Fuse, and H. Hashimoto. 1998. Insulin and glucagon secretion in lactating cows during heat ex-

- posture. *J. Anim. Sci.* 76:2182–2189. <https://doi.org/10.2527/1998.7682182x>.
- Jackson, R. J., C. U. T. Hellen, and T. V. Pestova. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. *Nat. Rev. Mol. Cell Biol.* 11:113. <https://doi.org/10.1038/nrm2838>.
- Jacob, S. T. 1995. Regulation of ribosomal gene transcription. *Biochem. J.* 306:617–626.
- Kaufman, J. D., K. R. Kassube, and A. G. Rius. 2017. Lowering rumen-degradable protein maintained energy-corrected milk yield and improved nitrogen-use efficiency in multiparous lactating dairy cows exposed to heat stress. *J. Dairy Sci.* 100:8132–8145. <https://doi.org/10.3168/jds.2017-13026>.
- Kaul, G., G. Pattan, and T. Rafeequi. 2011. Eukaryotic elongation factor-2 (eEF2): Its regulation and peptide chain elongation. *Cell Biochem. Funct.* 29:227–234. <https://doi.org/10.1002/cbf.1740>.
- Khamzina, L., A. Veilleux, S. b. Bergeron, and A. Marette. 2005. Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: Possible involvement in obesity-linked insulin resistance. *Endocrinology* 146:1473–1481. <https://doi.org/10.1210/en.2004-0921>.
- Konishi, H., H. Matsuzaki, M. Tanaka, Y. Ono, C. Tokunaga, S. Kuroda, and U. Kikkawa. 1996. Activation of RAC-protein kinase by heat shock and hyperosmolarity stress through a pathway independent of phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* 93:7639–7643.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Li, J., D. N. Grigoryev, S. Q. Ye, L. Thorne, A. R. Schwartz, P. L. Smith, C. P. O'Donnell, and V. Y. Polotsky. 2005. Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice. *J. Appl. Physiol.* 99:1643–1648. <https://doi.org/10.1152/japplphysiol.00522.2005>.
- Li, L., Y. Sun, J. Wu, X. Li, M. Luo, and G. Wang. 2015. The global effect of heat on gene expression in cultured bovine mammary epithelial cells. *Cell Stress Chaperones* 20:381–389. <https://doi.org/10.1007/s12192-014-0559-7>.
- Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2[−] $\Delta\Delta$ CT method. *Methods* 25:402–408. <https://doi.org/10.1006/meth.2001.1262>.
- Lund, S., P. R. Pryor, S. Østergaard, O. Schmitz, O. Pedersen, and G. D. Holman. 1998. Evidence against protein kinase B as a mediator of contraction-induced glucose transport and GLUT4 translocation in rat skeletal muscle. *FEBS Lett.* 425:472–474. [https://doi.org/10.1016/S0014-5793\(98\)00293-2](https://doi.org/10.1016/S0014-5793(98)00293-2).
- Manikandan, S. 2010. Data transformation. *J. Pharmacol. Pharmacother.* 1:126–127. <https://doi.org/10.4103/0976-500X.72373>.
- Menzies, K. K., C. Lefevre, K. L. Macmillan, and K. R. Nicholas. 2009. Insulin regulates milk protein synthesis at multiple levels in the bovine mammary gland. *Funct. Integr. Genomics* 9:197–217. <https://doi.org/10.1007/s10142-008-0103-x>.
- Mieulet, V., M. Roceri, C. Espeillac, A. Sotiropoulos, M. Ohanna, V. Oorschot, J. Klumperman, M. Sandri, and M. Pende. 2007. S6 kinase inactivation impairs growth and translational target phosphorylation in muscle cells maintaining proper regulation of protein turnover. *Am. J. Physiol. Cell Physiol.* 293:C712–C722. <https://doi.org/10.1152/ajpcell.00499.2006>.
- Morales, A., S. E. M. Cota, N. O. Ibarra, N. Arce, J. K. Htoo, and M. Cervantes. 2016. Effect of heat stress on the serum concentrations of free amino acids and some of their metabolites in growing pigs. *J. Anim. Sci.* 94:2835–2842. <https://doi.org/10.2527/jas.2015-0073>.
- Navé, B. T., D. M. Ouwens, D. J. Withers, D. R. Alessi, and P. R. Shepherd. 1999. Mammalian target of rapamycin is a direct target for protein kinase B: Identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem. J.* 344:427–431. <https://doi.org/10.1042/bj3440427>.
- Nicklin, P., P. Bergman, B. Zhang, E. Triantafellow, H. Wang, B. Nyfeler, H. Yang, M. Hild, C. Kung, C. Wilson, V. E. Myer, J. P. MacKeigan, J. A. Porter, Y. K. Wang, L. C. Cantley, P. M. Finan, and L. O. Murphy. 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. *Cell* 136:521–534. <https://doi.org/10.1016/j.cell.2008.11.044>.
- Pardo, O. E., and M. J. Seckl. 2013. S6K2: The neglected S6 kinase family member. *Front. Oncol.* 3:191. <https://doi.org/10.3389/fonc.2013.00191>.
- Rius, A. G., J. A. D. R. N. Appuhamy, J. Cyriac, D. Kirovski, O. Becvar, J. Escobar, M. L. McGilliard, B. J. Bequette, R. M. Akers, and M. D. Hanigan. 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. *J. Dairy Sci.* 93:3114–3127. <https://doi.org/10.3168/jds.2009-2743>.
- Ruvinsky, I., N. Sharon, T. Lerer, H. Cohen, M. Stolovich-Rain, T. Nir, Y. Dor, P. Zisman, and O. Meyuhas. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev.* 19:2199–2211. <https://doi.org/10.1101/gad.351605>.
- Schneider, C. A., W. S. Rasband, and K. W. Eliceiri. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* 9:671–675. <https://doi.org/10.1038/nmeth.2089>.
- Wang, X., and C. G. Proud. 2006. The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* 21:362–369. <https://doi.org/10.1152/physiol.00024.2006>.
- Wheelock, J. B., R. P. Rhoads, M. J. VanBaale, S. R. Sanders, and L. H. Baumgard. 2010. Effects of heat stress on energetic metabolism in lactating Holstein cows. *J. Dairy Sci.* 93:644–655. <https://doi.org/10.3168/jds.2009-2295>.
- Yoshihara, T., H. Naito, R. Kakigi, N. Ichinoseki-Sekine, Y. Ogura, T. Sugiura, and S. Katamoto. 2013. Heat stress activates the Akt/mTOR signalling pathway in rat skeletal muscle. *Acta Physiol. (Oxf.)* 207:416–426. <https://doi.org/10.1111/apha.12040>.