Meat and Muscle Biology™

Arginine and Lysine Promote Skeletal Muscle Hypertrophy by Regulating the mTOR Signaling Pathway in Bovine Myocytes

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Abstract: The objective of this study was to assess the effects of additional arginine (ARG) and lysine (LYS) on the regulatory factors associated with myogenic differentiation of bovine satellite cells and hypertrophy of myotubes. Bovine satellite cells were isolated from 3-mo-old Holstein bull calves ($n = 3$) and subjected to a process of myogenic differentiation while exposed to varying doses of ARG or LYS. The experimental groups were as follows: (1) ARG dose-dependent (ARG: 0 [control], 60, and 120 μM) with the presence of basal LYS (800 μM) and (2) LYS dose-dependent (LYS: 0 [control], 50, and 100 μM) with basal ARG (400 μM) for the duration of 48 or 96 h. As a result of their alteration of myogenic regulatory factors (MRF), ARG and LYS might promote myogenic differentiation. Myoblast determination protein 1 (MyoD) and myogenin (MyoG) gene expression and protein abundance levels were elevated ($P < 0.01$) in myocytes treated with ARG or LYS compared to the untreated group. Additionally, supplementation with ARG or LYS phosphorated the proteins associated with the mechanistic target of rapamycin (mTOR) signaling pathway in a dose-dependent manner without affecting insulin-like growth factor 1 (IGF-1). These findings suggest that ARG and LYS may promote myofiber hypertrophy by directly modulating the mTOR signaling pathway. Furthermore, our observations indicate that the supplementation of ARG or LYS may have the potential to induce a transformation of muscle fiber types toward oxidative muscle fibers, as evidenced by the altered ($P < 0.01$) expression of myosin heavy chain (MHC) isoforms, specifically MHC I and MHC IIA. Taken together, our findings provide evidence that ARG and LYS have the capacity to enhance myotube hypertrophy through the regulation of MRF and mTOR signaling pathways.

Keywords: arginine, lysine, beef cattle, mTOR pathway, muscle hypertrophy
Meat and Muscle Biology 7(1): 16876, 1–13 (2023) **doi:10.22175/mmb.16876**
Submitted 21 June 2023 Accepted 11 August 2023

Introduction

In bovines, the numerical density of muscle fibers is primarily determined at birth, with subsequent growth predominantly arising from hypertrophy of these pre-established fibers (Rehfeldt et al., 2000; Wegner et al., 2000). Numerous methodologies can be adopted to augment the mass of the skeletal muscle, such as interventions targeting the activation of satellite cells via exogenous stimuli to amplify cellular proliferation and modulate myogenic regulatory factors (MRF). Ensuring the optimal delivery of crucial nutrients to support muscular growth is essential. Among these, amino acids play a paramount role as they are directly implicated in cell signaling-mediated muscle growth and protein biosynthesis. Enhanced availability of these amino acids has been associated with the activation of cellular modulators integral to the commencement of translation, leading to augmented protein synthesis within the skeletal muscle (O'Connor et al., 2003; Watford and Wu, 2005).

In cattle, arginine (ARG) is categorized as an essential amino acid. Besides its role as a fundamental

precursor for urea, nitric oxide, and polyamines synthesis, it also orchestrates several metabolic pathways pivotal for health, growth, reproductive processes, and homeostasis (Morris Jr, 2009). In corn-based beef cattle diets, lysine (LYS) is the primary limiting amino acid for protein synthesis (National Academies of Sciences, Engineering, and Medicine, 2016). Both ARG and LYS are implicated in the Akt/mammalian target of rapamycin (mTOR) signaling pathway, a pathway known for promoting protein biosynthesis and concurrently reducing protein degradation in the skeletal muscle (Satterfield et al., 2013; Sales et al., 2016; Ma et al., 2017). As for skeletal muscle, molecular mechanisms for the action of ARG may involve directly activating the mTOR complex and promoting protein synthesis (Meijer and Dubbelhuis, 2004; Suryawan et al., 2009). Additionally, LYS has been documented to modulate the mTOR signaling mechanism in dairy cattle, subsequently augmenting milk protein biosynthesis (Nan et al., 2014; Lin et al., 2018). The mTOR signaling pathway functions as an integral modulator of cellular metabolism, growth, and proliferation, with pronounced activity in skeletal muscles (Park et al., 2005; Laplante and Sabatini, 2009), underlining its significance in promoting protein biosynthesis and muscle hypertrophy. Prior research has elucidated that ARG and LYS influence this pathway, facilitating the enhancement of muscle growth and regeneration (Yao et al., 2008; Jin et al., 2019). The modulation of mTOR signaling by these amino acids triggers a physiological cascade culminating in augmented muscle fusion index, myotube diameter, and protein biosynthesis. The intricate interplay between ARG, LYS, and the mTOR pathways may be pivotal for optimizing muscular growth performance.

Nonetheless, it remains unclear how supplementary ARG and LYS may influence the myogenic regulation of mature skeletal muscle in beef cattle. This study postulates that supplementary ARG and LYS potentiate the myogenic differentiation of satellite cells and muscle fusion rate by influencing MRF activity and stimulating the mTOR/Akt pathway. Therefore, our research aimed to unravel the mechanisms underlying skeletal muscle growth and the implications of supplementary ARG and LYS on satellite cell myogenic differentiation.

Materials and Methods

Bovine satellite cell isolation

The satellite cell isolation protocol (PROTO202000294) for this research project was thoroughly reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University. Bovine satellite cells (BSC) were isolated from 3-mo-old Holstein bull calves ($n = 3$, body weight: 77.10 ± 2.02 kg). The calves were harvested at the Michigan State University Meat Laboratory in East Lansing, Michigan, and the entire process was conducted under the supervision and inspection of the United States Department of Agriculture (USDA). The protocol for isolating and cultivating BSC was conducted in reference to Johnson et al. (1998) and Kim et al. (2018). *Longissimus* muscle tissue was procured and transported to the Muscle Biology Laboratory at Michigan State University (East Lansing, MI). The tissue was dissected to eliminate blood vessels, connective tissues, and adipose tissue. Subsequently, the dissected *Longissimus* muscle tissue was ground using a sterile meat grinder. The resulting ground muscle tissue was placed in tubes and subjected to incubation for 1 h at 37°C using a solution containing 0.1% of Pronase® (Calbiochem, La Jolla, CA) in Earl's Balanced Salt Solution (Sigma Aldrich, St. Louis, MO). Following the incubation period, centrifugation was performed at room temperature at a force of $1,500 \times g$ for 4 min. The supernatant was discarded, and the resulting pellet was reconstituted in phosphate-buffered saline (PBS; Sigma Aldrich). The suspension was subjected to a further centrifugation step at room temperature, employing a force of $500 \times g$ for 10 min. As before, the supernatant was removed, and the pellet containing mononucleated cells was resuspended using PBS, followed by another centrifugation step at $500 \times g$ for 10 min at room temperature to obtain a pellet consisting of mononucleated cells.

Cell culture and treatments

Isolated satellite cells were plated and incubated in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Waltham, MA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), and 1x Antibiotic-Antimycotic (Gibco) at 38°C under a humidified atmosphere of 95% O_2 and 5% CO_2. Based on the confluency of BSC, serial passages were made for purification. Cells at the exponential phase with passage numbers 2–4 were used for all experiments. These BSC were seeded on the 6-well plate at a density of 1×10^5 cells/mL in DMEM with 10% FBS and 1x Antibiotic-Antimycotic (Gibco) at 38°C under a humidified atmosphere of 95% O_2 and 5% CO_2. Upon reaching 80% to 90% confluence, the growth medium

was replaced with a differentiation medium composed of SILAC DMEM Flex Media (DMEM without D-glucose, phenol red, L-ARG, L-LYS, L-glutamine; Gibco) supplemented with D-glucose (25 mM, Sigma Aldrich), phenol red (40 µM, Sigma Aldrich), L-glutamine (4 mM, Gibco), 2% horse serum, and 1× Antibiotic-Antimycotic. Concurrently, cells were treated with different doses of ARG and LYS: (1) ARG only treated (ARG: 0; CON, 60, and 120 µM; Thermo Fisher Scientific) with L-LYS (800 µM, Thermo Fisher Scientific) and (2) LYS only treated (LYS: 0; CON, 50, and 100 µM; Thermo Fisher Scientific) with L-ARG (400 µM, Thermo Fisher Scientific) (Figure 1). The cells were incubated either for 48 h or for 96 h and harvested for further analysis. The selection of ARG and LYS concentrations was determined according to the blood AA concentrations documented in previous *in vivo* investigations (Supplement 1).

mRNA extraction and real-time quantitative PCR

After 48 h of treatment, total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA). The concentration and purity of messenger RNA (mRNA) were determined with a spectrophotometer at an absorbance of 260 nm and 280 nm using a NanoDrop™ One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). An acceptable range of 2.01 to 2.11 was used for the 260/280 ratio. Genomic DNA removal and complementary DNA (cDNA) synthesis were performed using QuantiTect reverse transcription kit (Qiagen, Germantown, MD) according to manufacturer recommendations. Real-time quantitative PCR (QuantStudio 6Pro, Applied Biosystems) was used to measure the quantity of the genes of interest relative to the quantity of ribosomal protein subunit 9 (RPS9) and hydroxymethylbilane synthase (HMBS) mRNA in total RNA. The expression of RPS9 and HMBS is not different across bovine tissues and is widely used as a housekeeping gene in cattle (Bionaz and Loor, 2007; Janovick-Guretzky et al., 2007; Pérez et al., 2008). The expression of genes was normalized using RPS9 and HMBS as endogenous controls. Measurement of the relative quantification of the cDNA of interest was carried out using TaqMan Fast Advanced Master Mix (Applied Biosystems) and TaqMan Gene expression Assays (Thermo Fisher Scientific) (Tables 1 and 2). Assays were performed in triplicate and using the thermal cycling parameters recommended by the manufacturer (45 cycles of 15 s at 95°C and 1 min at 60°C). All real-time reactions and Ct values analyses were conducted using the QuantStudio 6 Pro System from Applied Biosystems.

Western blotting

After 96 h of treatment, cellular lysates were prepared using mammalian protein extraction buffer (M-PER™) lysate buffer (Thermo Fisher Scientific) supplemented with protein inhibitor (Thermo Fisher Scientific). The total protein content of the lysates was determined using the bicinchoninic acid assay (Thermo Fisher Scientific) with a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) at 562 nm. Samples containing 20 µg of protein were denatured at 70°C for 10 min followed by 85°C for 2 min, and then resolved on a Bolt 4%–12% Bis-Tris Plus gel (Thermo Fisher Scientific) using a voltage of 200 V for 22 min. Subsequently, the proteins were transferred onto a nitrocellulose membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). The membranes were blocked with iBind Flex Solution (iBind Flex Buffer, Thermo

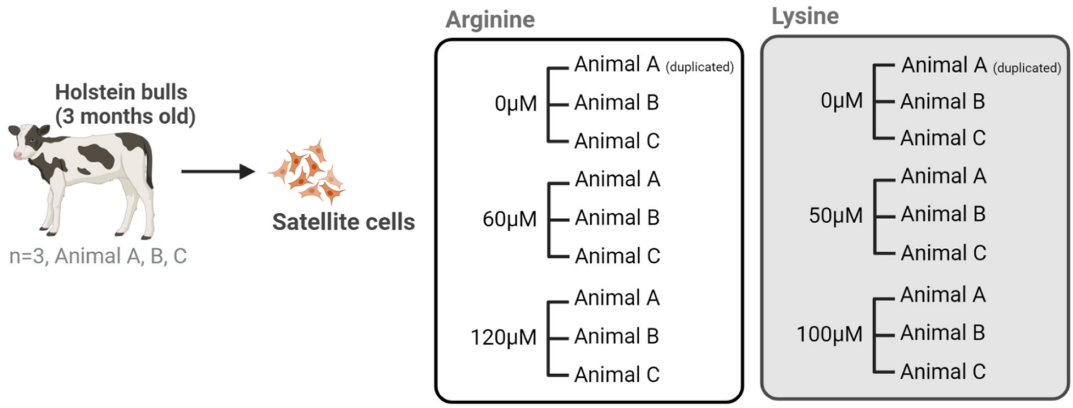

Figure 1. Each individual animal is regarded as a distinct and independent replication. Repetitive measurements are conducted for each animal to ensure rigorous data collection and enhance the statistical reliability of the study.

Table 1. TaqMan® probes and primers used for reverse transcription quantitative real-time PCR (RT-qPCR) assays

Gene[1]	TaqMan® probe assay	Manufacturer
RPS9	Bt03272016_m1	Thermo Fisher
HMBS	Bt03234763_m1	
MyoD	Bt03244740_m1	
MyoG	Bt03258928_m1	
IGF1	Bt03252282_m1	
MHC I	Bt03224257_m1	
P70S6K	Bt00923436_m1	

[1]*RPS9*: ribosomal protein subunit 9; *HMBS*: hydroxymethylbilane synthase; *HSP*: heat shock protein; *MyoD*: myoblast determination protein 1; *MyoG*: myogenin; *IGF1*: insulin-like growth factor 1; *MHC*: myosin heavy chain; *P70*^{S6K}: ribosomal protein S6 kinase beta 1.

Table 2. TaqMan® probes and primers used for reverse transcription quantitative real-time PCR (RT-qPCR) assays

Gene[1]	Primer and probe sequence (5' to 3')	Manufacturer
MHC IIA		Thermo Fisher
Forward	GCAATGTGGAAACGATCTCTAAAGC	
Reverse	GCTGCTGCTCCTCCTCCTG	
Probe	6FAM-TCTGGAGGACCAAGTGAA CGAGCTGA-TAMRA	
MHC IIX		
Forward	GGCCCACTTCTCCCTCATTC	
Reverse	CCGACCACCGTCTCATTCA	
Probe	6FAM-CGGGCACTGTGGACTACAA CATTACT-TAMRA	

[1]*MHC*: myosin heavy chain.

Fisher Scientific) for 10 min at room temperature to prevent non-specific binding. Primary antibodies including anti-myogenic differentiation 1 (MyoD), rabbit monoclonal, dilution of 1:1000 (Cell Signaling, Danvers, MA), anti-myogenin (MyoG), mouse monoclonal, dilution of 1:1000 (Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), mouse monoclonal, dilution 1:1000 (DSHB), anti-mTOR, rabbit monoclonal, dilution of 1:1000 (Cell Signaling), anti-phospho-mTOR, rabbit polyclonal, dilution of 1:1000 (Cell Signaling), anti-p70^{S6K}, rabbit polyclonal, dilution of 1:1000 (Cell Signaling), anti-phospho-p70^{S6K}, rabbit monoclonal, dilution of 1:1000 (Cell Signaling), anti-Akt, mouse monoclonal, dilution of 1:1000 (DSHB), and anti-phospho-Akt, rabbit polyclonal, dilution of 1:1000 (Cell Signaling) were applied to the membranes. Secondary antibodies, including Goat anti-mouse IgG H&L (HRP), mouse polyclonal, dilution of 1:1000 (Abcam Inc.) and Goat anti-rabbit IgG H&L (HRP), rabbit polyclonal, dilution of 1:2000 (Abcam Inc.), were applied using the iBind Flex Western System (Thermo Fisher Scientific) at room temperature for 4 h. Protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and detected using an imager (FluoChem, Alpha Innotech, San Leandro, CA). The expression levels of the proteins were normalized to the reference protein GAPDH or phosphor-protein level. Relative protein expression was quantified using ImageJ software (NIH, Bethesda, MD).

Differentiation index, myotube diameter, and protein synthesis

For immunofluorescence analysis, cells were seeded onto 4-well Lab-Tek chamber slides (Thermo Fisher Scientific). Following a 96-h treatment, the slides were fixed with 4% paraformaldehyde (Thermo Fisher Scientific) at room temperature for 15 min. After 3 washes with PBS, the cells were permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific) in PBS for 15 min. In order to block non-specific binding, cells were treated with 2% bovine serum albumin (Thermo Fisher Scientific) in PBS for 1 h at 4°C. Subsequently, the cells were incubated overnight at 4°C with the following primary antibody: anti-myosin heavy chain (MHC) (mouse monoclonal, dilution 1:1000, DSHB). After 3 washes with PBS, the cells were incubated with the secondary antibody, goat anti-mouse IgG Alexa Fluor™ 488 (1:1000; Thermo Fisher Scientific) for 30 min at room temperature. Slides were washed thrice with PBS and counterstained with 4′6-diamidino-2-phenylindole (DAPI; 1:1000; Thermo Fisher Scientific) in PBS for 5 min at room temperature. Coverslips were mounted onto glass slides using a Fluoromount-G™ Mounting Medium (Thermo Fisher Scientific) and sealed with nail polish. The slides were imaged at a magnification of 20× using fluorescent microscopy (EVOS M5000, Thermo Fisher Scientific). The differentiation index (%) for cells was defined as the percentage of nuclei number in differentiated myotubes (harboring more than 2 nuclei per myotube) divided by the total nuclei number (Ren et al., 2010). Myotube diameter was measured using a modified method of the previous study (Williamson et al., 2009). Myotubes in each well were measured at randomly selected 5 locations taken along the length using the ImageJ software (NIH, Bethesda, MD). Five values per repetition were obtained as the mean

and standard deviation for each fusion index and myotube diameter.

The protein synthesis rate was determined using Protein Synthesis Assay Kit (Abcam). Cells were plated on 4-well chamber slides (Thermo Fisher Scientific), and then ARG and LYS treatment for 96 h after inducing differentiation. At 96 h of treatment, cells were treated with O-propargyl-puromycin to label nascent polypeptides and incubated for 1 h in an incubator. After 1-h incubation, cells were fixed with a fixative solution for 15 min at room temperature. Cells were washed once with washing buffer and permeabilized with permeabilization buffer for 10 min at room temperature. Following rinsing steps, cells were incubated with a protein reaction cocktail including PBS, copper reagent, Alexa Fluor™ 488 fluorescent azide, and reducing agent for 30 min at room temperature protected from light. Following the reaction, DNA staining was conducted for 20 min. The slides were imaged at a magnification of 10× using fluorescent microscopy (EVOS M5000, Thermo Fisher Scientific). Labeled proteins in each well were measured at randomly selected 5 locations, and images were analyzed using a modified method of the previous study (Cooke et al., 2019) by ImageJ software (NIH) to quantify mean fluorescence per cell.

Statistical analysis

The data obtained from the experiment were analyzed and graphed using GraphPad Prism Version 9.4.1 (Graph Pad Software, San Diego, CA). One-way analysis of variance (ANOVA) was performed to evaluate the impact of ARG and LYS on gene expressions, protein levels, differentiation index, myotube diameter, and protein synthesis. In the event of a significant difference, Tukey's honest significant difference (HSD) test was utilized as a *post hoc* analysis to compare the means among different ARG and LYS treatments. The statistical model incorporated the fixed effect of ARG (0, 60, and 120 μM) and LYS (0, 50, and 100 μM) and the random effect of cell's donors, with each culture well serving as an experimental unit. All experiments were triplicated using cells derived from distinct animals. The data are presented as standard errors of means. A significance level (α) of 0.05 was employed to determine statistical significance, while tendencies were discussed for P values ranging from 0.05 to 0.10.

Results

The gene expression of MyoD exhibited a dose-dependent increase upon the addition of ARG and LYS (Figure 2). The highest expression of MyoD was observed at a concentration of 120 μM ARG, followed by 60 μM ARG, whereas the CON group displayed the lowest expression ($P < 0.01$). Similarly, cells treated with 100 μM LYS showed significantly higher MyoD expression ($P < 0.01$) compared to those treated with 50 μM LYS and the CON group. With an increase in ARG concentration to 60 μM and 120 μM, the gene expression of MyoG also increased by 44.9% and 56.6%, respectively, relative to the CON group. Additionally, the addition of LYS demonstrated a dose-dependent increase ($P < 0.01$) in MyoG expression. The analysis of protein abundance further supported this trend. The protein levels of MyoD exhibited an increase ($P < 0.05$) when cells were incubated with ARG at concentrations of 60 and 120 μM (Figure 3). Furthermore, the addition of LYS at concentrations of 50 and 100 μM also resulted in an elevated protein level of MyoD compared to the CON. Similarly, the protein levels of MyoG exhibited an increase ($P < 0.01$) when cells were incubated with ARG at concentrations of 60 and 120 μM (Figure 4). Additionally, the addition of LYS at concentration of 100 μM also resulted in an elevated ($P < 0.05$) protein level of MyoG compared to the control group and 50 μM (Figure 4).

No significant difference was observed in mRNA gene expression of insulin-like growth factor 1 (IGF-1) following cell incubation with ARG ($P = 0.25$) or LYS ($P = 0.91$) (Figure 2). However, the addition of LYS resulted in a significant increase ($P < 0.01$) in the expression of $P70^{S6K}$ compared to CON (Figure 2). Notably, a dose-dependent trend was observed, with the expression level of $P70^{S6K}$ being greater at a concentration of 100 μM compared to 50 μM.

Three predominant isoforms of MHC in bovine skeletal muscle, namely MHC I, IIA, and IIX, were subjected to analysis. The highest concentration of ARG (120 μM) resulted in a significant upregulation of MHC I ($P < 0.01$) and MHC IIA ($P < 0.01$), whereas the expression of MHC IIX remained unaffected ($P = 0.69$) (Figure 4). Cells incubated with 100 μM of LYS exhibited overexpression of MHC I ($P < 0.01$), but no significant change was observed in MHC IIA ($P = 0.47$). A tendency toward alteration was noted in MHC IIX ($P = 0.09$).

The phosphorylation status of key regulatory proteins was examined to assess the mTOR pathway's activity. The phosphorylation of Akt was found to be significantly increased ($P < 0.05$) in response to 120 μM of ARG, compared to both 60 μM and CON (Figure 5). Furthermore, the addition of 100 μM of

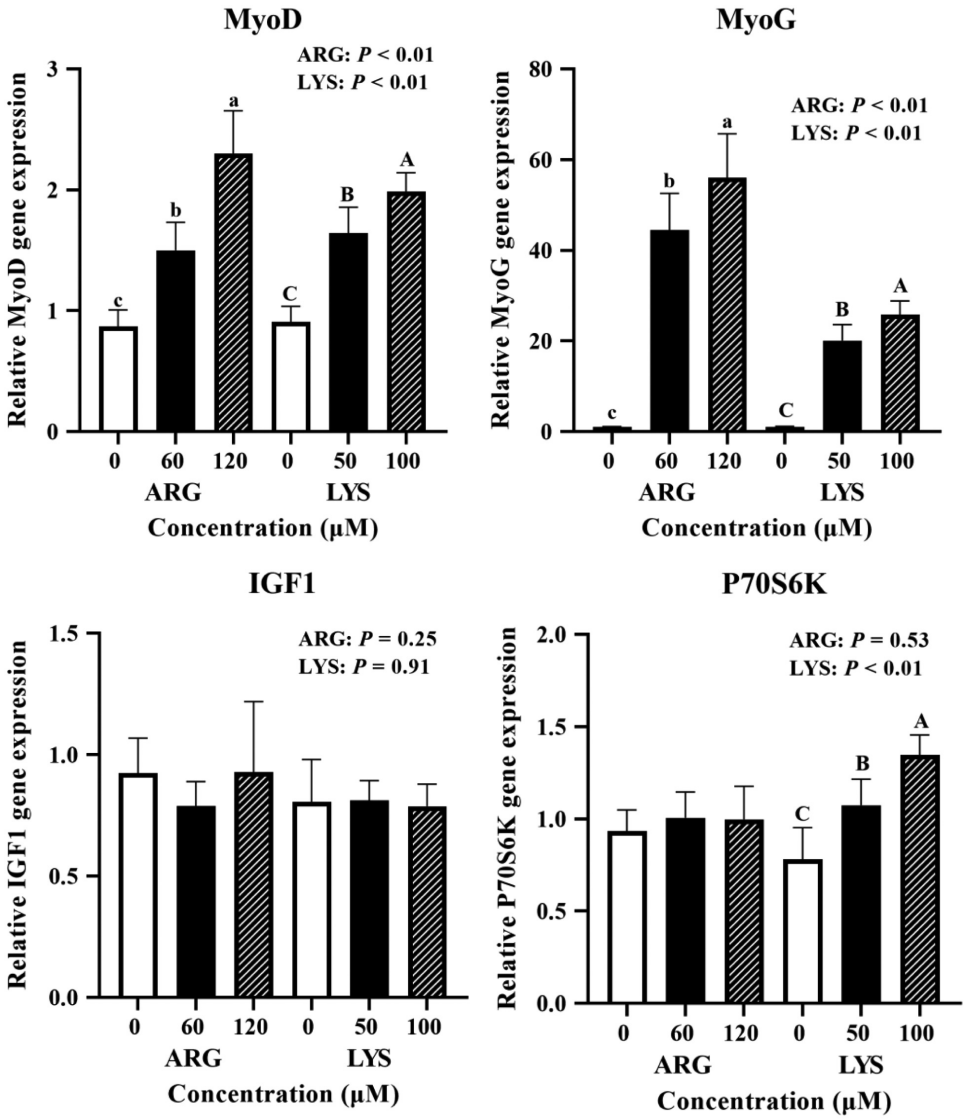

Figure 2. Impact of arginine (ARG) and lysine (LYS) on myogenic regulatory factors, IGF-1, and $P70^{S6K}$ gene expression during myogenic differentiation. Error bars in the graph represent the standard error. The statistical significance was determined using one-way ANOVA for each ARG and LYS group. Different letters ($^{a–c}$ for ARG groups and $^{A–C}$ for LYS groups) indicate significant differences in mean values. IGF-1, insulin-like growth factor 1; MyoD, myoblast determination protein 1; MyoG, myogenin; $P70^{S6K}$, ribosomal protein S6 kinase beta-1.

LYS enhanced ($P < 0.01$) the phosphorylation of Akt. The phosphorylation of mTOR and $P70^{S6K}$ relative to their native forms were significantly increased ($P < 0.01$) upon exposure to 120 μM of ARG (Figure 5). Furthermore, the addition of 100 μM of LYS resulted in an increase ($P < 0.05$) in the phosphorylation of mTOR and $P70^{S6K}$ compared to CON, whereas no significant difference was observed between 50 μM and 100 μM of LYS.

The introduction of ARG at concentrations of 60 μM and 120 μM led to a significant increase ($P < 0.01$) in the differentiation index of myofibers after 96 h of incubation (Figure 6). A dose-dependent effect was observed in cells treated with LYS (Figure 6). Specifically, cells incubated with 100 μM of LYS exhibited the highest ($P < 0.01$) differentiation index, followed by 50 μM, whereas the CON showed the lowest level of differentiation. Myotube diameter showed a similar pattern. Myotubes treated with 120 μM of ARG displayed the largest diameter, followed by those treated with 60 μM, whereas the CON group exhibited the smallest myotube diameter (Figure 6). A significant increase ($P < 0.01$) in myotube diameter was observed in myotubes incubated with both 50 μM and 100 μM of LYS compared to CON (Figure 6).

In the same context, ARG ($P < 0.01$) and LYS ($P < 0.01$) were found to enhance protein synthesis (Figure 7). Notably, both amino acids stimulated protein synthesis in a dose-dependent manner.

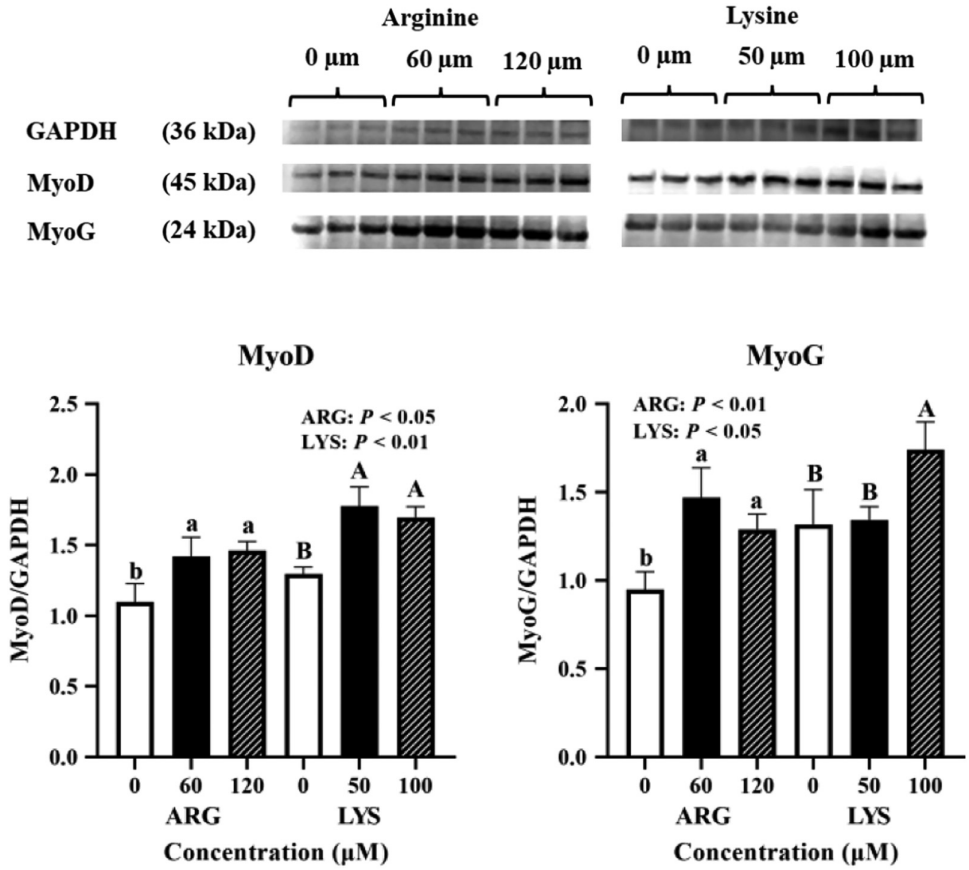

Figure 3. Effect of arginine (ARG) and lysine (LYS) treatment on the protein expression of MyoD and MyoG during myogenic differentiation in bovine satellite cells. Western blots (top) and quantification of protein levels (bottom) of MyoD and MyoG in ARG and LYS treatment groups. Error bars indicate the standard error. The *P* values are determined by one-way ANOVA for each ARG and LYS group. [a,b]Mean values with different letters differ significantly in ARG groups. [A,B]Mean values with different letters differ significantly in LYS groups. MyoD, myoblast determination protein 1; MyoG, myogenin.

Discussion

Myogenic differentiation involves participating in numerous transcription factors, with MRF being prominent examples. These transcription factors, including myoblast MyoD and MyoG, belong to the basic helix-loop-helix transcription factors family (Hernández-Hernández et al., 2017). They play a crucial role in the activation of the myogenic program, formation, maturation, and differentiation of myoblasts. Our data denoted a significant increase in the gene expression of MyoD and MyoG in myocytes treated with ARG or LYS compared to the CON group. Specifically, cells treated with 60 μM of ARG exhibited over 40-fold higher expression of MyoG, whereas cells treated with 120 μM of ARG showed over 55-fold higher expression, both relative to CON. Furthermore, the addition of LYS resulted in over 20-fold higher expression of MyoG compared to the CON group. Considering that MyoG plays a pivotal role as a transcription factor in myogenic differentiation, it is plausible that both ARG and LYS may contribute to the formation of myotubes. These findings are in agreement with those of previous studies. Despite the well-known effects of ARG and LYS on protein synthesis in muscle, the direct regulation of MRF by these amino acids remains inadequately studied. It has been proposed that mTOR controls MyoD expression via muscle-specific microRNA; however, the exact mechanism remains unclear (Wallace et al., 2016). The activation of MRF by amino acids may occur indirectly through the mTOR pathway.

Our findings show that the ARG and LYS mediate the Akt/mTOR/P70S6k signaling pathways. The mTOR signaling pathway serves as a major pathway for protein synthesis, and amino acids play a crucial role in its regulation (Wang and Proud, 2006). The mTOR pathway serves as a pivotal regulator of cellular metabolism, growth, proliferation, and survival. Among its functions are the stimulation of protein synthesis and the promotion of muscle hypertrophy by regulating downstream cell signaling cascades within skeletal muscles (Bodine et al., 2001).

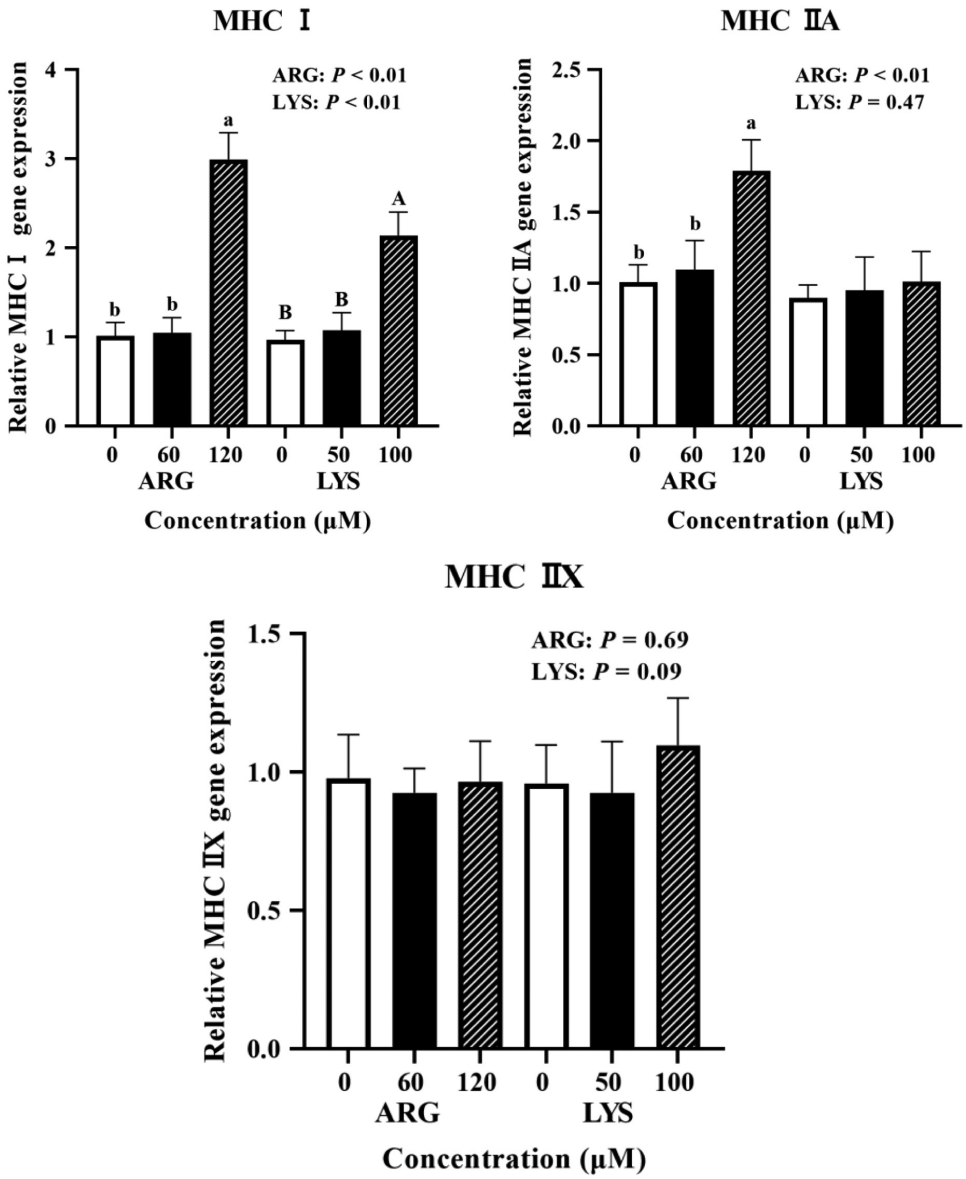

Figure 4. Impact of arginine (ARG) and lysine (LYS) on myosin heavy chain (MHC) gene expression during myogenic differentiation. Error bars in the graph represent the standard error. The statistical significance was determined using one-way ANOVA for each ARG and LYS group. Different letters ($^{a–c}$ for ARG groups and $^{A–C}$ for LYS groups) indicate significant differences in mean values.

Studies have shown that ARG and LYS, in particular, are involved in modulating the mTOR signaling pathway, ultimately promoting protein synthesis (Yao et al., 2008; Gao et al., 2015). $P70^{S6K}$, a protein kinase, has been identified as a significant factor contributing to protein synthesis and cell proliferation, highlighting its fundamental role in cell growth and development (Xiao et al., 2009). It operates as a downstream target of the mTOR signaling pathway, one of the principal regulators of cell growth and metabolism. By controlling this pathway, ARG and LYS may promote muscle hypertrophy.

Insulin-like growth factor 1 (IGF-1) is widely recognized for its pivotal role in activating the Akt-mTOR pathway, a prominent cellular signaling cascade (Han et al., 2008; Yoshida and Delafontaine, 2020). Despite the evidence indicating that ARG and LYS activate the mTOR pathway, we did not observe any significant changes in IGF-1 levels. These findings suggest that ARG or LYS may regulate the mTOR pathway independently of IGF-1. Two distinct types of IGF-1 production occur within the organism, one localized within the cell (local IGF-1) and the other in circulation in the blood stream (Dobrowolny et al., 2005). Each production type plays a unique role in the regulation of various biological processes. We only observed local IGF-1; however, the previous *in vivo* studies showed that LYS and ARG may not alter circulating IGF-1 as well.

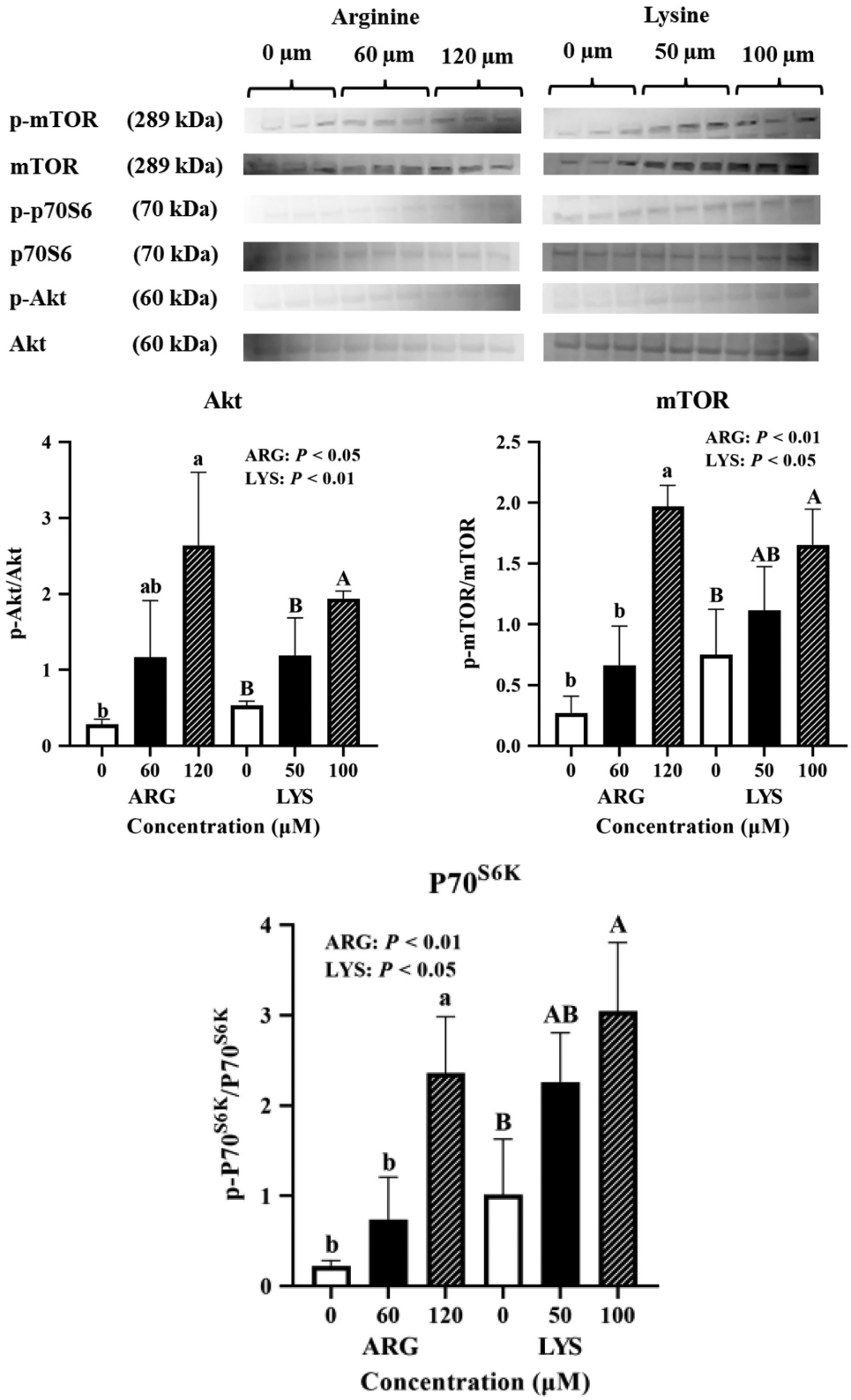

Figure 5. Effect of arginine (ARG) and lysine (LYS) treatment on the protein level of mTOR/S6 signaling pathway-related protein during myogenic differentiation in the cells. Western blots (top) and quantification of protein levels (bottom) of mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase beta-1 ($P70^{S6K}$), and Akt in ARG and LYS treatment groups. Error bars indicate the standard error. The *P* values are determined by one-way ANOVA for each ARG and LYS groups. [a, b]Mean values with different letters differ significantly in ARG groups. [A, B]Mean values with different letters differ significantly in LYS groups.

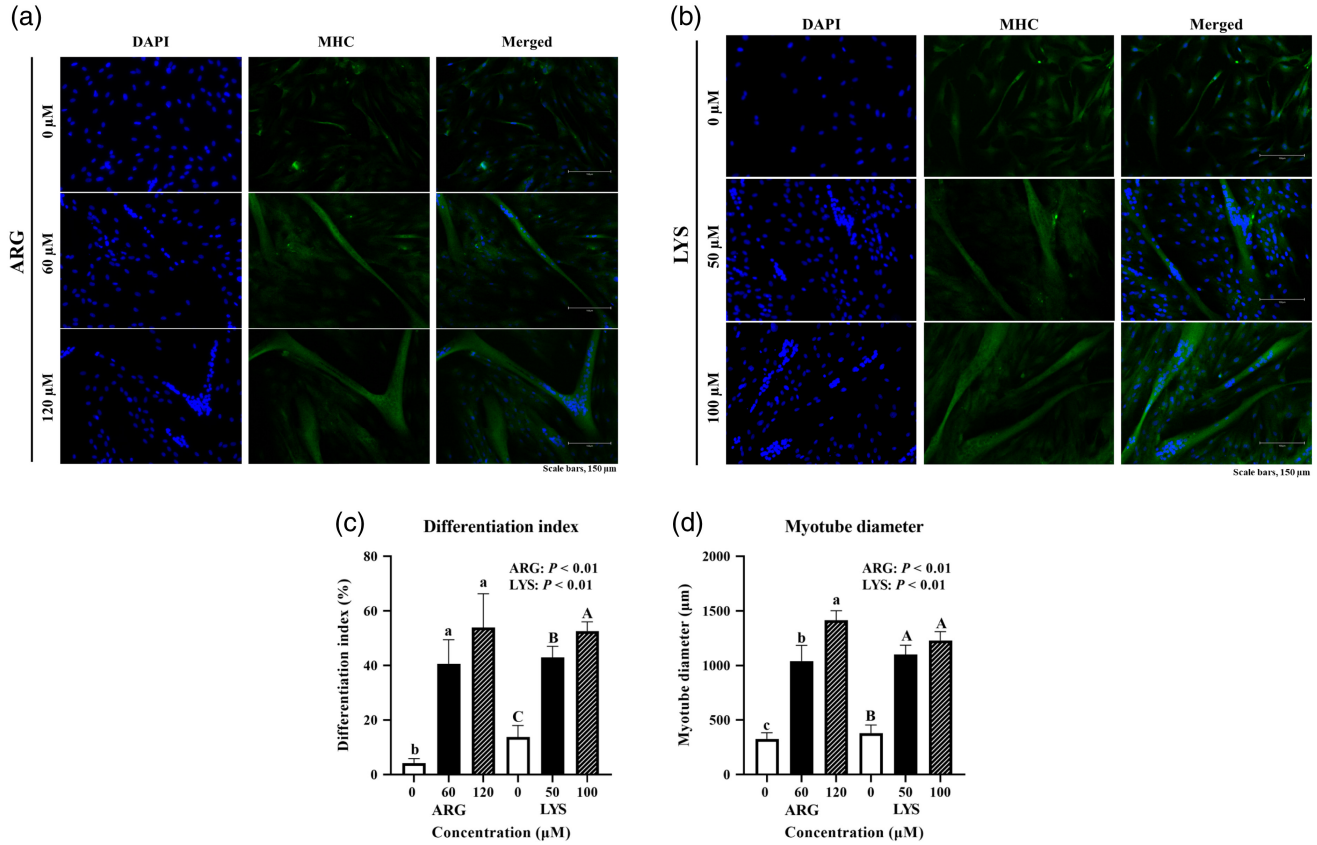

Figure 6. Differentiation index and myotube diameter increased in both arginine (ARG) and lysine (LYS) treatment. (A, B) immunohistochemistry of myosin heavy chain (MHC) expression during myogenic differentiation in cells. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI, blue). MHC was stained with a mouse monoclonal anti-MHC (green): scale bars, 150 μm. (C) Differentiation index was calculated as the percentage of nuclei within the myotube area to the total number of nuclei produced from immunohistochemistry. (D) The diameters of myotube were measured at randomly selected 3 locations taken along the length using the ImageJ software. For each differentiation index and myotube diameter, 5 values per repetition were expressed as the mean and standard deviation. Error bars indicate the standard error. The *P* values are determined by one-way ANOVA for each ARG and LYS group. $^{a–c}$Mean values with different letters differ significantly in ARG groups. $^{A–C}$Mean values with different letters differ significantly in LYS groups.

A pig study showed that feeding LYS improved growth performance in pigs without altering plasma IGF-1 concentrations (Hasan et al., 2020). This previous study suggested that the compromised growth performance, resulting from changes in total protein and amino acid profile by deficient dietary LYS levels, might be associated with certain cell signaling and metabolic pathways that are independent of the growth hormone/IGF-1 axis (Hasan et al., 2020). Another pig study observed that dietary supplementation with ARG led to increased body weight gain, even though no significant changes were observed in the circulating concentrations of plasma IGF-1 (Bass et al., 2017). Similarly, a meta-analysis conducted in humans indicated that acute and chronic ARG supplementation had no impact on levels of IGF-1 (Nejati et al., 2023). However, contrasting viewpoints exist, as some studies have suggested that ARG can induce the secretion of IGF-1 from the endoplasmic reticulum in a dose-dependent manner in rodent models (Tsugawa et al., 2019). The activation of the mTOR pathway by LYS and ARG appears to be independent of IGF-1. Further investigation is required, particularly given the scarcity of cattle-related studies.

Furthermore, it appears that ARG induces a transformation of muscle fibers from glycolytic to oxidative types. Our investigation revealed that elevated concentrations of ARG (120 μM) led to the overexpression of MHC I and IIA. This observation aligns with the findings of a previous study conducted on C2C12 myotubes by Chen et al. (2018). The administration of LYS did not impact on MHC isoforms, except for a tendency toward an increase in MHC IIX. The previous study reported that dietary LYS levels can significantly influence the increase of glycolytic muscle fibers in pigs (Morales et al., 2015). This effect is thought to be mediated by the stimulation of skeletal muscle protein synthesis at the transcriptional level. Veloso et al. (2018) also revealed that dietary LYS appears to trigger changes in the isoforms of MHC isoforms

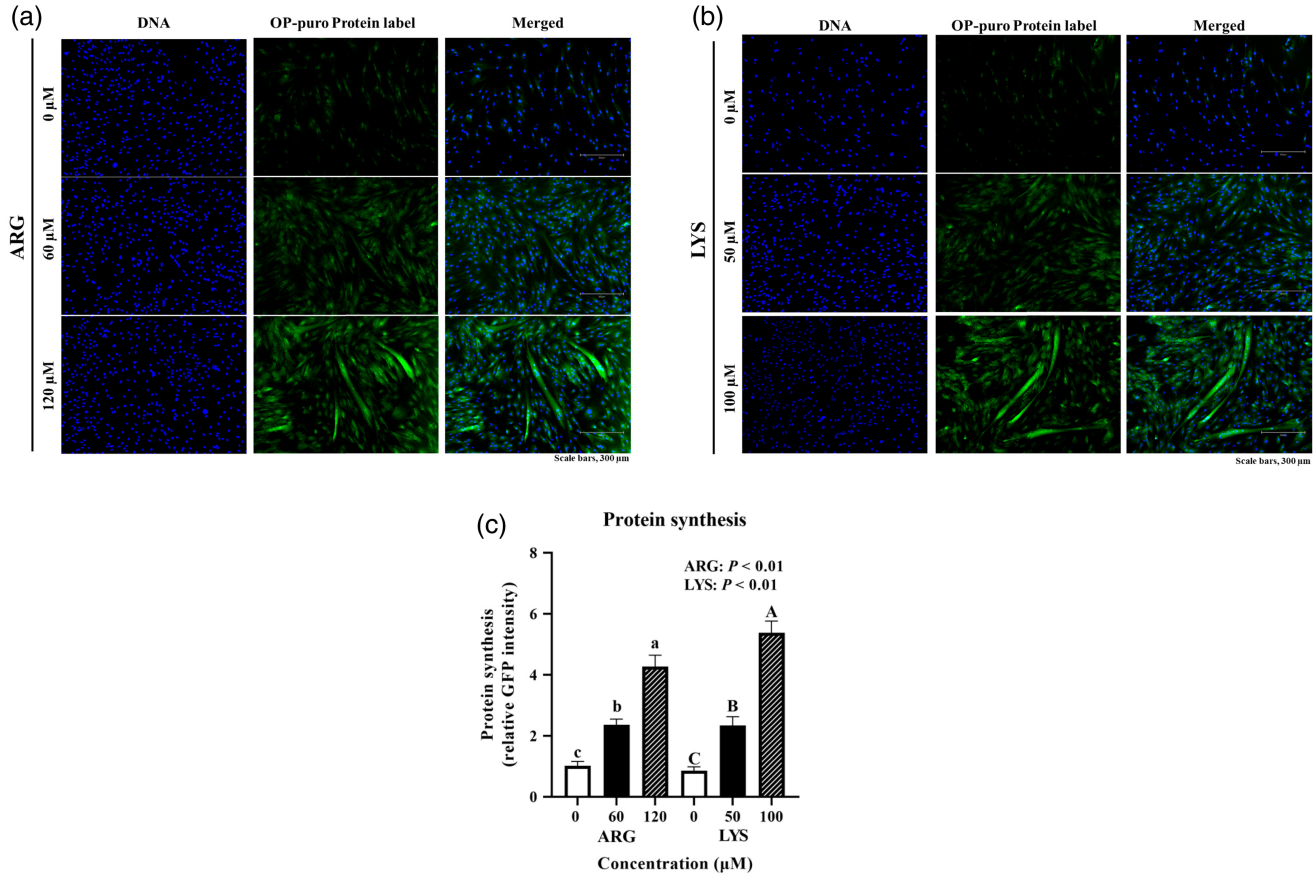

Figure 7. Differentiation index and myotube diameter increased in both arginine (ARG) and lysine (LYS) treatment. (A, B) Immunohistochemistry of labeled protein during myogenic differentiation in cells. Nuclei were stained with DNA staining solution in the kit (blue). Protein was stained with an O-propargyl-puromycin (OP-puro) protein label (green). Scale bars, 300 μm. (C) Green fluorescent protein (GFP) intensity relative to a without treatment well with an OP-puro protein labeled to quantify global protein synthesis. Error bars indicate the standard error. The *P* values are determined by one-way ANOVA for each ARG and LYS group. $^{a–c}$Mean values with different letters differ significantly in ARG groups. $^{A–C}$Mean values with different letters differ significantly in LYS groups.

in pigs. Our findings also suggest that LYS might specifically stimulate the transcription of the MHC IIX in bovine species.

In the present study, both ARG and LYS have shown the ability to increase the muscle fusion index and myotube diameter while enhancing protein synthesis. These findings suggest that both amino acids positively impact muscle growth processes. The fusion index and myotube diameter, used as indicators, reflect muscle hypertrophy in differentiated skeletal muscle cells. The previous studies emphasized the pivotal role of the mTOR signaling pathway in driving protein synthesis and promoting muscle hypertrophy (Hu et al., 2012; Kim et al., 2015). This pathway plays a critical role in coordinating the complex processes that result in skeletal muscle growth and enhanced protein synthesis, ultimately increasing muscle fiber size. Based on our current findings, the ARG and LYS serve as vital energy sources for the progression of skeletal muscle growth. The observed increase in protein synthesis and the enhancement of the fusion index can be interpreted as evidence that the increased availability of ARG and LYS serves as supplementary energy sources, fueling the ongoing process of muscle growth. This role of these amino acids, as both contributors to protein synthesis and as energy substrates, emphasizes their multifaceted importance in facilitating muscle development.

Conclusions

The activation of the mTOR pathway is a critical mechanism through which ARG and LYS exert their effects on skeletal muscle growth. Our findings support the notion that ARG and LYS mediate the Akt/mTOR/P70S6k signaling pathways, which are involved in promoting protein synthesis and muscle hypertrophy. While IGF-1 is known to activate the Akt-mTOR pathway, our study did not observe significant changes in

IGF-1 levels upon ARG or LYS treatment. This suggests that ARG and LYS may regulate the mTOR pathway independently of IGF-1. Although some studies have reported conflicting results regarding the effects of ARG and LYS on circulating IGF-1 levels, the specific effects of these amino acids on skeletal muscle growth in beef cattle remain largely unexplored. Furthermore, ARG appears to induce a transformation of muscle fibers from glycolytic to oxidative types, as indicated by the upregulation of specific MHC isoforms.

Further research is necessary to better understand how these amino acids influence beef cattle muscle development, including their interaction with growth factors, signaling pathways, and metabolic pathways.

Acknowledgments

A portion of the salary and research support for Jongkyoo Kim and Won Seob Kim has been provided by Michigan AgBio Research, Michigan State University (Project #MICL02732) and the Multistate Research Project NC-1184 (Project #MICL04228). Disclosure: The authors declare no conflicts of interest.

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