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L-Leucine stimulates proliferation and casein secretion in porcine mammary epithelial cells through mTOR-p70S6K-4EBP1 signalling pathway

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Abstract: Milk production determines health status and optimal growth of suckling neonates. Recent studies revealed that the secretion of leucine into sow milk from the mammary gland is significantly lower than the amount taken up by the mammary gland from the blood, suggesting the potential and fundamental role in udder metabolism. However, little is known about the underlying mechanism. In this study, porcine mammary epithelial cells (PMECs) were employed to investigate the effects of leucine on protein synthesis and mammalian target of rapamycin (mTOR) signalling. Results showed that leucine supplementation significantly increased the proliferation of PMECs in a dose-dependent manner from 0 to 1 mmol/l. Secretions of four casein subunits were significantly increased by addition of leucine. The phosphorylation of proteins related to mTOR signalling, including mTOR, eukaryotic translation initiation factor 4E-binding protein, and p70 ribosomal protein S6 kinase, were accordingly upregulated by leucine supplementation. Casein secretion was not affected by leucine treatment when mTOR signalling was inhibited by rapamycin. This suggests that leucine stimulates protein synthesis through the mTOR signalling pathway. These findings facilitate the utilization of dietary leucine supplementation to improve milk production in lactating sows.

Keywords: leucine; sow; milk protein; mTOR signalling

Milk production by the mothers often determines the optimal growth and health of suckling neonates to a large extent (Lei et al. 2012a). Milk is the sole source of nourishment, through which neonates acquire nutrients necessary for the development and growth. Therefore, adequate milk production is vital to evaluate the status of mother and the survival rate of babies. Previous studies concentrated on qualitative milk parameters such as lipid and protein concentrations (Makovicky et al. 2015, 2019), however, casein has received less attention.

Branched-chain amino acids (BCAAs), including leucine (Leu), isoleucine and valine, are considered to be not only building blocks for protein synthesis (Shimomura and Kitaura 2018) but also molecules with functional diversity (Ma et al. 2020a, b). The stimulation of protein synthesis in muscle tissue by Leu supplementation has been proved (Suryawan et al. 2011). The quantities of extraction for Leu by the mammary gland of sows were higher than those of secretion in milk protein (Manjarin et al. 2014). Similar results were shown in lactating sows (Trottier et al.

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1997). These studies indicate that a fraction of Leu taken up by the mammary gland is excreted through the milk. Consequently, Leu is proportionally retained in the pool of the mammary gland. The retention in udder conferred on Leu a new potential function and may involve the synthesis of structural proteins and the development of mammary gland (Wang et al. 2014). In addition, net daily mammary uptake for Leu increased with the increase of litter size (Nielsen et al. 2002). Another experiment with lactating sows showed that dietary supplementation of BCAAs significantly increased milk protein content (Dunshea et al. 2005). In milk, casein and whey are two fundamental components of proteins (Zhou et al. 2015). Casein is considered as an ideal source of protein in young animals, and suckling piglets could fully digest casein as well (Rafiee Tari et al. 2019).

Previous studies have demonstrated that Leu regulates casein synthesis via the mammalian target of rapamycin (mTOR) signalling pathway in bovine mammary epithelial cells (Gao et al. 2015). However, the influence of Leu on casein secretion in porcine mammary epithelial cells (PMECs) and its mechanism are not known yet. In the present study, we used isolated and characterized PMECs (Dahanayaka et al. 2015) to investigate the influence of Leu on cell proliferation and mTOR signalling pathways.

MATERIAL AND METHODS

Reagents

Penicillin-streptomycin solution, SDS-PAGE sample loading buffer, cell lysis buffer for western blot, insulin and DAPI staining solution were all purchased from Beyotime (Shanghai, China). Foetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) were purchased from HyClone (Logan, UT, USA). Peroxidase-conjugated goat anti-rabbit IgG was bought from KeyGEN BioTECH (Nanjing, China). Other reagents were bought from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The PMECs were kindly provided by Dr. Guoyao Wu (Texas A&M University, College Station, USA). Cells were cultured as described previously (Dunshea et al. 2005). Briefly, cells were cultured in DMEM/F-12 containing 10% FBS and 5 μg/ml insulin at 37 °C in a humidified atmosphere containing 5% CO_2 and then differentiated in the medium containing 2 μg/ml prolactin.

Cell growth and milk protein determination

The PMECs were inoculated in the cell culture plate. When the growth of the cells reached 50% confluence, 2 μg/ml prolactin was used to treat the cells for the differentiation for two days. After being differentiated, the cells were starved for six hours in DMEM without Leu. The PMECs were continuously cultured in a medium containing 0, 0.25, 0.5, 1, 2, 4 mmol/l Leu, respectively. The cells were collected on day 2 or 4. The numbers of PMECs was measured by the CCK-8 kit, and the OD value of PMECS was measured at 450 nm. The cell supernatant was used to determine the abundance of αs1-casein (αs1-CN), αs2-casein (αs2-CN), β-casein (β-CN), and k-casein (k-CN) via ELISA kits (Shanghai Enzyme-linked Biotechnology Co, Ltd, China). The cell sample was lysed with the cell lysis buffer and centrifuged at 10 000 × *g* to determine the protein expressions of mTOR pathway. In another experiment, after starvation for 6 h in a medium without Leu, the PMECs were cultured in the medium containing 0, 1 mmol/l Leu, 0.000 3 mmol/l rapamycin, and 1 mmol/l Leu plus 0.000 3 mmol/l rapamycin. Casein secretion and protein expressions of mTOR signalling pathway were determined.

Western blot analysis

The PMECs after treatment were harvested and lysed in lysis buffer (Ma et al. 2018). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. The samples were added to a 12% polyacrylamide gel for electrophoresis. The protein was first transferred to the polyvinylidene fluoride (PVDF) membrane, which was placed in a tris-buffered saline with 1% Triton X-100 (TBST) blocking solution containing 5% skim milk powder and sealed for 2 hours. The PVDF membrane was placed in the primary antibody at 4 °C overnight. Primary antibodies were as follows: phos-

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phorylated (P)-mTOR (S2448) (1 : 500 dilution), p70 ribosomal protein S6 kinase (p70S6K) (Thr389) (1 : 1 000 dilution), eukaryotic translation initiation factor 4E-binding protein (4EBP1) (1 : 500 dilution), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1 : 1 000 dilution), which were obtained from Cell Signaling Technology (Danvers, MA, USA). The PVDF membranes were washed five times by 1× TBST for 10 minutes. The membranes were incubated in the secondary antibody (Cell Signaling) at 1 : 5 000 dilution for 2 h at room temperature. The PVDF membranes were washed three times by 1× TBST for 10 minutes. After washing, a chemiluminescent detection reagent ECL-plus from Beyotime Biotechnology (Shanghai, China) was added to the PVDF membrane, and then a BioRad scan was used to detect the fluorescent gel detection system. The results of the grey value analysis were performed using ImageJ software v1.52 (National Institutes of Health, Bethesda, MA, USA).

Immunofluorescence detection

The PMECs were treated with Leu or rapamycin in the 96-well cell plates as described above and stationary liquid was added. The cell plates were washed with PBS three times and were placed in the primary antibody at 37 °C for 1 hour. Primary antibodies bought from Bioss Inc (Woburn, MA, USA) were as follows: phosphorylated (P)-mTOR (S2448) (1 : 100 dilution) and P-4EBP1 (1 : 100 dilution) and P-p70S6K (Thr389) (1 : 100 dilution) was bought from Affinity Biosciences (Cincinnati, OH, USA). The plates were washed with 1× TBST three times for 5 min and were incubated in the secondary antibody at 37 °C for 2 hours. After washing three times, they were stained with DAPI for 10 minutes. The cells and the retained pattern were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using SPSS software v22.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed by one-way analysis of variance and regression analysis followed by the Duncan multiple comparison test for individual comparisons. The level of significance was set at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

Leu supplementation and PMEC proliferation

As shown in Figure 1, addition of Leu significantly improved the relative growth rate of PMECs at both 24 h and 48 h. The highest growth rate was observed in the group of 1 mmol/l Leu. Specifically, a significant increase in numbers of PMECs was observed in a dose-dependent manner from 0 to 1 mmol/l.

Leu treatment and casein secretion

As seen from Figure 2, secretion of four casein subunits αs1-casein, αs2-casein, β-casein, and k-casein was elevated by Leu treatment. The trend

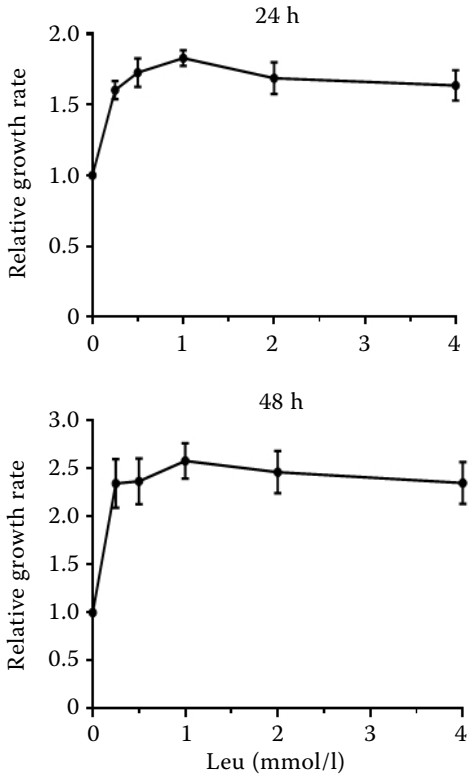

Figure 1. Effect of leucine (Leu) supplementation on the relative growth rate of porcine mammary epithelial cells (PMECs)

Data represent the mean ± SD in three independent experiments. PMECs were starved for 6 h in Dulbecco's modified eagle medium (DMEM) without Leu and then incubated in DMEM containing 0, 0.25, 0.5, 1, 2, 4 mmol/l Leu. Thereafter, the OD value was measured by the CCK-8 kit. Relative growth rate means the percentage of cell proliferation in the Leu-treated groups relative to the control group

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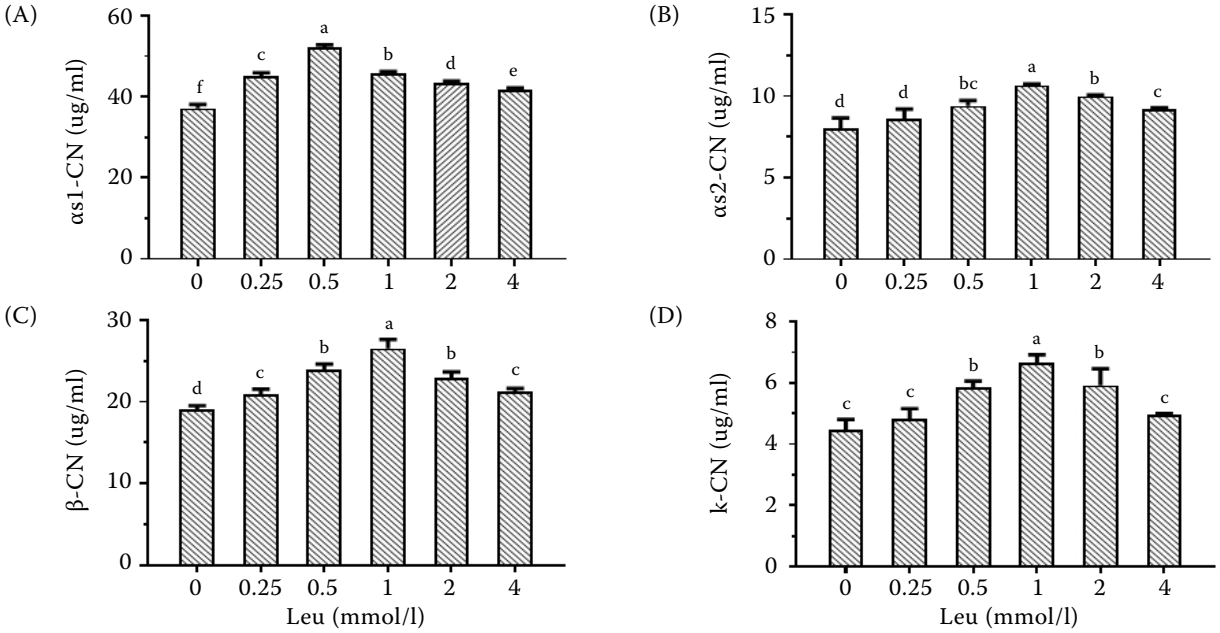

Figure 2. Influence of leucine (Leu) on abundance of four casein subunits in porcine mammary epithelial cells (PMECs)
(A) αs1-casein (αs1-CN), (B) αs2-casein (αs2-CN), (C) β-casein (β-CN), (D) k-casein (k-CN)
[a–f]Different lowercase letters indicate statistically significant differences ($P < 0.05$)
Data represent the mean ± SD in three independent experiments. PMECs were starved in Dulbecco's modified eagle medium (DMEM) without Leu for 6 hours and then they were cultured in DMEM containing 0, 0.5, 1 or 2 mmol/l Leu. The abundance of caseins was detected via ELISA kits

was to increase and then to decline. At 1 mmol/l, the secretion reached the peak. Moreover, as shown in Figure 3, addition of Leu significantly enhanced the expressions of phosphorylation of mTOR, p70S6K and 4EBP1 in a dose-dependent manner from 0 to 1 mmol/l.

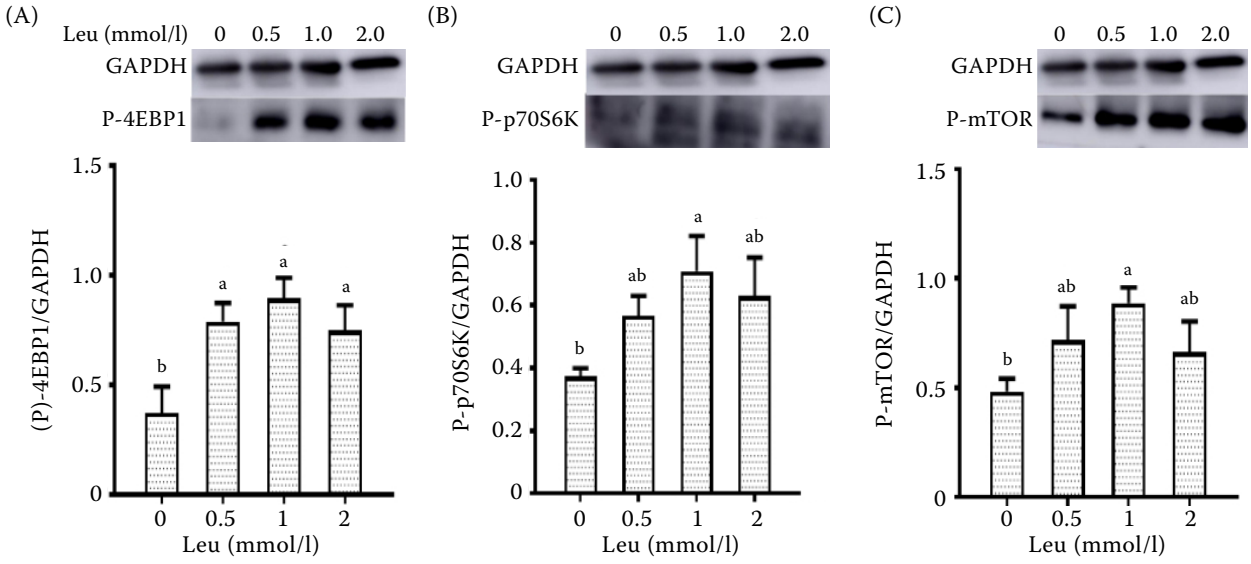

Figure 3. Influence of leucine (Leu) on expression of (A) phosphorylated (P)-4EBP1, (B) P-p70S6K, and (C) P-mTOR in porcine mammary epithelial cells (PMECs)
[a,b]Different letters represent significant differences ($P < 0.05$)
Data represent the mean ± SD in three independent experiments. PMECs were starved in Dulbecco's modified eagle medium (DMEM) without Leu for 6 h and then they were cultured in DMEM containing 0, 0.5, 1 or 2 mmol/l Leu. Finally, the cells were collected for western blot analysis

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Rapamycin and mTOR signalling

The relative growth rate of PMECs decreased with the increase of rapamycin concentration in a dose-dependent manner from 0 to 700 nmol/l. At the concentration of 300 nmol/l rapamycin, the cell proliferation rate was reduced by about 20%. A dose of 300 nmol/l rapamycin significantly inhibited the phosphorylation of mTOR and p70S6K (Figure 4). Furthermore, addition of 300 nmol/l rapamycin significantly decreased the expressions of αs1-casein, αs2-casein, β-casein, and k-casein (Figure 5).

Casein secretion under mTOR inhibitor

The phosphorylation of mTOR, p70S6K, and 4EBP1 was upregulated by Leu supplementation but downregulated by rapamycin treatment (Figure 4). Supplementation of both Leu and rapamycin did not stimulate the phosphorylation of mTOR, p70S6K, and 4EBP1. These results were further confirmed by an immunofluorescence assay (Figure 6). As seen from Figure 5, the staining intensity of supplementation of both Leu and rapamycin was not significantly increased compared to that of rapamycin treatment. Similarly, supplementation of both Leu and rapamycin did not stimulate the secretion of casein proteins (Figure 4).

DISCUSSION

As a functional AA, Leu showed functional diversity. L-Leucine excreted from the udder of lactating sows is far lower than that obtained by the mammary gland from the mammary artery. This suggests that leucine may be conferred a new function in udder metabolism. The morphology was still intact and active under the treatment with Leu for up to four days as shown in Figure 6. This guarantees PMECs to secrete milk production normally (Zheng et al. 2010). The efficiency of protein synthesis in the mammary gland is closely related to the quantity of mammary epithelial cells. Previous studies have noted the importance of Leu for breast cell growth and development (Mercier and Gaye 1982). It is

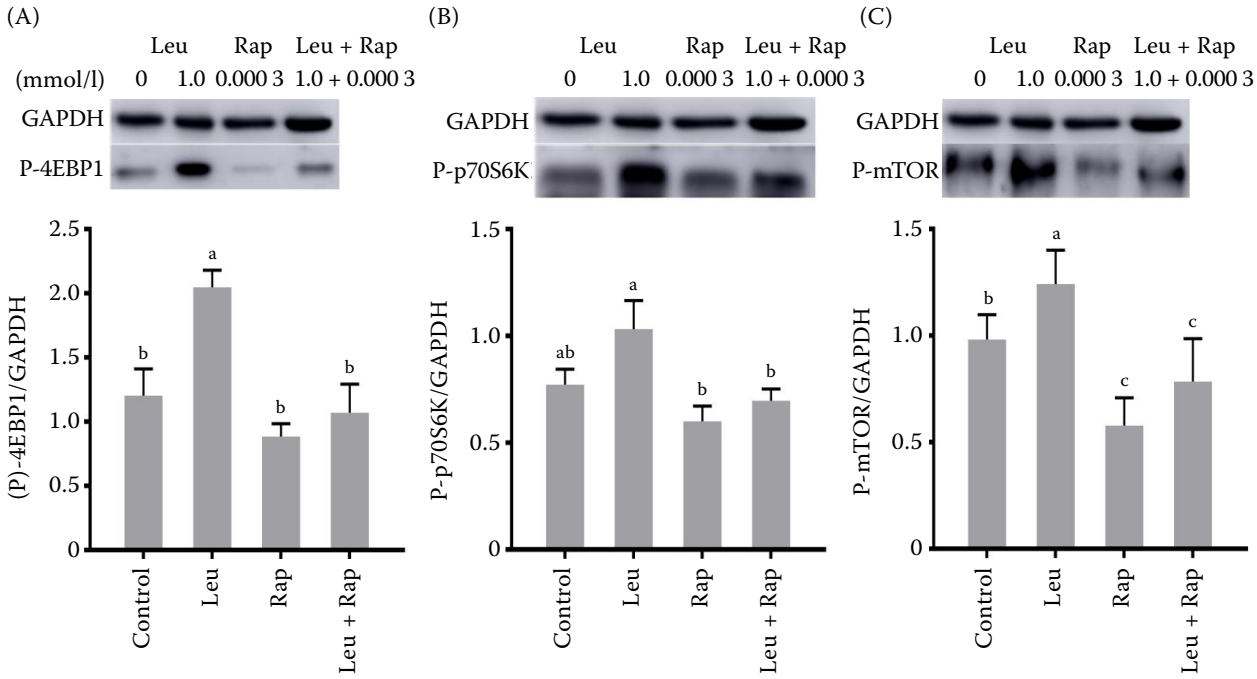

Figure 4. Influence of leucine (Leu) and rapamycin (Rap) on expression of (A) phosphorylated (P)-4EBP1, (B) P-p70S6K, and (C) P-mTOR in porcine mammary epithelial cells (PMECs)

[a–c]Different letters represent significant differences ($P < 0.05$)

Data represent the mean ± SD in three independent experiments. PMECs were starved in Dulbecco's modified eagle medium (DMEM) without Leu for 6 h and then they were cultured in DMEM containing 0, 1 mmol/l Leu, 300 nmol/l Rap, and both of them. Finally, the cells were collected for western blot analysis

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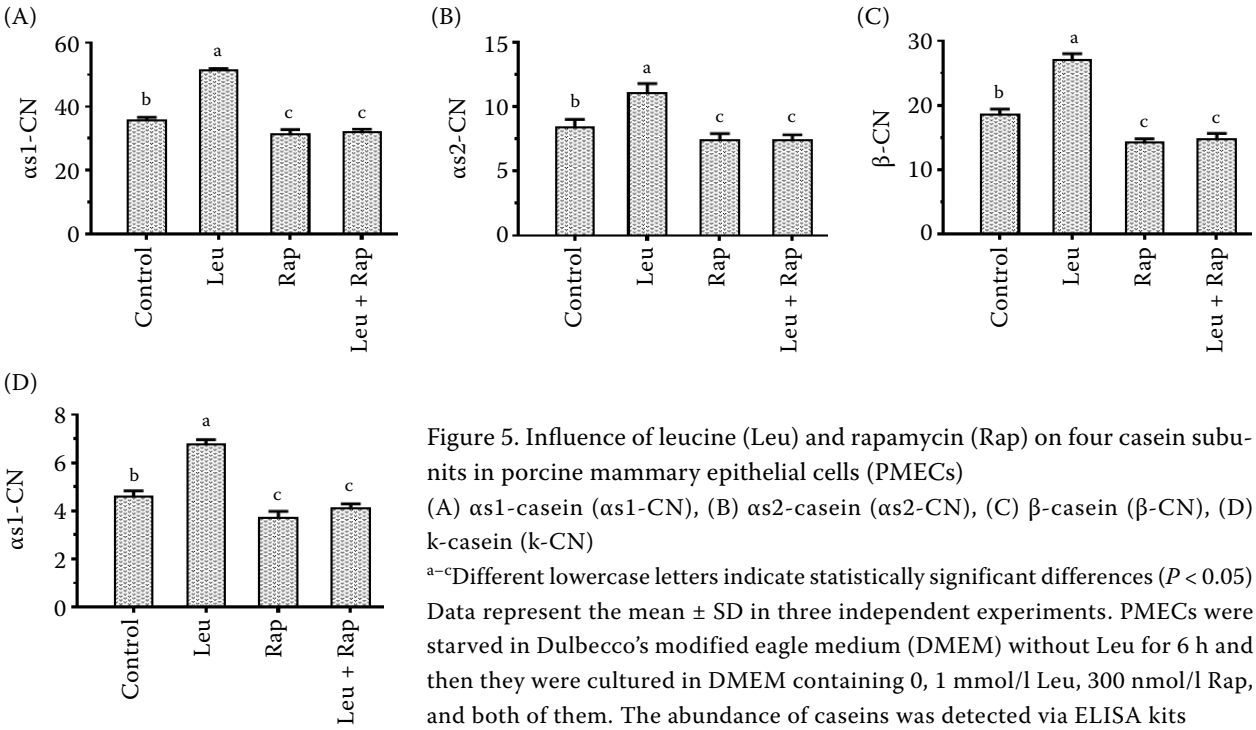

Figure 5. Influence of leucine (Leu) and rapamycin (Rap) on four casein subunits in porcine mammary epithelial cells (PMECs)

(A) αs1-casein (αs1-CN), (B) αs2-casein (αs2-CN), (C) β-casein (β-CN), (D) k-casein (k-CN)

[a–c]Different lowercase letters indicate statistically significant differences ($P < 0.05$)

Data represent the mean ± SD in three independent experiments. PMECs were starved in Dulbecco's modified eagle medium (DMEM) without Leu for 6 h and then they were cultured in DMEM containing 0, 1 mmol/l Leu, 300 nmol/l Rap, and both of them. The abundance of caseins was detected via ELISA kits

widely accepted that Leu is not only one of the building blocks of protein but also an important regulatory factor for protein synthesis (Manjarin et al. 2014), animal growth, and lactation (Zhang et al. 2017). In this study, we investigate the impact of Leu on cell proliferation, casein secretion, and mTOR signalling of PMECs. In order to eliminate the interference of other amino acids, a customized medium containing physiological concentrations of all other amino acids except Leu was used to mimic the *in vivo* state (Kim et al. 2013).

Casein is a family of related phosphoproteins that make up the majority of the proteins in mammalian milk. The results indicated that Leu supplementation significantly improved cell proliferation and increased the secretion of caseins. Our results are consistent with previous reports on bovine mammary epithelial cells (Appuhamy et al. 2012; Gao et al. 2015). In bovine mammary epithelial cells, the supplementation of Leu significantly increased the expressions of four casein subunits in a dose-dependent manner (Gao et al. 2015). However, increasing dietary isoleucine or Leu had no effects on milk dry matter, fat, ash and lactose (Moser et al. 2000).

It has been recognized that the mTOR signalling pathway mediates casein synthesis induced by amino acids (Prizant and Barash 2008; Appuhamy et al. 2014; Arriola Apelo et al. 2014). BCAAs, especially Leu, have been proved to promote the cell proliferation, increase the longevity, and enhance the fractional protein synthesis rates in bovine mammary epithelial cells by the phosphorylation of mTOR, 4EBP1 and S6K1 (Lei et al. 2012b; Appuhamy et al. 2014). In the present study, we used rapamycin, an allosteric mTORC1 inhibitor (Benjamin et al. 2011), to further verify the underlying mechanism for the regulatory role of Leu in porcine mammary cells. The results showed that the stimulating role of Leu in casein synthesis was positively correlated with the activation of phosphorylated mTOR, p70S6K1 and 4EBP1. The mTOR is a key component of protein complexes, mTORC1 and mTORC2 (Saxton and Sabatini 2017), and plays an important role in cell growth and survival, transcription, and protein synthesis (Lipton and Sahin 2014). Previous study showed that the phosphorylation of mTOR (Ser2481) was enhanced with the increase of Leu concentrations in bovine mammary epithelial cells (Gao et al. 2015), and our results are consistent with this.

The p70S6K and 4EBP1 are two downstream targets of mTOR (Hay and Sonenberg 2004). It is generally accepted that translation of 5' terminal oligopyrimidine mRNAs requires the activation of p70S6K (Pullen and Thomas 1997), the widely recognized of which is the phosphorylation. One of the p70S6K substrates is ribosomal protein S6

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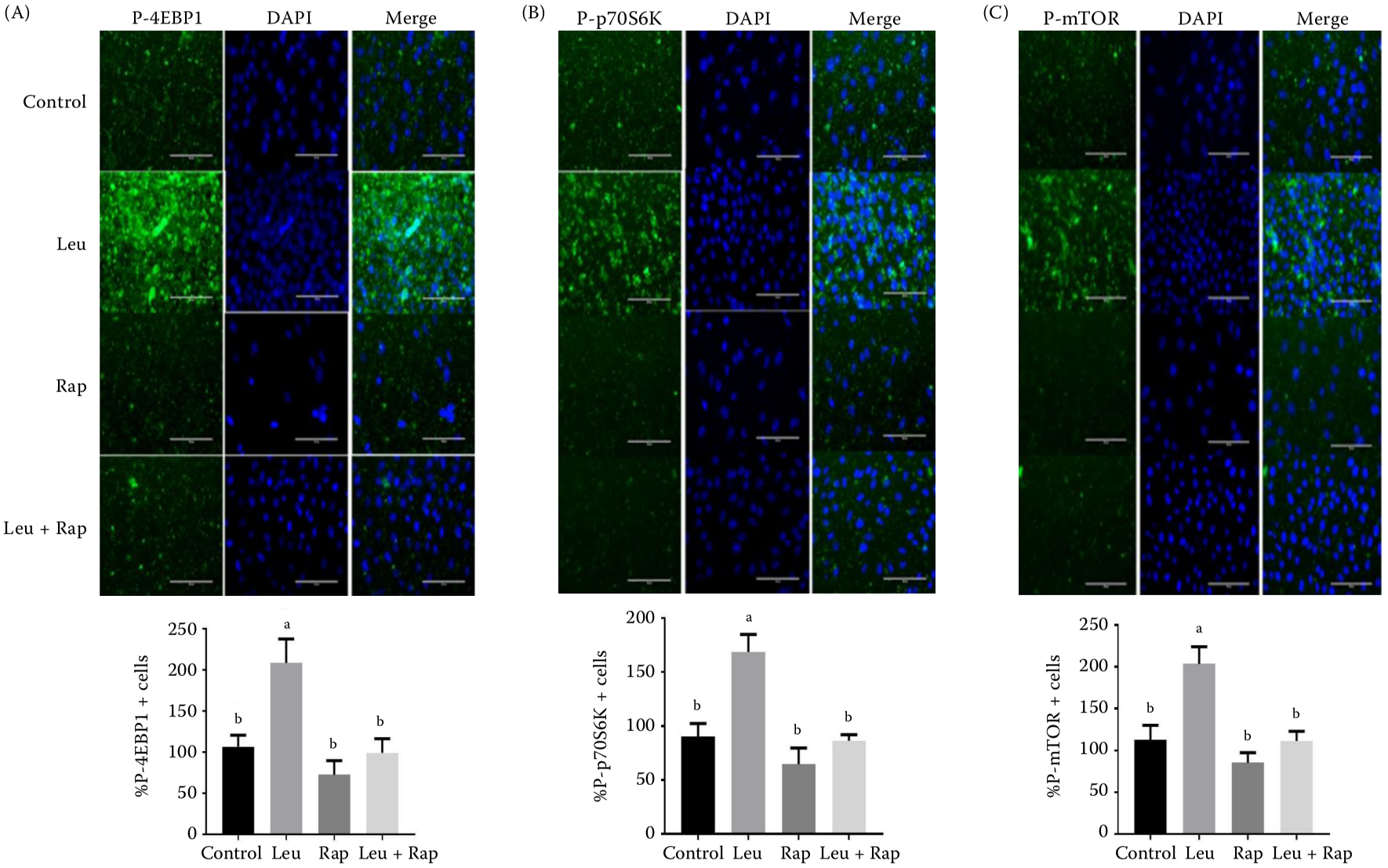

Figure 6. Influence of leucine (Leu) and rapamycin (Rap) on expression of (A) phosphorylated (P)-4EBP1, (B) P-p70S6K, and (C) P-mTOR in PMECs by immunofluorescence method

Porcine mammary epithelial cells were starved in Dulbecco's modified eagle medium (DMEM) without Leu for 6 h and then they were cultured in DMEM containing 0, 1 mmol/l Leu, 300 nmol/l Rap, and both of them. Finally, the cells were observed under a fluorescence microscope. P-4EBP1, P-p70S6K and P-mTOR (green), DAPI (blue)

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of the 40S ribosomal subunit, phosphorylation of which stimulates protein synthesis at the ribosome. As another important downstream target of mTOR, 4EBP1 plays a regulatory role in the interaction between eIF4E and eIF4G and inhibits cap-dependent translation by binding with high affinity to eIF4E. Hyperphosphorylated 4EBP1 prevents the interaction and finally results in the activation of cap-dependent translation (Pause et al. 1994). Our results showed that addition of Leu significantly increased both phosphorylated p70S6K and 4EBP1, which was in agreement with the previous report on bovine mammary epithelial cells (Gao et al. 2015). Recent studies found that Leu signals to mTORC1 via its metabolite acetyl-coenzyme A through EP300-mediated acetylation of raptor (Son et al. 2019).

In conclusion, the results of the present study indicated Leu supplementation promoted cell proliferation and milk protein (casein) synthesis. The underlying mechanism was closely related to the mTOR signalling pathway, specifically the activation of phosphorylation of mTOR, p70S6K, and 4EBP1. These findings contribute to the dietary Leu supplementation, thereby increasing the milk production of lactating sows. However, further animal models are needed to explore the regulatory effects of Leu on the synthesis and secretion of milk components including protein, fatty acids, and lactose. This study provides a valuable reference for the use of Leu to improve casein secretion in sows.

Conflict of interest

The authors declare no conflict of interest.

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