Animal Nutrition 2 (2016) 218–224

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original research article

Leucine increases mucin 2 and occludin production in LS174T cells partially via PI3K-Akt-mTOR pathway

Xiangbing Mao [a, b, *, 1], Haiyan Hu [a, b, 1], Jun Tang [a, b], Daiwen Chen [a, b], Bing Yu [a, b]

[a] Animal Nutrition Institute, Sichuan Agricultural University, Ya'an 625014, China
[b] Key Laboratory of Animal Disease-Resistance Nutrition, Ministry of Education, Ya'an 625014, China

ARTICLE INFO

Article history:
Received 6 April 2016
Accepted 25 May 2016
Available online 2 June 2016

Keywords:
Mucin 2
Occludin
Leucine
LS174T cells
PI3K-Akt-mTOR

ABSTRACT

Mucin 2 and occludin play a crucial role in preserving the intestinal mucosal integrity. However, the role for leucine mediating intestinal mucin 2 and occludin expression has little been investigated. The current study was conducted to test the hypothesis that leucine treatment could increase mucin 2 and occludin levels in LS174T cells. The LS174T cells were incubated in the Dulbecco's Modified Eagle Medium (DMEM) supplementing 0, 0.5 and 5 mmol/L L-leucine for the various durations. Two hours after the leucine treatment, the inhibitor of mammalian target of rapamycin (mTOR) and protein kinase B (Akt) phosphorylation in LS174T cells were significantly increased ($P < 0.05$), and the mucin 2 and occludin levels were also significantly enhanced ($P < 0.05$). However, the pretreatment of 10 nmol/L rapamycin, which was an mTOR inhibitor, or 1 μmol/L wortmanin, which was an inhibitor of phosphatidylinositol 3-kinase (PI3K), completely inhibited leucine-induced mTOR or Akt phosphorylation ($P < 0.05$), and significantly reduced leucine-stimulated mucin 2 and occludin levels ($P < 0.05$). These results suggest that leucine treatment promotes the mucin 2 and occludin levels in LS174T cells partially through the PI3K-Akt-mTOR signaling pathway.

1. Introduction

The mucus gel layer and the intercellular junctions between the intestinal epithelial cells are the crucial components of the non-specific barrier mechanisms in gut, which benefits the maintenance of intestinal mucosal integrity in human (Jankowski et al., 1994). The intercellular junctions between the intestinal epithelial cells mainly maintain through some transmembrane and non-membrane proteins, including occludin (Jankowski et al., 1994; Furuse et al., 1993). In addition, mucins, such as mucin 2, are secreted by goblet cells, which are the key components of the mucus gel layer in gut mucosa (Mao et al., 2011a). The intercellular junctions and mucins are important for maintaining animal and human health. Many studies have indicated that some nutrients, including essential amino acids, may affect the intestinal non-specific barrier mechanisms (Mao et al., 2011a, 2012; Chen et al., 2013; Wang et al., 2010). Our recent study also showed that dietary leucine supplementation significantly increased the mucin 2 level in the intestinal mucosa of weaned pigs (Mao et al., 2015). However, it is little known whether leucine can regulate the mucin 2 and occludin expression in intestinal cells.

The previous studies have also shown that leucine may stimulate specific protein expression in various cells (Zhang et al., 2014; Pérez de Obanos et al., 2006; Roh et al., 2003; Ijichi et al., 2003; Mao et al., 2011b). Moreover, during the protein synthesis in mammalian cells, leucine acts as the energy supply and

* Corresponding author.
E-mail address: acatmxb2003@163.com (X. Mao).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.aninu.2016.05.004

substrates, and also regulates some intracellular signaling pathways, including the PI3K-Akt-mTOR signaling pathway (Kimball, 2002). LS174T cells can produce the tight junction protein (Elamin et al., 2014; Resta-Lenert et al., 2011), which is appropriate as a model to determine the barrier of function of the intestinal mucosa. Therefore, the present study hypothesized that leucine treatment can increase the mucin 2 and occludin levels in LS174T cells, and PI3K-Akt-mTOR pathway plays a potential role in regulating the mucin 2 and occludin levels by leucine.

2. Materials and methods

2.1. Cell culture

The LS174T cells from the American Type Culture collection (ATCC; Rockville, MD) were used as an *in vitro* model for intestinal epithelium. The cells (1×10^6 cells/well) were seeded in six-well plates in 2 mL of Dulbecco's Modified Eagle Medium (DMEM; Hyclone Laboratories Inc., Logan, UT) containing 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% fetal calf serum (FCS; Gibco Laboratories Life Technologies Inc., Grand Island, NY) and antibiotics (100 units penicillin/mL and 100 μg streptomycin/mL; Gibco Laboratories Life Technologies Inc., Grand Island, NY) at 37°C and in a 5% CO_2 atmosphere. When cells were grown to 90% confluence, they were starved for 12 h in serum and antibiotic-free DMEM.

2.1.1. Experiment one

The LS174T cells were incubated for 2 h in the presence of 0, 0.5 and 5 mmol/L leucine, and there were 6 replications for each leucine-treatment dose ($n = 6$). Following 2 h, the cells were collected and used to determine mTOR and Akt phosphorylation states and the levels of mucin 2, occludin and β-tubulin.

2.1.2. Experiment two

The time course (0, 1, 2, 4 and 6 h) of leucine treatment was administrated by incubating cells in the presence of the proper dose that was derived from Exp. 1, and there were 6 replications for each leucine-treatment duration ($n = 6$). Following the various culture durations, the cells were collected to examine mTOR and Akt phosphorylation states and the levels of mucin 2, occludin and β-tubulin.

2.1.3. Experiment three

After the starvation, LS174T cells were incubated in starvation media containing 10 nmol/L rapamycin or 1 μmol/L wortmanin. Following a 30 min treatment period, the proper dose of L-leucine that was derived from Exp. 1 was added to the media for the proper duration that was derived from Exp. 2, and there were 6 replications for each treatment ($n = 6$). Then, all cells were collected and used to determine mTOR and Akt phosphorylation states and the levels of mucin 2, occludin and β-tubulin.

2.2. The enzyme-linked immunosorbent assay (ELISA) analysis of mucin 2

The mucin 2 level was measured with ELISA analysis in the collected LS174T cells of each experiment, and there were 6 replications for each treatment ($n = 6$).

Cell protein isolation was performed as described previously (Mao et al., 2013). Briefly, following collection, the LS174T cells were suspended in 200 μL of fresh medium, and subjected to three cycles of freezing and thawing. The cell lysates were centrifuged at 10,000 × *g* and 4°C for 10 min. The supernatants were isolated and stored at −80°C until analyzed.

Then, mucin 2 protein levels were determined by ELISA as described previously with minor modifications (Devine et al., 1992; Elamin et al., 2014). Briefly, rabbit anti-mucin 2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was coated in the Falcon Microtest III ELISA 96-well plate (Becton–Dickinson, CA) by incubating 50 μL/well of a 1:100 dilution in the blocking solution (1% bovine serum albumin [BSA] in phosphate buffered saline [PBS]) overnight at 4°C. After expelling unbound antibody, the plate was blocked with 75 μL/well blocking solution at room temperature for 2 h. Following washing the plate 3 times with PBS containing 0.05% Tween-20, 50 μL/well of the cell protein sample diluted 1/2 in 2 × PBS containing 0.05% Tween-20 was added to each well and incubated overnight at 4°C. After washing the plate as above, mouse anti-mucin 2 (Abcam Inc., Cambridge, MA) was added by incubating 50 μL/well of a 1:100 dilution in the blocking solution at room temperature for 2 h. After washing the plate as above, goat anti-mouse horseradish peroxidase (HRP)-conjugated IgG (Jackson Immuno-Research Laboratories Inc., West Grove, PA) was added by incubating 25 μL/well of a 1:100 dilution in the blocking solution at room temperature for 1 h. Following washing the plate as above, 100 μL of 3,3′,5,5′-tetramethylbenzidine was added to each well and incubated with shaking in the dark at room temperature for 30 min. Finally, 25 μL of stop solution was added to each well, and the optical density was read at 450 nm using a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT).

2.3. Western blot analysis of occludin, β-tubulin and phosphorylation of mTOR and Akt

Protein levels for occludin, β-tubulin and the mTOR and Akt phosphorylation in LS174T cells were determined by Western blot analysis in the collected LS174T cells of each experiment, and there were 6 replications for each treatment ($n = 6$) as described previously with some modification (Mao et al., 2011b). Briefly, following lysing, the cell lysates were centrifuged for 15 min at 12,000 × *g* and 4°C. The supernatant was isolated. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical Co., Rockland, IL). The supernatants containing equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 7.5% or 12% gel and transferred to polyvinylidene difluoride membranes. After the transfer, membranes were blocked with 5% non-fat dry milk in the buffer (0.1% Tween-20, 50 mmol/L Tris–HCl, pH 7.6, and 150 mmol/L NaCl) for 1 h at room temperature. Proteins were visualized with specific antibodies (Cell Signaling Technology, Beverly, MA), horseradish peroxidase conjugated secondary antibodies (Jackson Immuno-Research Laboratories Inc., West Grove, PA), and the Western Blotting Luminol Reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The optical density of the bands for the occludin and the mTOR and Akt phosphorylation were normalized to their respective β-tubulin band using the Alpha Imager 2200 (Alpha Innotech, San Leandro, CA) software.

2.4. Statistical analysis

All data were expressed as means ± SE, analyzed using one-way ANOVA, and followed by Duncan's Multiple Range test. All analyses were performed using SAS (Version 8.1; SAS Institute, Cary, NC). $P < 0.05$ was considered statistical significance.

3. Results

3.1. Effect of leucine treatment on the phosphorylation of mTOR and Akt and the levels of mucin 2 and occludin

The levels of mTOR and Akt phosphorylation were significantly stimulated by the leucine treatment ($P < 0.05$), which changing was larger under 5 mmol/L leucine treatment (Fig. 1). Furthermore, as shown in Fig. 1, leucine treatment could significantly increase the levels of mucin 2 and occludin ($P < 0.05$), and that changing was also larger under 5 mmol/L leucine treatment (Fig. 1). Therefore, the dose of leucine treatment was 5 mmol/L in the remaining experiments of this study.

The mTOR and Akt phosphorylation levels were significantly stimulated at 1 h following 5 mmol/L leucine treatment ($P < 0.05$), and reached its maximum at 2 h (Fig. 2). Additionally, as shown in Fig. 2, the levels of mucin 2 and occludin were remarkably stimulated in 1 h following 5 mmol/L leucine treatment ($P < 0.05$), and reached a plateau by 2 h. Thus, the duration of leucine treatment was 2 h in the remaining experiments of this study.

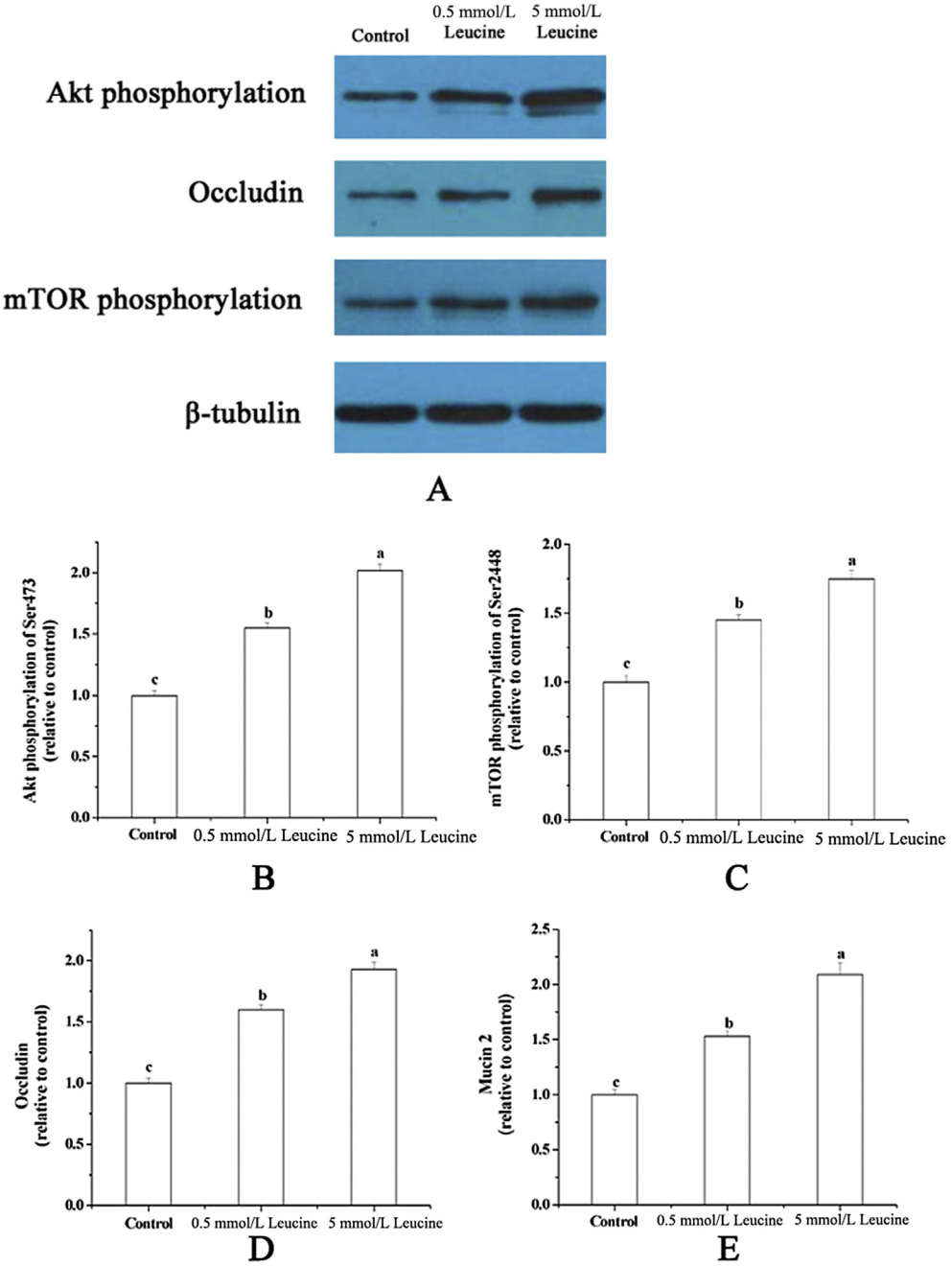

Fig. 1. Effect of supplementing different doses of leucine in medium on the phosphorylation of mammalian target of rapamycin (mTOR) and protein kinase B (Akt), and the levels of mucin 2 and occludin in LS174T cells. The LS174T cells were incubated with 0, 0.5 and 5 mmol/L leucine for 2 h. Representative western blots (A) for occludin, β-tubulin and phosphorylation of mTOR and Akt in LS174T cells were showed. Results were expressed as the amount of Akt phosphorylation (B), mTOR phosphorylation (C) and occludin (D) to β-tubulin and the amount of mucin 2 (E) in each treatment as a ratio of the treatment to the control. Values are means ± SE; $n = 6$. ^{a,b,c} Bars with different letters are significantly different ($P < 0.05$).

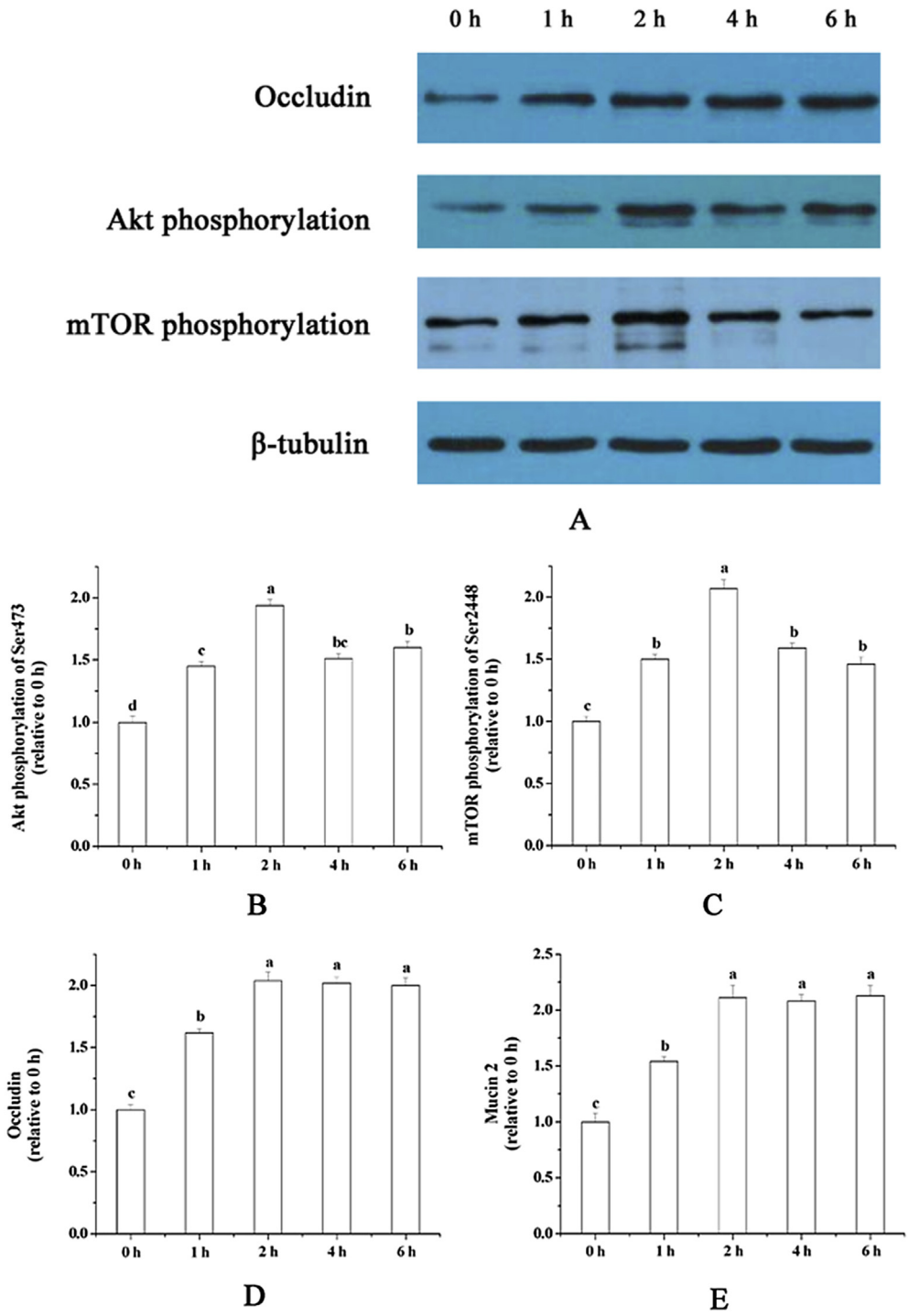

Fig. 2. Effect of 5 mmol/L leucine treatment in medium on the phosphorylation of mammalian target of rapamycin (mTOR) and protein kinase B (Akt), and the levels of mucin 2 and occludin for the various duration in LS174T cells. The LS174T cells were incubated with 5 mmol/L leucine for 0, 1, 2, 4 and 6 h. Representative western blots (A) for occludin, β-tubulin and phosphorylation of mTOR and Akt in LS174T cells were showed. Results were expressed as the amount of Akt phosphorylation (B), mTOR phosphorylation (C) and occludin to β-tubulin (D) and the amount of mucin 2 (E) in each treatment as a ratio of the treatment to the control. Values are means ± SE; $n = 6$. ^{a,b,c,d} Bars with different letters are significantly different ($P < 0.05$).

3.2. *Effect of leucine and/or inhibitor on phosphorylation of mTOR and/or Akt in LS174T cells*

As shown in Fig. 3, the levels of mTOR phosphorylated on Ser2448 and Akt phosphorylated on Ser473 in LS174T cells were significantly increased by supplementing 5 mmol/L leucine ($P < 0.05$). Rapamycin, the inhibitor of the mTOR activation, significantly decreased the mTOR phosphorylation that was enhanced by supplementing leucine ($P < 0.05$, Fig. 3). Moreover, wortmanin, the phosphatidylinositol 3-kinase (PI3K) inhibitor, significantly inhibited the Akt phosphorylation that was stimulated by supplementing leucine ($P < 0.05$, Fig. 3).

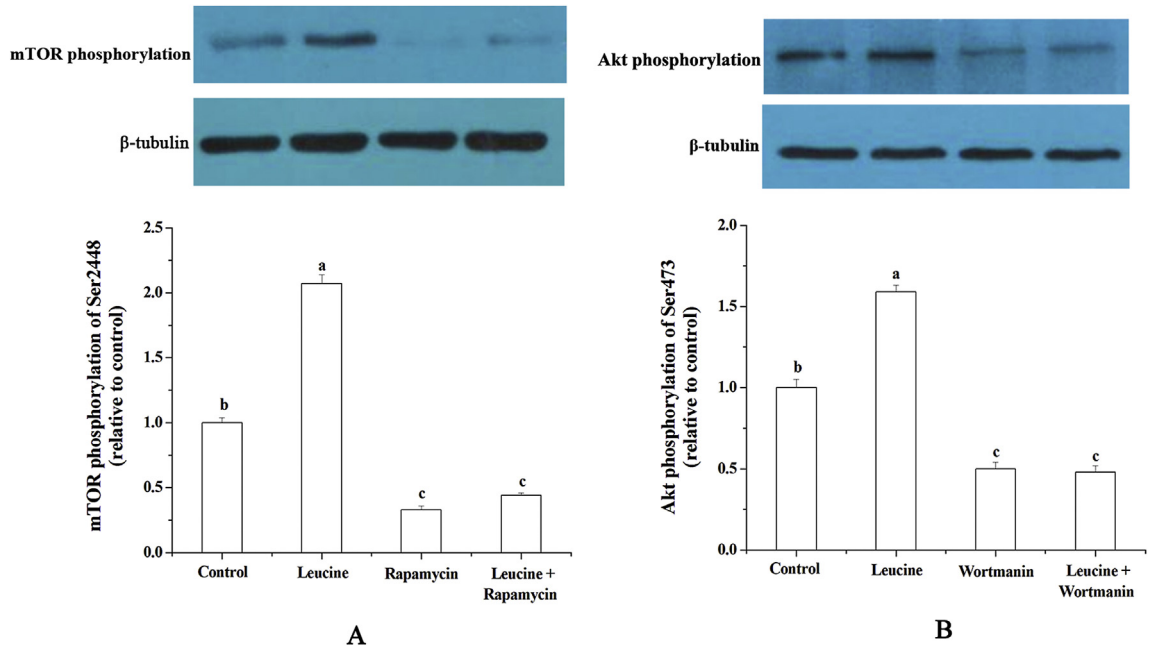

Fig. 3. Effect of rapamycin or wortmanin on phosphorylation of mammalian target of rapamycin (mTOR) or protein kinase B (Akt) in LS174T cells cultured in medium supplemented with leucine. The LS174T cells were subjected to pre-incubation with or without 10 nmol/L rapamycin (A) or 1 μmol/L wortmanin (B), and then treated with 5 mmol/L leucine for 2 h. Results are expressed as the amount of mTOR and Akt phosphorylation to β-tubulin in each treatment as a ratio of the treatment to the control. Values are means ± SE; $n = 6$. [a,b,c] Bars with different letters are significantly different ($P < 0.05$).

3.3. *Effect of leucine and/or inhibitor on the mucin 2 level and the occludin expression in LS174T cells*

As shown in Fig. 4, when LS174T cells were incubated in starvation media added 5 mmol/L leucine, the levels of mucin 2 and occludin were significantly enhanced ($P < 0.05$). However, the increasing level of mucin 2 and occludin in response to the addition of leucine was significantly inhibited by pretreatment with rapamycin or wortmanin ($P < 0.05$, Fig. 4).

4. Discussion

Our previous study showed that dietary leucine supplementation could increase the number of goblet cell and the mucin 2 level in the jejunum of piglets (Mao et al., 2015). Similar to that study, the finding of the present work is that leucine treatment significantly increased mucin 2 and occludin levels in LS174T cells (Figs. 1, 2 and 4). Furthermore, the effect of leucine treatment on mucin 2 and occludin levels might be relative to the PI3K-Akt-mTOR signaling pathway (Fig. 4).

Mucin 2, an important component of the mucus gel layer in the intestine, is mainly synthesized and secreted by the goblet cells, which is the important component of non-specific barrier mechanisms in the intestinal mucosa (Mao et al., 2011a). In addition, as a component of intestinal epithelial tight junction, occludin plays an important role in protecting animals and humans against enteric pathogens (Jankowski et al., 1994; Furuse et al., 1993). In the present study, leucine treatment significantly increased the mucin 2 and occludin levels in LS174T cells (Figs. 1, 2 and 4). Recent studies have also shown that LS174T cells, a colorectal cell line derived from human, have the intestinal epithelial goblet-like characteristics, and can produce the tight junction protein, including occludin (Elamin et al., 2014; Resta-Lenert et al., 2011). Therefore, in the current study, we used LS174T cells as a model to determine the effect of leucine on mucin 2 and occludin levels in the intestinal mucosa. Therefore, it is possible that leucine supplementation might be able to improve the barrier function of intestinal mucosa via increasing the mucin 2 and occludin levels.

The previous studies have shown that leucine could increase the specific protein production in some cells via the PI3K-Akt-mTOR signaling pathway (Zhang et al., 2014; Pérez de Obanos et al., 2006; Roh et al., 2003; Ijichi et al., 2003; Mao et al., 2011b). In this study, the addition of leucine significantly increased the activity of PI3K-Akt-mTOR signaling pathway and the levels of mucin 2 and occludin (Figs. 1, 2 and 4). In addition, rapamycin or wortmanin, the specific inhibitor of mTOR or PI3K, completely inhibited the phosphorylation of mTOR or Akt (Fig. 3), and significantly reduced the increase of mucin 2 and occludin levels that was promoted by leucine treatment (Fig. 4). Therefore, these results suggest that leucine increasing mucin 2 and occludin production of LS174T cells could at least partially be regulated by the PI3K-Akt-mTOR signaling pathway.

5. Conclusions

The results of the present study show that leucine treatment increased mucin 2 and occludin levels in LS174T cells through the PI3K-Akt-mTOR signaling pathway. However, the inhibitors of PI3K-Akt-mTOR signaling pathway could not completely inhibit the increase of mucin 2 and occludin levels that was promoted by leucine treatment, which reveals that leucine mediates the production of specific proteins possibly via other modes or pathways.

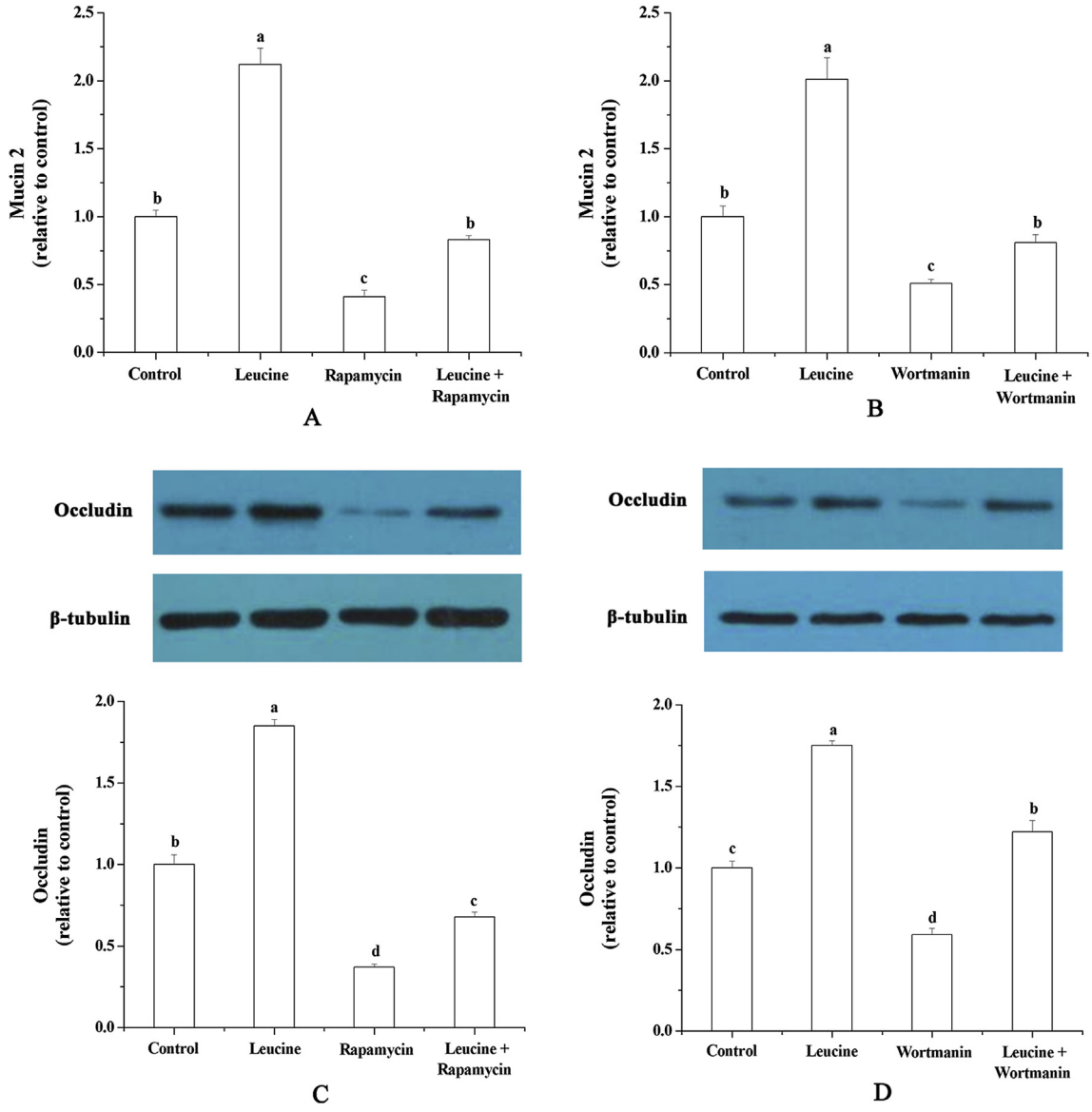

Fig. 4. Effect of rapamycin or wortmanin on the mucin 2 and occludin levels in LS174T cells cultured in medium supplemented with leucine. The cells were subjected to pre-incubation with or without 10 nmol/L rapamycin (A and C) or 1 μmol/L wortmanin (B and D), and then treated with 5 mmol/L leucine for 2 h. Results are expressed as the amount of mucin 2 (A and B) and the amount of occludin to β-tubulin (C and D) in each treatment as a ratio of the treatment to the control. Values are means ± SE; $n = 6$. ^{a,b,c,d} Bars with different letters are significantly different ($P < 0.05$).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This study was financially supported by the grant from the National Natural Science Foundation of China (31201812), the earmarked fund for the China Agriculture Research System (CARS-36), and the grant from the Science and Technology Support Program of Sichuan Province (13ZC2237). Special thanks to Professor De Wu from Sichuan Agricultural University for editing the manuscript.

References

Chen H, Mao X, He J, Yu B, Huang Z, Yu J, et al. Dietary fibre affects intestinal mucosal barrier function and regulates intestinal bacteria in weaning piglets. Br J Nutr 2013;110:1837–48.

Devine PL, Birrell GW, Whitehead RH, Harada H, Xing P, McKenzie IFC. Expression of MUC1 and MUC2 mucins by human tumor cell lines. Tumor Biol 1992;13:268–77.

Elamin E, Masclee A, Troost F, Dekker J, Jonkers D. Cytotoxicity and metabolic stress induced by acetaldehyde in human intestinal LS174T goblet-like cells. Am J Physiol Gastrointest Liver Physiol 2014;307:G286–94.

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993;123:1777–88.

Ijichi C, Matsumura T, Tsuji T, Eto Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. Biochem Biophys Res Commun 2003;303:59–64.

Jankowski JA, Goodlad RA, Wright NA. Maintenance of normal intestinal mucosa: function, structure, and adaptation. Gut 1994;35:S1–4.

Kimball SR. Regulation of global and specific mRNA translation by amino acids. J Nutr 2002;132:883–6.

Mao X, Liu M, Tang J, Chen H, Chen D, Yu B, et al. Dietary leucine supplementation improves the mucin production in the jejunal mucosa of the weaned pigs challenged by porcine rotavirus. PLoS One 2015;10:e0137380.

Mao X, Qi S, Yu B, Huang Z, Chen H, Mao Q, et al. Dietary L-arginine supplementation enhances porcine β-defensins gene expression in some tissues of weaned pigs. Livest Sci 2012;148:103–8.

Mao X, Qi S, Yu B, He J, Yu J, Chen D. Zn^{2+} and L-isoleucine induce the expressions of porcine β-defensins in IPEC-J2 cells. Mol Biol Rep 2013;40:1547–52.

Mao X, Zeng X, Qiao S, Wu G, Li D. Specific roles of threonine in intestinal mucosal integrity and barrier function. Front Biosci 2011a;E3:1192–200.
Mao X, Zeng X, Wang J, Qiao S. Leucine promotes leptin receptor expression in mouse C2C12 myotubes through the mTOR pathway. Mol Biol Rep 2011b;38:3201–6.
Pérez de Obanos MP, López Zabalza MJ, Prieto J, Herraiz MT, Iraburu MJ. Leucine stimulates procollagen alpha1 (I) translation on hepatic stellate cells through ERK and PI3K/Akt/mTOR activation. J Cell Physiol 2006;209:580–6.
Resta-Lenert S, Das S, Batra SK, Ho SB. Muc17 protects intestinal epithelial cells from enteroinvasive *E. coli* infection by promoting epithelial barrier integrity. Am J Physiol Gastrointest Liver Physiol 2011;300:G1144–55.
Roh C, Han J, Tzatsos A, Kandror KV. Nutrient-sensing mTOR-mediated pathway regulates leptin productin in isolated rat adipocytes. Am J Physiol Endocrinol Metab 2003;284:E322–30.
Wang W, Zeng X, Mao X, Wu G, Qiao S. Optimal dietary true ileal digestible threonine for supporting the mucosal barrier in small intestine of weanling pigs. J Nutr 2010;140:981–6.
Zhang S, Ren M, Zeng X, He P, Ma X, Qiao S. Leucine stimulates ASCT2 amino acid transporter expression in porcine jejunal epithelial cell line (IPEC-J2) through PI3K/Akt/mTOR and ERK signaling pathways. Amino Acids 2014;46:2633–42.