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Original research article

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

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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

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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.