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Autophagy alleviates LPS induced Hyper-Permeability of Caco-2 cells by regulating claudin-2 protein

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Abstract: **To investigate the effect of autophagy on claudin-2 in lipopolysaccharide (LPS) induced hyper-permeability of intestinal epithelial cell. In human colon cancer cell line (Caco-2), the model of intestinal epithelial injury induced by LPS was established, which were LPS group, LPS combined with inhibitor of autophagy (3-Methyladenine, 3-MA) and control group. Transepithelial resistance (TER) was detected by Millicell-ERS and monolayer permeability was evaluated by paracellular transport of fluorescein isothiocyanate dextran (FITC-dextran).Expression of LC3II and claudins-2 in Caco-2 cells were analyzed by Western blot. Expression of mRNA of CL-2 was detected by RT-PCR. The results showed that LPSinduced the expression of LC3II was increased, and TER was decreased compared with control group. Moreover, claudin-2 and monolayer permeability were up-regulated in LPS + 3-MA group compared with LPS group, and TER was significantly decreased compared with LPS group and control group. Autophagy may down regulated the expression of CL-2 in intestinal epithelial cells induced by LPS, and reduce intestinal epithelial permeability to protect intestinal mucosal barrier.**

Keywords: Autophagy; Claudin-2; Intestinal epithelial cells

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1. Introduction

Intestinal epithelial cells play a crucial role in intestinal barrier, separated hundreds of millions of microorganisms [1,2]. Increasing evidence suggests that intestinal epithelial barrier is associated with many diseases, such as inflammatory bowel disease (Crohn's disease, ulcerative colitis), irritable bowel syndrome and bacterial translocation caused by severe acute pancreatitis [2,3].Animal experiment and clinical research show that decreased tight junction protein was associated with the progression of inflammatory bowel disease [4]. LPS has been described as endotoxin, which is an important component of gram negative bacteria, for its not through the normal intestinal epithelial barrier, and under normal circumstances are mostly distributed in the intestine, and endotoxin was not detected in plasma of healthy people [5]. Studies have shown that LPS can be detected in animal models and clinical studies, when intestinal epithelial TJ was reduced or damaged, which can lead to systemic inflammatory response. Moreover, previous studies have shown that LPS can increase the permeability of Intestinal epithelial barrier and reduced the expression of TJ in intestinal epithelial cells [6,7], and reported that LPS induces activation of autophagy in intestinal cells [8-10]. Autophagy is an intracellular lysosomal degradation pathway, which is involved in neurodegeneration, tumor formation and cardiovascular disease [11,12]. Studies have reported that activation of intestinal epithelial autophagy can reduce the inflammatory response induced by endotoxin [11, 13-15]. Recent studies have shown that TJ can be regulated by autophagy induced by starvation in vitro [16,17], but the effect of autophagy on LPS induced intestinal epithelial damage is unclear. In this study, we investigated the effects of autophagy on the expression of tight junction proteins (CL-2) using LPS induced intestinal epithelial injury in vitro.

2. Materials and Methods

2.1. Cell culture

Caco-2 cells (passage 20) were purchased from the American TypeCulture Collection (Manassas, VA) and maintained at 37˚C in a culturemedium composed of Dulbecco's modified Eagle's medium with 4.5mg/mL glucose, 50U/mL penicillin, 50U/mL streptomycin, 4mmol/L glutamine, 25mmol/L HEPES, and 10%fetal bovine serum as previously described.Caco-2 cells were used between passages 21 and 26 in this study, and the cells were kept at 37˚C in a 5%CO_2 environment. The Caco-2 cells was treated withalone LPS (Sigma-Aldrich) or combined with 3-MA (LPS+ 3-MA, Sigma-Aldrich) for 12h. Meanwhile, the blank control group was established.

2.2. Determination of TER and paracellular permeability

Caco-2 transepithelial electrical resistance (TER)

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was measured using anepithelial Millicell-ERS as previously reported. Electrical resistance was measured until similar valueswere recorded on three consecutive measurements at 0h, 6h, 12h, 24h. Caco-2 cell monolayers were grown on Trans-well plates and treated as described above. By the end of TER measurements, 1g/L fluorescein isothiocyanatelabeled dextran 4kDa (FITC-D4; Sigma-Aldrich) was added to the apicalcompartment. Then, 50 mL of mediumfrom the apical and basal compartments were collected in 96-well plates, and FITC-D4 was measured spectrophotometrically at an excitationwavelength of 498nm and an emission wavelength of 540nm. Monolayer permeability was quantified as percentage of FITC-D4 permeatingfrom the apical to the basal compartment.

2.3. Western blot analysis

Protein expression from Caco-2 cells was assessed byWestern blot, as previously described. Cells lysedwith lysis buffer (50mM Tris-HCl, 150 mM NaCl, 500 mM NaF, 2mM EDTA, 100 mM vanadate, 100mM PMSF, 1mg/ml leupeptin) on ice for 30min. Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad,Hercules, CA). Equivalent total proteincontent were resolved by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (50μgtotal protein/lane). Proteins from the gelwere transferred to the membrane overnight. The membrane was incubated for 2h in blocking solution (5% dry milk in TBSTween 20 buffer). The membrane was incubated with 1μg/ml ofrabbit antibodies to LC3, claudin-2 and β -actin(Sigma–Aldrich).After a wash in TBS-1% Tween buffer, the membrane was incubated in secondaryantibody of LC3 and claudin-2 (Sigma–Aldrich) and developed using Santa Cruz Western Blotting Reagents (Santa Cruz Biotechnology). Densities of the bands werequantified using ImageJ.

2.4. Quantitative Real-Time PCR

Total RNA was extracted from the collected cellswith TRIzol reagent (Invitrogen) according to the manufacturer's instructions and reverse transcribed tocomplementary DNA using Multiscribe reverse transcriptase (Applied Biosystems). Primer sequences: CL-2, F:5'-CTC CTG GGA TTC ATT CCT GTTG-3', R: 5'-TCAGGCACCAGTGGTGAGTAGA-3';GAPDH,F :5'-GCACCGTCAAGGCTGAGAAC,F:5-TGGTGAA GACGCCAGTGGA-3'. Real-time quantitativePCR was performed using the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen) anda StepOne Real-Time PCR apparatus (Applied Biosystems). The expression level of the CL-2 genes was normalized relativeto levels of the GAPDH, which was used as an endogenous control.

2.5. Statistical analysis

The data were expressed by mean ± SD. Student-t test was used to compare the two groups. Statistical analysis was performed using SPSS 17.0, and P <0.05was regarded as statistically significant.

3. Results

3.1. Effects of autophagy on TER and permeability of intestinal epithelial cells

In the LPS group, LPS+3-MA group and control group, Caco-2 cells were induced 0h, 6h, 12h, 24h in three groups of cells TER values (Figure 1A). The TER value of LPS+ group 3-MA was lower than that of LPS group and control group (P<0.05, P<0.05). The permeability of FITC-D4 of the three groups of cells (Figure 1B), the permeability of monolayer Caco-2 cells in LPS+ 3-MA group was higher than that in LPS group and control group (P<0.05, P<0.05).

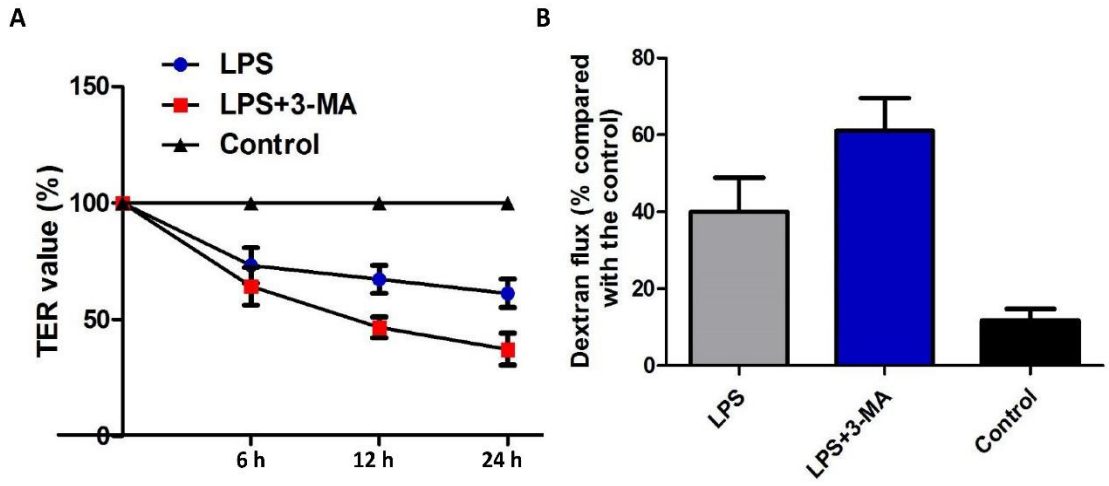


Figure 1. In the LPS group, LPS+ 3-MA group and control group, the TER value (A),

permeability of FITC-D4 (B), (P<0.05).

3.2. Effects of autophagy on CL-2 protein expression

The expression levels of LC3II and CL-2 protein in LPS+ 3-MA group, LPS group and control group were shown in Figure 3. The level of autophagy in LPS+ 3-MA group was significantly lower than that in LPS group and control group, and the expression of CL-2 was higher than that of LPS group (P < 0.05).

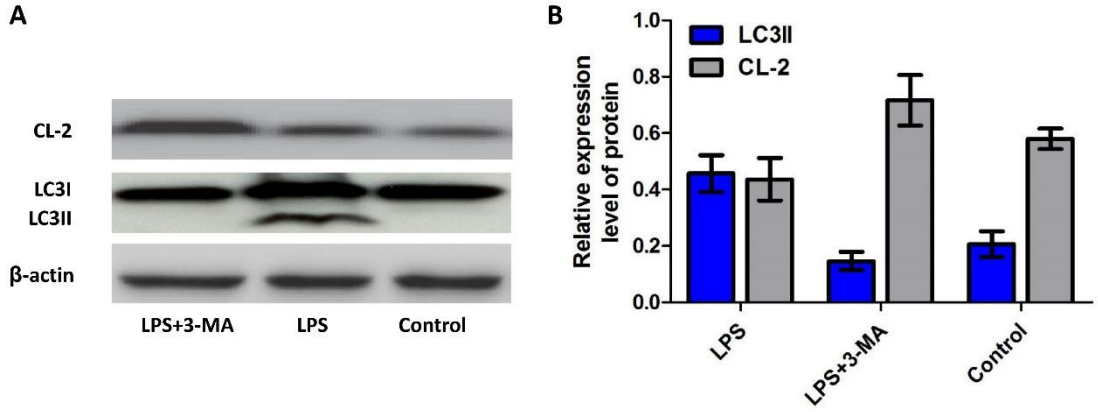


Figure 2. In the LPS group, LPS+ 3-MA group and control group, detection of LC3II and CL-2protein by Western blot (A, B), (P<0.05).

3.3.Effect of autophagy inhibition on the expression of CL-2 mRNA

In epithelial Caco-2 cells, the expression of CL-2 mRNA in LPS+ 3-MA group, LPS group and control group, as shown in Figure 4, in LPS+ 3-MA group was higher than that in LPS group (P < 0.05).

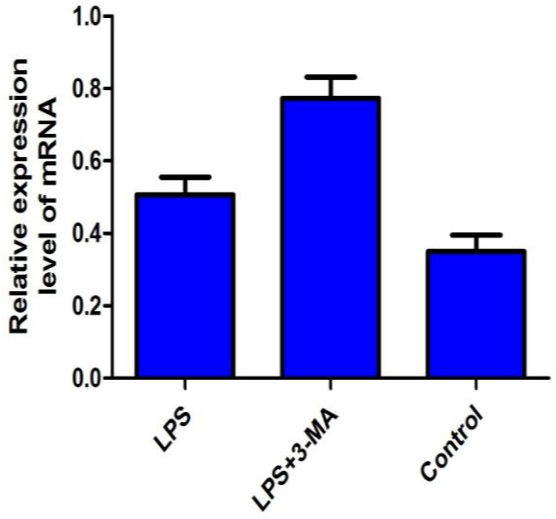


Figure 3. Expression of CL-2 mRNA, in LPS+ 3-MA group, LPS group and control group. (P<0.05).

4. Discussion

In the gut, intestinal epithelial TJ of intestinal epithelial barrier closed the gap between epithelial cells, regulating absorption of dietary nutrients, to defense from intestinal pathogens, allergens and endotoxin. To maintain the integrity of the intestinal epithelial barrier is essential to the health of the people, and studies shown that intestinal epithelial permeability was associated with obesity and obesity related insulin resistance, inflammatory bowel disease and intestinal bacterial translocation [18]. In a variety of pathological conditions such as severe acute pancreatitis, sepsis and necrotizing enteritis and other diseases, intestinal epithelial TJ were damaged [19]. However, the regulatory mechanism of intestinal epithelial tight junction is not fully understood. It has been reported that LPS can increase the permeability of intestinal barrier [6] by change the TJ of epithelial cells [20]. Recent study have shown that autophagy in intestinal epithelial cells can regulate the expression of TJ, but the effect of autophagy on LPS induced intestinal epithelial permeability and TJ is unclear.In the study, we use LPS to establish the induced intestinal epithelial cell damage model in Caco-2 cells by autophagy inhibitor 3-MA to block intestinal epithelial autophagy, to study effect of autophagy on intestinal epithelial permeability and CL-2.

Caco-2 cells are epithelial cells of human intestinal tumor, which can be cultured into a monolayer epithelial cells, and have TJ structure. It are used to study epithelial permeability, drug absorption and TJ damage in vitro. TJ proteins were broadly separated into transmembrane proteins, cytosolic plaque proteins and regulatory proteins. Claudins is one of transmembranetight junction proteins, the tetraspanning claudins arethe most important, as the extracellular domains of claudins on adjacent cells

forming pores to regulate selectivity of tight junction for ion [21]. Previous study determined that expression of a single claudin family member, claudin-2,is strong responsible transepithelialresistance inkidney cells.Subsequent analyses have shown thatepithelial barrier function was decreased by claudin-2-driven for increasing in paracellular ion conductance [22]. The TER value of intestinal epithelial monolayer cells is one of the main indexes of monolayer intestinal epithelial integrity. This study shows that the decrease of Caco-2 cell monolayer TER after induced by LPS, while in LPS TER induced autophagy inhibition were decreased compared with alone LPS-induced, andwe speculate that autophagy has protective against LPS induced intestinal Caco-2 damage. In Transwell FITC labeled dextran into the lower compartment shown that it enters the lower compartment via intercellular TJ, consistent with previous studies [23,24]. In this study, the Caco-2 treated LPS+ 3-MA cells was also significantly increased compared with that in LPS induced group, suggesting that autophagy has a protective effect on LPS induced increasing intestinal epithelial permeability. Autophagy is one of the basic homeostatic processes in cells, and it is the characteristics of the formation of the double membrane structure of autophagy by removing the aging mitochondria and the damaged proteins [25]. Autophagy was associated with tumor development, cell senescence, and immune function. Studies have shown that activation of autophagy to intestinal epithelium is crucial for infection and resistance of enteric bacteria [15,26]. It has been reported that autophagy can reduce the inflammatory response induced by endotoxin in vitro. In vitro, it has been proved that autophagy can regulate the expression of tight junction proteins [17]. 3-MA was a commonly used inhibitor of autophagy pathway, and studies have shown that inhibition of PI3Ks pathway, blocking the formation of autophagy [27]. In this study, 3-MA was used to block autophagy in LPS induced Caco- 2, and the increasing intestinal epithelial CL-2 protein were detected compared with cells treated with LPS alone. The increased expression of CL-2 was closely associated with TER and permeability of tight junctions in intestinal monolayer cells. Consistent with this study, cell monolayers were treated TNF-alpha, leading increase of CL-2 levels and intestinal epithelial tight junction dysfunction, which are partly caused by the inhibition of autophagic degradation [22].

In this study, in LPS induced Caco-2 cell damage, autophagy was blocked by 3-MA, and we proved that autophagy may protect intestinal epithelial cells by regulating the expression of intestinal epithelial tight junction proteins CL-2. This study may provides a new theoretical basis and therapeutic targets for the regulation and treatment of intestinal epithelial damage.

Acknowledgements

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