Porcine Glucagon-Like Peptide-2 Regulates Tight Junction Protein Expression in Piglet Jejunal Epithelial Cells via Extracellular Signal-Regulated Kinase 1/2 Postprint

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Abstract

This study investigated the regulatory effects of porcine glucagon-like peptide-2 (pGLP-2) on tight junction protein expression and its signaling mechanisms in cultured porcine small intestinal epithelial cells from jejunum (IPEC-J2). The culture medium was supplemented with 10^{-9} mol/L pGLP-2 (pGLP-2 group) and 10^{-9} mol/L pGLP-2 plus 10 μmol/L U0126 (pGLP-2+U0126 group), respectively, while the control group received none of these reagents, with 3 replicates per group and one culture well per replicate. The protein expression levels of zonula occludens-1 (ZO-1), occludin, claudin-1, and extracellular regulated kinase 1/2 (ERK1/2) in IPEC-J2 cells were determined. The results showed that compared with the control group, the addition of pGLP-2 to IPEC-J2 cell culture medium significantly increased the protein expression of ZO-1, occludin, claudin-1, p42-ERK1/2, and p44-ERK1/2 ($P<0.05$); compared with the pGLP-2 group, the addition of the ERK1/2 inhibitor U0126 to IPEC-J2 cell culture medium significantly decreased the protein expression of ZO-1, occludin, claudin-1, p42-ERK1/2, and p44-ERK1/2 ($P<0.05$). In conclusion, the ERK1/2 pathway is an important signaling pathway through which pGLP-2 regulates tight junction protein expression in intestinal epithelial cells.

Full Text

Introduction

GLP-2 has a short half-life and is rapidly degraded by dipeptidyl peptidase-IV (DPP-IV), which is abundantly present in the blood [2]. GLP-2 protects intestinal cells by acting on the GLP-2 receptor (GLP-2R) to regulate the proliferation and inhibit apoptosis of intestinal epithelial cells [3-4]. The regulation of GLP-2'

s effects on promoting cell proliferation, inhibiting apoptosis, and cytoprotection involves multiple cell signaling pathways, primarily including the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) or phosphatidylinositol-3 kinase/protein kinase B (PI-3K/Akt) pathways, the Wingless-type MMTV integration site/β-catenin (Wnt/β-catenin) pathway, and the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway, among others. However, the signal transduction mechanisms of GLP-2 remain controversial [5-8]. The diversity of GLP-2 functions and the different cell models used in research are the main reasons for discrepancies in study results. Current in vitro models for studying GLP-2 mechanisms mainly include Caco-2 cells [5], BHK fibroblasts [6], HeLa cells [7], and HEK293 cells [8]. This study utilized porcine small intestinal epithelial cells from jejunum (IPEC-J2) as the research model, providing an intestinal-derived cell model for investigating the application of exogenous pGLP-2 in piglet intestinal injury and functional disorders. Based on investigating the effects of pGLP-2 on tight junction protein expression, this study examined the signaling pathways through which pGLP-2 regulates tight junction protein expression by adding the ERK1/2 signaling pathway inhibitor U0126.

Materials and Methods

1.1 Experimental Materials

The IPEC-J2 cell line was kindly provided by Dr. Wang Junjun from the College of Animal Science, China Agricultural University. [Gly2]pGLP-2 (peptide sequence: HGDGSFSDEMNTVLDNLATRDFINWLLHTKITDSL) was synthesized by Hangzhou Zhongtai Biochemical Co., Ltd. The main reagents included: DMEM/F12 (Gibco, C11330500BT), fetal bovine serum (FBS) (Gibco, 10099133), 100× insulin-transferrin-selenium (ITS) (Sigma, I3146), penicillin-streptomycin (Gibco, 15140122), epidermal growth factor (EGF) (Sigma, E4127), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, 25200056), porcine ERK1/2 antibody (Cell Signaling, 4376s), β-actin antibody (Santa Cruz, Sc47778), zonula occludens-1 (ZO-1) antibody (Santa Cruz, Sc10804), occludin antibody (Abcam, Ab312721), claudin-1 antibody (Abcam, Ab15099), U0126 (Cell Signaling, 9903), and goat anti-mouse immunoglobulin G conjugated with horseradish peroxidase (IgG-HRP) (Pierce, PA128568). Other routine reagents and kits were purchased from Huadong Medicine Group Co., Ltd.

1.2 Experimental Design and Cell Treatment

IPEC-J2 cells were cultured in 75 cm^2 cell culture flasks at 37 °C with 5% CO_2 and 95% humidity using complete medium (93% DMEM/F12, 5% FBS, 1% ITS, 1% penicillin-streptomycin, 10 ng/mL EGF). The medium was changed every other day. When cells reached 80% confluence, they were digested with 0.25% trypsin-EDTA solution and seeded into 6-well plates at 1$×10^{5}$ cells per well. When cells reached 80% confluence again, they were cultured with

serum-free medium. The experiment employed a single-factor design with three treatment groups: control group, pGLP-2 group, and pGLP-2+U0126 group. Each treatment had three replicates with one culture well per replicate. The control and pGLP-2 groups were treated with serum-free culture medium containing 0 or 10^{-9} mol/L pGLP-2 overnight, respectively. The pGLP-2+U0126 group was pretreated with 10 μmol/L U0126 for 1 h, followed by the addition of serum-free culture medium containing 10^{-9} mol/L pGLP-2. After 24 h, the culture medium was removed, cells were washed twice with pre-warmed phosphate-buffered saline (PBS), and lysed with RIPA buffer.

1.3 Western Blotting Analysis

Total protein was extracted from samples using a total protein extraction kit, and protein concentration was determined using a bicinchoninic acid (BCA) assay kit. A 10% separating gel and 5% stacking gel were prepared. Total protein (60 μg) was loaded per well in a volume of 10–15 μL. Electrophoresis was performed at 60 V for the stacking gel and 80 V for the separating gel for approximately 5 h. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was equilibrated in Tris-glycine transfer buffer for 30 min, and proteins were transferred to a membrane at a constant voltage of 100 V for 2 h under cooling conditions. After transfer, the polyvinylidene fluoride (PVDF) membrane was blocked with 5% skim milk in Tween-TBS (T-TBS) at room temperature for 1 h. Porcine ERK1/2 antibody, ZO-1 antibody, occludin antibody, claudin-1 antibody, and β-actin antibody were diluted at 1:1,000, 1:500, 1:1,000, 1:800, and 1:2,000, respectively, in T-TBS containing 3% skim milk and incubated overnight at 4 °C. The membrane was washed with T-TBS for 5 min, repeated four times. Secondary antibody (1:2,000) was added and incubated at room temperature for 1 h, followed by washing with T-TBS for 5 min, repeated five times. The SuperSignal® West Dura Extended Duration Substrate kit was used according to the manufacturer' s instructions. Approximately 1 mL of ECL working solution was prepared and incubated with the blot at room temperature for 1 min. The membrane was sealed with plastic wrap, exposed to X-ray film in a dark box for 5–10 min, and then developed and fixed. Band densities were analyzed using Bandscan 5.0 software, with each band measured three times. Target protein expression was relatively quantified using the following formula: target protein expression = optical density of target protein / optical density of β-actin.

1.4 Data Processing and Statistical Analysis

Data were analyzed using SAS 6.12 software by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison test. Results are expressed as 'mean ± standard deviation' .

Results

2.1 Effects of pGLP-2 on Tight Junction Proteins and ERK1/2 Expression

As shown in Figure 1 [Figure 1: see original paper], compared with the control group, the addition of pGLP-2 to the culture medium significantly increased the protein expression levels of the tight junction proteins ZO-1, occludin, and claudin-1, as well as p42-ERK1/2 and p44-ERK1/2 in IPEC-J2 cells ($P < 0.05$).

2.2 Effects of the Inhibitor U0126 on Tight Junction Proteins and ERK1/2 Expression

As shown in Figure 1, compared with the pGLP-2 group, the addition of pGLP-2 to IPEC-J2 cells pretreated with the ERK1/2 inhibitor U0126 significantly decreased the protein expression levels of ZO-1, occludin, claudin-1, p42-ERK1/2, and p44-ERK1/2 ($P < 0.05$). &: significant difference compared with control group ($P < 0.05$); #: significant difference compared with pGLP-2 group ($P < 0.05$).

Discussion

GLP-2 can effectively promote the expression of tight junction proteins in intestinal epithelial cells and enhance intestinal barrier function. Dong et al. [9] reported that GLP-2 significantly increased the expression of key tight junction protein genes occludin, claudin-3, and claudin-7 in mouse jejunum. Moran et al. [10] found that GLP-2 not only significantly increased the protein expression of key tight junction proteins occludin and ZO-1 in Caco-2 cells, but also effectively inhibited the TNF-α-induced decrease in occludin and ZO-1 protein expression. Wu et al. [11] demonstrated that a single injection of [Gly2]pGLP-2 microspheres effectively inhibited the dextran sulfate sodium (DSS)-induced decrease in colonic occludin expression in mice. Studies using IPEC-J2 as a model showed that GLP-2 significantly improved cell morphology under normal culture conditions and increased the expression of key tight junction protein genes occludin, claudin-1, and ZO-1 [12]; GLP-2 also effectively inhibited LPS-induced disruption of IPEC-J2 cell morphology and decreased expression of key tight junction protein genes. Consistent with these results, our study found that addition of pGLP-2 to IPEC-J2 cells significantly increased the protein expression of ZO-1, occludin, and claudin-1.

Studies on the regulation of tight junction proteins have shown that numerous growth factors can regulate tight junction protein expression through the mitogen-activated protein kinase (MAPK) signaling pathway to modulate barrier function [13]. The ERK1/2 signaling pathway is a classical MAPK signaling pathway, and GLP-2 can directly promote epithelial cell proliferation through the ERK1/2 signaling pathway. Jasleen et al. [14] used 10 nmol/L GLP-2 to stimulate human intestinal epithelial Caco-2 cells and found that the content

of activated ERK1/2 in cells increased significantly, with a 10-fold increase in the proliferative response; the MEK inhibitor PD98059 blocked the proliferative effect of GLP-2 in a dose-dependent manner. Subsequently, Jasleen et al. [15] again demonstrated that GLP-2-induced proliferation of Caco-2 cells was accompanied by a transient increase in ERK phosphorylation, which was blocked by the MEK inhibitor PD98059. However, Yusta et al. [16] reported that 20 nmol/L GLP-2 did not increase ERK1/2 phosphorylation levels in baby hamster kidney fibroblasts (BHK), but instead decreased the basal phosphorylation level of ERK1/2, suggesting that GLP-2 signaling is not coupled to the p44/p42 MAPK pathway, which may be related to the use of kidney-derived cells in the study. Regarding the effect of whether the cell model used in experiments expresses GLP-2R on the signal transduction mechanism of GLP-2R, Koehler et al. [7] found in HeLa cells that GLP-2-mediated activation of ERK1/2 was not eliminated by reducing transfection of GLP-2R cDNA. Li et al. [17] also demonstrated that although mouse microglial BV-2 cells do not express GLP-2R, GLP-2 attenuation of LPS-induced inflammatory responses in BV-2 cells was also achieved through inhibition of the ERK1/2 signaling pathway. That is, different cell models used to study the signal transduction mechanisms of GLP-2 in regulating intestinal function are the main reason for controversial results in this area. U0126 is a highly selective inhibitor of the MAPK kinase MEK1/2, with 100 times higher inhibitory activity than PD98059. It inhibits MEK1/2 kinase activity in a non-competitive manner, thereby preventing activation of p42 MAPK and p44 MAPK encoded by respective genes. In this study, addition of U0126 significantly decreased the protein expression of p42-ERK1/2 and p44-ERK1/2. Moreover, the IPEC-J2 cell line used in this study is a normal physiological newborn piglet jejunal epithelial cell line and represents the most ideal cell model for studying porcine GLP-2 regulation of intestinal barrier function. Our results showed that treatment of IPEC-J2 with 10^{-9} mol/L pGLP-2 significantly increased the protein expression of tight junction proteins ZO-1, occludin, and claudin-1, as well as p42-ERK1/2 and p44-ERK1/2. However, pretreatment with the ERK1/2 inhibitor U0126 followed by pGLP-2 addition significantly inhibited the expression of these tight junction proteins and ERK1/2 isoforms, indicating that the ERK1/2 pathway is an important signaling pathway for pGLP-2 regulation of tight junction protein expression in intestinal epithelial cells.

Conclusion

The ERK1/2 inhibitor U0126 significantly inhibited the pGLP-2-induced increase in protein expression of ZO-1, occludin, claudin-1, p42-ERK1/2, and p44-ERK1/2, indicating that ERK1/2 is an important signaling pathway through which pGLP-2 regulates tight junction protein expression in intestinal epithelial cells.

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Abstract

Porcine Glucagon-like Peptide-2 Regulates Tight Junction Protein Expressions in Porcine Small Intestinal Epithelial Cell through Extracellular Regulated Kinase 1/2 Signaling Pathway

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Porcine small intestinal epithelial cells from jejunum (IPEC-J2) were used to study the regulation of porcine glucagon-like peptide-2 (pGLP-2) on protein expressions of tight junction proteins and its signaling pathway. Culture media were supplemented without (control group) or with 10^{-9} mol/L pGLP-2 (pGLP-2 group), and with 10^{-9} mol/L pGLP-2 and 10 μmol/L U0126 (pGLP-2+U0126 group), respectively. Each group had 3 replicates with 1 culture well per replicate. Protein expressions of zonula occludens-1 (ZO-1), occludin, claudin-1 and extracellular regulated kinase1/2 (ERK1/2) in the cytoplasm of IPEC-J2 were determined. The results showed as follows: compared with the control group, supplementation of pGLP-2 in the culture medium of IPEC-J2 significantly increased protein expressions of ZO-1, claudin-1, occludin, p42-ERK1/2 and p44-ERK1/2 ($P < 0.05$); compared with the pGLP-2 group, supplementation of U0126, an inhibitor of ERK1/2, in the culture medium of IPEC-J2 significantly decreased protein expressions of the above proteins ($P < 0.05$). The results suggest that the ERK1/2 pathway is an important signaling pathway for pGLP-2 regulating tight junction protein expressions in intestinal epithelial cells.

Key words: porcine glucagon-like peptide-2; extracellular regulated kinase1/2; tight junction protein

Note: Figure translations are in progress. See original paper for figures.