Enterotoxigenic *Escherichia coli* infection induces tight junction proteins expression in mice

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Summary

Enterotoxigenic *Escherichia coli* (ETEC) causes diarrhea in travelers, young children and piglets, but the precise pathogenesis of ETEC induced diarrhea is not fully known. Recent investigations have shown that tight junction (TJ) proteins and aquaporin 3 (AQP 3) are contributing factors in bacterial diarrhea. In this study, using immunoblotting and immunohistochemistry analyses, we found that ETEC increases the protein abundance of TJ proteins (occludin, claudin-1, zonula occludens-1) in mice. Enterotoxigenic *Escherichia coli* induced the expression of TJ proteins in mice through pathways by involving myosin light chain kinase (MLCK)-myosin II regulatory light chain (MLC20) pathways; however, ETEC has little effect on the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway. Enterotoxigenic *Escherichia coli* infection has little effect on the protein abundance of AQP 3. Collectively, ETEC infection affects the abundance of intestinal TJ protein, which suggests the importance of TJ proteins in ETEC induced diarrhea.

Key words: Aquaporin 3, Enterotoxigenic *Escherichia coli*, MLCK, Tight junction

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the principal agent responsible for diarrhea in travelers (Rowe *et al*., 1970), and is a leading cause of infectious diarrhea in young children in developing countries (Sears and Kaper, 1996). Porcine ETEC strains also cause severe secretory diarrhea in pigs (Berberov *et al*., 2004). Thus, ETEC is an important cause of human and porcine morbidity and mortality. Heat-labile (LT) and heat-stable (ST) toxins produced by ETEC lead to water and electrolyte loss from the intestine of infected subjects (Nataro and Kaper, 1998; Uzzau and Fasano, 2000). Enteric pathogens and enterotoxins disrupt the intestinal tight junctions (TJs), including claudins, occludin, and zonula occludens proteins, to activate the paracellular secretion pathway, which also has critical importance in diarrhea (Uzzau and Fasano, 2000; Guttman *et al*., 2006; Guignot *et al*., 2007). Increasing investigations have reported the influence of ETEC in intestinal TJ functions. For example, ETEC (O149 K91 K88) lowers tight junctional strand numbers, but has no effect on tight junctional depth and the permeability of the porcine jejunal epithelium in 3-week-old piglets (Egberts *et al*., 1993). ETEC (K88) decreases cell permeability based on the transepithelial electrical resistance (TER) and ^{14}C-inulin transfer in human Caco-2 enterocytes (Roselli *et al*., 2003). It also induces membrane damage by delocalizing zonulaoccludens (ZO)-1, reducing occludin amounts, and the dephosphorylation of occludin inintestinal porcine intestinal epithelial cells (Roselli *et al*., 2007). ETEC 2534-86 reduces TER, but has little effect on the total claudin-1 abundance and the disruption of occludin, ZO-1 and caludin-1 in the porcine intestinal epithelial cell line (IPEC-J2) (Johnson *et al*., 2010). ETEC (K88) affects the expression of TJ proteins (zonula occludens-1, claudin-1, and E-cadherin) in Caco-2 cells (Yu *et al*., 2012) and in Chinese native Jinhua pigs and European Landrace pigs (Gao *et al*., 2013; Yang *et al*., 2014). These discrepancies may relate to the difference in ETEC strains, infectious models and analysis methods used in the investigation. Most importantly, none of these studies tried to explore the influence of ETEC infection on the activation of signaling pathways associated with TJ functions, such as the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Li and Neu, 2009) and the myosin light chain kinase (MLCK)-myosin II regulatory light chain (MLC20) pathway (Edelblum and Turner, 2009). In the present work, we aimed to study the effect of ETEC infection on the expression of TJ proteins and the activation of its associated signaling pathway in a mouse model. We also studied the influence of ETEC infection on the expression of aquaporin 3 (AQP 3), which is a contributing factor in bacterial diarrhea (Guttman *et al*., 2007).

Materials and Methods

Bacterial strains and antibodies

This study used selected *E. coli* (SEC) 470 (serotype O4; oqxAB; F18; STa; STb; LT; SLT-IIe), SEC 298 (serotype O107; oqxAB; F18; STa; STb; SLT-IIe), SEC 817 (serotype O107; oqxAB; F18; STa; SLT-IIe) and C197 (serotype O4; SLT-IIe), which were originally isolated from piglets with diarrhea (Chen *et al*., 2014;

Tang *et al*., 2014). Antibodies specific against AQP 3 (ab125219), claudin-1 (ab15098), occludin (ab31721) and ZO1 (ab59720) were purchased from Abcam (Cambridge, MA, USA). Antibodies specific against p85 (sc-1637), p-Akt (Sc-7985-R), MLCK (sc365352), and MLC20 (sc15370) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA).

Animal model

The ETEC infection model was established according to the method introduced by Allen *et al*. (2006). Briefly, female ICR (Institute for Cancer Research) mice (6-week-old) were purchased from SLAC Laboratory Animal Central (Changsha, China). The mice were randomly assigned into groups with different ETEC strain infections (SEC470, SEC 298, SEC 817 or C197) or the control groups. All mice were orally inoculated with either 1×10^9 CFU of ETEC bacterial strain or sterile phosphate buffered saline (PBS). Jejunum were collected from ETEC infected or control mice at 24 h post infection. For the collection of jejunum tissues, the middle part of jejunum samples (about 2-3 cm) were collected after the PBS (pH = 7.2-7.4) washing. The jejunum tissues were fixed in fresh 4% paraformaldehyde for paraffin embedding or snap frozen in liquid nitrogen for protein extraction for western blotting. The dose for ETEC infection and time point for tissue collection was selected according to previous reports. This study was performed according to the guidelines of the Laboratory Animal Ethical Commission of the Hunan Agricultural University.

Immunoblotting

Western blot analysis was conducted according to previous studies (Ren *et al*., 2014a; Ren *et al*., 2017). Briefly, the proteins were transferred onto PVDF membranes (Millipore, MA, USA) after being separated by a reducing SDS-PAGE electrophoresis. After being blocked for 3 h in a blocking buffer (5% non-fat milk, 20 mM Tris, pH = 7.5, 150 mM NaCl, and 0.1% Tween-20), the PVDF membranes were incubated first with the primary antibodies at 4°C overnight and then with the HRP-conjugated secondary antibodies for 1 h at room temperature. The blots were developed using Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA).

Immunohistochemical analyses

Immunohistochemical analysis was conducted according to previous studies (Ren *et al*., 2014b; Ren *et al*., 2017). Firstly, after being fixed in 10% buffered formalin, the tissues were embedded in paraffin. Then the tissues were cut on 3 mm paraffin embedded slides, and subsequently experienced several treatments, including dewaxing and rehydrating. The treated sections were incubated with primary antibodies for 2 h at room temperature, and then with biotinylated secondary antibodies after washing, and subsequently with HRP-conjugated Streptavidin for 30 min (R&D Systems, London, UK).

Statistical analysis

Data were expressed as means ± standard error of means (SEM). All statistical analyses were performed using the SPSS 16.0 software (Chicago, IL, USA). Data were analyzed by one-way ANOVAs. Differences of $P<0.05$ were considered significant.

Results

Expression of tight junction proteins

We examined the effect of ETEC infection on expressions of the TJ proteins. First we quantified the abundance of occludin, claudin-1 and ZO-1 proteins. As shown in Fig. 1, SEC470, SEC817 and C197 significantly ($P<0.05$) increased the protein abundance of ZO-1, while ETEC 298 had little effect on the protein abundance of ZO-1 (Figs. 1A-B). Immunohistochemistry analyses also showed similar changes regarding the protein abundance of ZO-1 after ETEC infection (Fig. 1C). All ETEC strains significantly enhanced the protein abundance of claudin-1 with Western bolting and immunohistochemistry analysis compared to mice without ETEC infection (Figs. 2A-C). Meanwhile, as Fig. 2 shows, the protein abundance of claudin-1 in mice infected with C197 was much higher than those with SEC 470 and SEC 298 infections ($P<0.05$). Unlike claudin-1, there was no statistical difference among mice

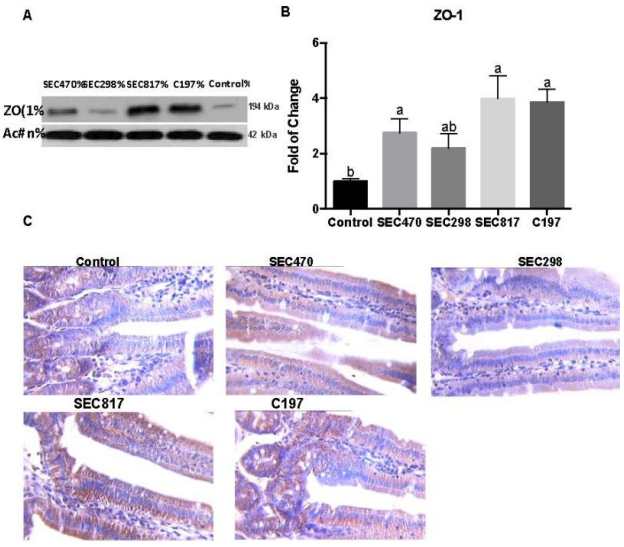

Fig. 1: Protein abundance of ZO-1. **A**: Immunoblotting of ZO-1 in the jejunumat 24 h post ETEC infection, **B**: Statistical analysis of relative abundance ZO-1 from data shown in A, and **C**: The protein abundance of ZO-1 in the jejunum at 24 h post ETEC infection by immunohistochemistry analyses. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data are presented as mean±SEM, n=5, with a-b used to indicate a statistically significant difference ($P<0.05$, one-way ANOVA). ZO-1: Zonula occludens-1

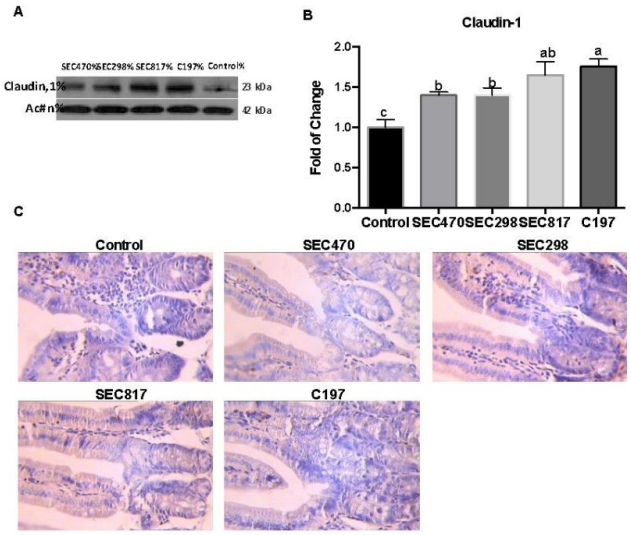

Fig. 2: Protein abundance of claudin-1. **A**: Immunoblotting of claudin-1 in the jejunum 24 h post ETEC infection, **B**: Statistical analysis of relative abundance of claudin-1 from data shown in A, and **C**: Protein abundance of claudin-1 in the jejunum 24 h post ETEC infection by immunohistochemistry analyses. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data are presented as mean±SEM, n=5, with a-c used to indicate a statistically significant difference (P<0.05, one-way ANOVA)

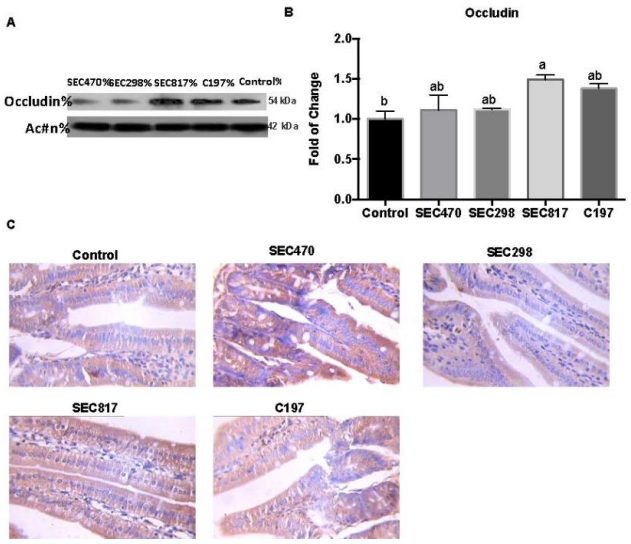

Fig. 3: Protein abundance of occludin. **A**: Immunoblotting of occludin in the jejunum 24 h post ETEC infection, **B**: Statistical analysis of relative abundance of occludin from data shown in A, and **C**: Protein abundance of occludin in the jejunum 24 h post ETEC infection by immunohistochemistry analyses. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data are presented as mean±SEM, n=5, with a-b used to indicate a statistically significant difference (P<0.05, one-way ANOVA method)

infected with SEC 470, or SEC 298, or C197 or PBS (Figs. 3A-C). However, a higher (P<0.05) protein abundance of occludin in mice infected with SEC 817 was found, compared to those infected with PBS (Fig. 3).

Expression of aquaporin 3

Aquaporin 3 water channels have been proposed to play critical roles in the normal dehydration of faecal contents and EPEC induced diarrhea (Guttman *et al*., 2007). Thus, its expression is also validated during ETEC infection. No significant difference was detected between the ETEC infected and control groups (Figs. 4A-C). However, as shown in Fig. 4 the protein abundance of AQP 3 was much lower in mice infected with SEC470 than those infected with SEC817 and C197 (P<0.05).

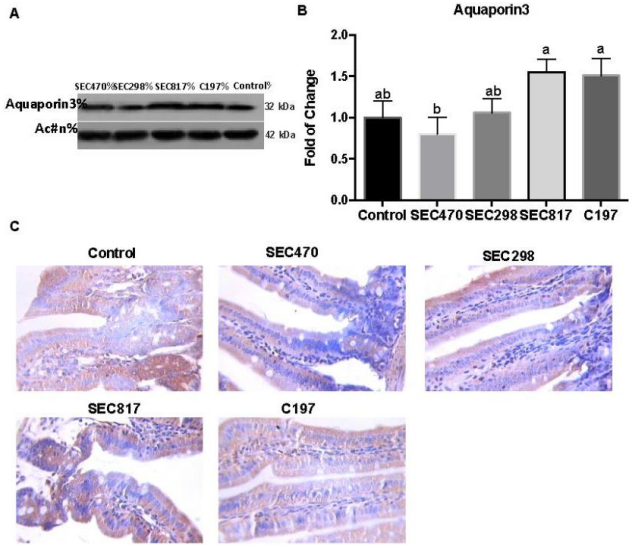

Fig. 4: Protein abundance of aquaporin 3. **A**: Immunoblotting of AQP 3 in the jejunum 24 h post ETEC infection, **B**: Statistical analysis of relative abundance of AQP 3 from data shown in A, and **C**: The protein abundance of AQP 3 in the jejunum 24 h post ETEC infection by immunohistochemistry analyses. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data are presented as mean±SEM, n=5, with a-b used to indicate a statistically significant difference (P<0.05, one-way ANOVA).

Activation of the PI3K-Akt pathway

Although previous researh has showed that enteropathogenic *E. coli* (EPEC) inhibits PI3K-mediated pathways to prevent its uptake by phagocytic cells (Celli *et al*., 2001), there is still a lack of substantial evidence to verify the effect of ETEC on this pathway. Therefore, we quantified PI3K, p-Akt protein abundance in the jejunum 24 h post ETEC infection. We also found that C197 infection significantly (P<0.05) promoted the protein abundance of p-Akt compared to the controls, while ETEC had little effect on PI3K and p-Akt protein abundance (Figs. 5A-C).

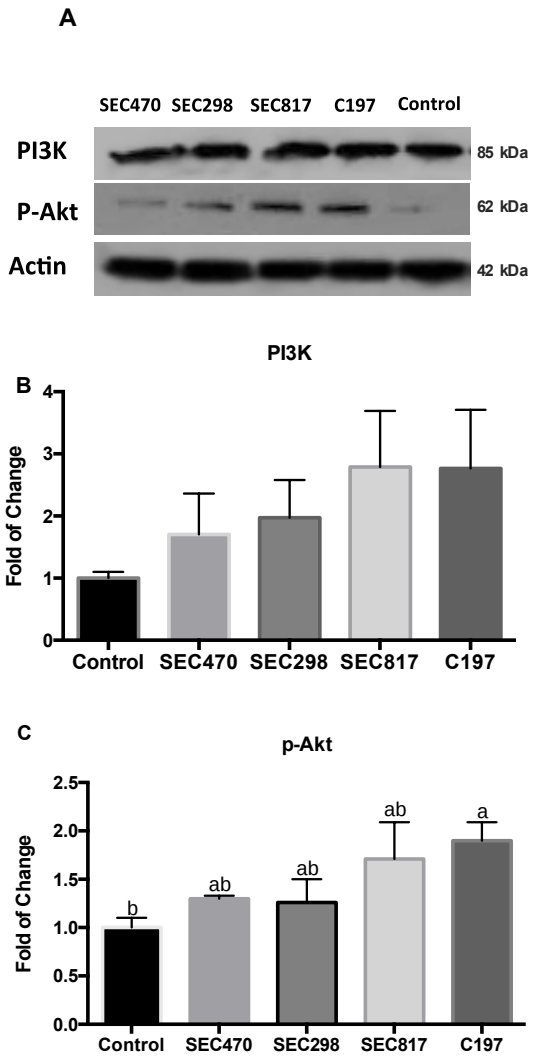

Fig. 5: Activation of PI3K–p-Akt pathway. **A**: Immunoblotting of PI3K and p-Akt in the jejunum 24 h post ETEC infection, **B**: Statistical analysis of relative abundance of PI3K from data shown in A, and **C**: Statistical analysis of relative abundance of PI3K from data shown in A. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data are presented as mean±SEM, n=5, with a-b used to indicate a statistically significant difference (P<0.05, one-way ANOVA)

Activation of MLCK-MLC20 pathway

One pathway involved in the cytokine-mediated regulation of the TJ regulation is MLCK, which phosphorylates MLC, resulting in the reorganization of TJ proteins, including endocytic removal from the apical junctional complex (Edelblum and Turner, 2009). Thus, we investigated the abundance of MLCK and found that ETEC infection significantly (P<0.05) increased MLCK protein content in the jejunum (Figs. 6A-C). We also examined the MLC20 content in the jejunum and found that ETEC infection also significantly (P<0.05) enhanced MLC20 protein abundance (Fig. 6).

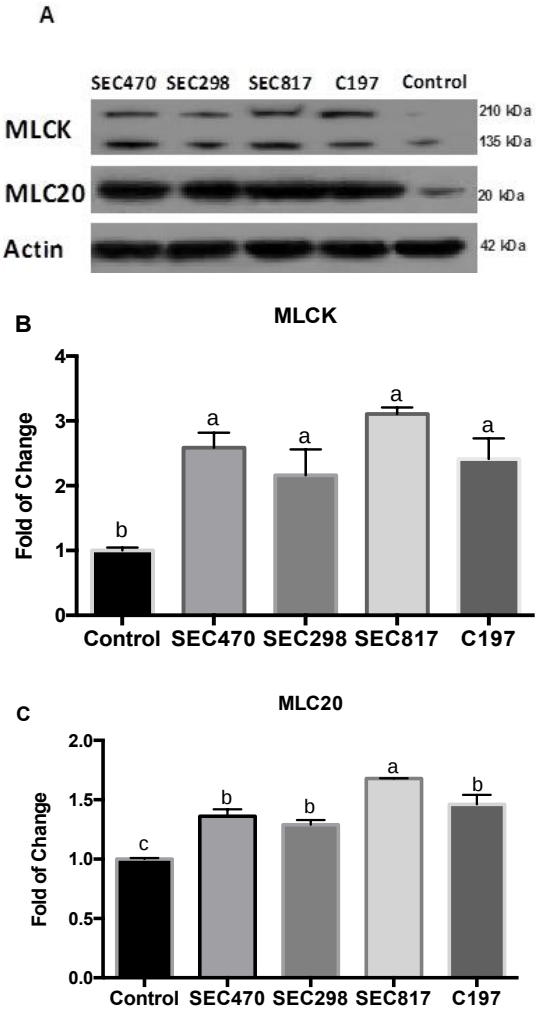

Fig. 6: Activation of MLCK/MLC20 pathway. **A**: Immunoblotting of MLCK and MLC20 in the jejunum at 24 h post ETEC infection, **B**: Statistical analysis of relative abundance of MLCK from data shown in A, and **C**: Statistical analysis of relative abundance of MLC20 from data shown in A. SEC470: Mice infected with SEC470 strain with a dose of 1×10^9 CFU; SEC298: Mice infected with SEC298 strain with a dose of 1×10^9 CFU; SEC817: Mice infected with SEC817 strain with a dose of 1×10^9 CFU; C197: Mice infected with C197 strain with a dose of 1×10^9 CFU; Control: Mice infected with same volume of sterile PBS. Data presented as mean±SEM, n=5, with a-c used to indicate a statistically significant difference (P<0.05, one-way ANOVA)

Discussion

Tight junctions form the continuous intercellular barrier between intestinal epithelial cells, and this barrier acts as the first physical barrier against a variety of pathogens, as well as maintaining the homeostasis in the gastrointestinal tract (Suzuki, 2013). All epithelial cells express junction complex proteins including occludin, claudin-1 and ZO-1 proteins, and form cell junctions such as adherens and TJs. The TJs, located most apically on the lateral membrane, are required to sustain the mucosal barrier. TJ functions are determined by four types of protein, namely claudins, occludin, junctional adhesion molecules (JAMs), and other cytoplasmic TJ

proteins such as ZO-1, ZO-2, and ZO-3, which bind to claudins and determine whether and where claudins are polymerized to make TJs (Umeda *et al*., 2006; Van Itallie *et al*., 2008). As the TJ defines the functional barrier between columnar epithelial cells, it becomes the target for various intestinal pathogens, such as *S. typhimurium* (Kohler *et al*., 2007), EPEC (Muza-Moons *et al*., 2004) and ETEC (Johnson *et al*., 2010). Unlike previous studies (Roselli *et al*., 2003; Roselli *et al*., 2007; Johnson *et al*., 2010; Yu *et al*., 2012; Yang *et al*., 2014), this study has found that ETEC infection enhances the protein abundance of TJ proteins. A possible reason for this difference might be the ETEC strain (F18 vs. K88) and the infectious model (mice and porcine or human cell line) (Roselli *et al*., 2003; Roselli *et al*., 2007; Johnson *et al*., 2010; Yu *et al*., 2012; Yang *et al*., 2014). However, it would be interesting to study the localization of these proteins after ETEC infection *in vivo*, because previous investigations have showed that even though these TJ proteins remain largely unchanged in many diseases, including diarrhea, their subcelular distributions are often altered (Guttman *et al*., 2006; Shen *et al*., 2011).

Several compelling studies have indicated that some bacterial pathogens require or activate PI3K-mediated signaling pathways to gain entry into non-phagocytic cells (Mecsas *et al*., 1998; Martinez *et al*., 2000). In addition, several lines of evidence have implicated that the PI3K-Akt pathway plays a vital role in intestinal barrier functions, i.e. TJs (Li and Neu, 2009). Mechanically, the PI3K-Akt pathway is widely documented to associate with TJ function and protein (Li and Neu, 2009; Lin *et al*., 2013; Kim and Kim, 2014). Deprivation of exogenous and endogenous glutamine decreases TER and protein abundance of claudin-1, and increases permeability in Caco-2 cells, while both wortmannin and LY294002 (PI3K inhibitors) prevent the decrease of TER and claudin-1, and the increase of permeability induced by glutamine deprivation (Li and Neu, 2009). Nevertheless, with the exception of C197, ETEC infection has little effect on the PI3K-Akt pathway. Besides the PI3K-Akt pathway, another critical signaling pathway in TJ function is the MLCK-MLC20 pathway (Edelblum and Turner, 2009). Enteropathogenic bacteria usually modulate TJ by activating the MLCK-MLC20 pathway (Barreau and Hugot, 2014). In this study, the MLCK/MLC20 pathway was activated after different ETEC infections. To the best of our knowledge, this is the first study to show that ETEC infections activate the MLCK-MLC20 pathway.

Aquaporin 3 has been proposed to play roles in the normal dehydration of faecal contents, and EPEC infection induces the mislocalization of AQP 3 from its normal location along cell membranes to the cell cytoplasm in mouse models (Guttman *et al*., 2007). More importantly, this mislocalization is associated with the diarrhea-like phenotype in infected mice, and mice recovering from infection regain normal membrane localization (Guttman *et al*., 2007). Also, the expression of AQP 3 is associated with the pathogenesis of *Helicobacter pylori* infection (Wang *et al*., 2012). However, no study has explored the function of AQP 3 in ETEC-induced diarrhea. In this study, different ETEC strain infections have shown little effects on the protein abundance of AQP 3 in mice. However, it is still interesting to explore the localization of AQP 3 after ETEC infections because the altered AQP 3 localization is a contributing factor in bacterial diarrhea (Guttman *et al*., 2007).

In conclusion, ETEC infection promotes the protein abundance of TJ proteins, but has little effect on the protein abundance of AQP 3. Enterotoxigenic *Escherichia coli* induces TJ protein change in mice through a pathway involving MLCK/MLC20.

Acknowledgements

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