Early weaning increases intestinal permeability, alters expression of cytokine and tight junction proteins, and activates mitogen-activated protein kinases in pigs

C. H. Hu, K. Xiao, Z. S. Luan, and J. Song

ABSTRACT: Although weaning stress has been reported to impair intestinal barrier function, the mechanisms have not yet been elucidated. In the present study, the intestinal morphology and permeability and mRNA expressions of tight junction proteins and cytokines in the intestine of piglets during the 2 wk after weaning were assessed. The phosphorylated (activated) ratios of p38, c-Jun NH₂-terminal kinase (JNK), and extracellular regulated kinases (ERK1/2) were determined to investigate whether mitogen-activated protein kinase (MAPK) signaling pathways are involved in the early weaning process. A shorter villus and deeper crypt were observed on d 3 and 7 postweaning. Although damaged intestinal morphology recovered to preweaning values on d 14 postweaning, the intestinal mucosal barrier, which was reflected by transepithelial electrical resistance (TER) and paracellular flux of dextran (4 kDa) in the Ussing chamber and tight junction protein expression, was not recovered. Compared with the preweaning stage (d 0), jejunal TER and mRNA expressions of occludin and claudin-1 on d 3, 7, and 14 postweaning and Zonula occludens-1 (ZO-1) mRNA on d 3 and 7 postweaning were reduced, and paracellular flux of dextran on d 3, 7, and 14 postweaning was increased. An increase \((P < 0.05)\) of tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-6 (IL-6) mRNA on d 3 and d 7 postweaning and an increase \((P < 0.05)\) of interferon-\(\gamma\) (IFN-\(\gamma\)) mRNA on d 3 postweaning were observed compared with d 0. No significant increase of transforming growth factor \(\beta\) 1 (TGF-\(\beta\)1) and interleukin-10 (IL-10) mRNA after weaning was observed. The phosphorylated (activated) ratios of JNK and p38 on d 3 and 7 postweaning and the phosphorylated ratio of ERK1/2 on d 3 postweaning were increased \((P < 0.05)\) compared with d 0. The results indicated that early weaning induced sustained impairment in the intestinal barrier, decreased mRNA expression of tight junction proteins, and upregulated the expression of proinflammatory cytokines, but anti-inflammatory cytokines were not affected in the intestine of piglets. The recovery of the intestinal barrier function was slower than that of the intestinal mucosal morphology. The weaning stress activated MAPK signaling pathways in the intestine, which may be an important mechanism of weaning-associated enteric disorders of piglets.

Key words: cytokine expression, intestinal barrier, mitogen-activated protein kinases, tight junction proteins, weaning pig

INTRODUCTION

Weaning piglets are abruptly forced to combined stressors, such as removal from sow and littermates, transportation to a new environment, fighting and establishment of social hierarchy, and abrupt changes in diet (Moeser et al., 2007). Although weaning stress is reported to impair intestinal barrier function (Moeser et al., 2007; Smith et al., 2010; Wijtten et al., 2011a; Hu et al., 2012), the mechanisms have not yet been
elucidated. The intestinal barrier is mainly formed by a layer of epithelial cells joined together by tight junctions (Li et al., 2012). Tight junction proteins form a dynamic seal between epithelial cells, becoming the rate-limiting step in the paracellular pathway and forming the principle physical barrier (Li et al., 2012). It would be of interest to investigate whether the compromised paracellular barrier function after weaning might be attributed to the alterations of tight junctions. However, few data are available regarding the effects of early weaning on tight junction protein expression in piglets. Cytokines have an important physiological and pathological effect on the intestinal tight junction barrier (Al-Sadi et al., 2009). Pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-6 and anti-inflammatory cytokines like IL-10 and transforming growth factor β (TGF-β) regulate intestinal barrier differently (Al-Sadi et al., 2009). Changes in the intestinal cytokine network may be expected in weaning pigs. Only a few studies have investigated the gene expression of inflammatory cytokines in the intestine of piglets after weaning (Pié et al., 2004). Moreover, few data are available regarding the effect of early weaning on anti-inflammatory cytokines. Mitogen-activated protein kinase (MAPK) pathways, a family of serine-threonine kinases, transduce signals from a diverse array of extracellular stimuli (Pearson et al., 2001; Shiflett et al., 2004). The 3 primary MAPK signaling pathways are the extracellular regulated kinases (ERK1/2), p38 MAPK, and c-Jun NH2-terminal kinase (JNK; Shiflett et al., 2004). Whether early weaning results in MAPK activation remains unknown. It would be of interest to determine the involvement of MAPK signaling pathway in early weaning piglets.

In the present study, the intestinal morphology and permeability and mRNA expressions of tight junction proteins and cytokines in the intestine of piglets during the 2 wk after weaning were assessed. The phosphorylated (activated) ratios of p38, JNK, and ERK1/2 were determined to investigate whether MAPK signaling pathways are involved in the early weaning process.

**MATERIALS AND METHODS**

All procedures were approved by the Zhejiang University Animal Care and Use Committee.

**Animals, Housing, and Diet**

Six litters (Duroc × Landrace × Yorkshire, 9 to 11 piglets per litter) were selected. At 20 d of age (preweaning stage), 1 piglet from each of 6 different litters was killed. At weaning day (21 d of age), 3 piglets from each of 6 different litters were allocated to 1 of the 3 experimental groups killed at 3, 7, and 14 d postweaning. For each group, 6 piglets from 6 different litters were removed from the sow, mixed, and housed in nursery pens. The 3 pens had an equal numbers of males and females, with an average BW of the piglets of 5.7 ± 0.2 kg (mean ± SE). The weaned piglets were given ad libitum access to feed and water. Diet was formulated to meet requirements suggested by the NRC (1998; Table 1).

**Table 1. Ingredient and chemical composition of the weaned diet on an as-fed basis**

<table>
<thead>
<tr>
<th>Item</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients, g/kg</td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>553.5</td>
</tr>
<tr>
<td>Soybean meal, CP 460</td>
<td>165</td>
</tr>
<tr>
<td>Extruded full-fat soybean, CP 355</td>
<td>120</td>
</tr>
<tr>
<td>Fish meal, CP 628</td>
<td>50</td>
</tr>
<tr>
<td>Dried whey, CP 120</td>
<td>60</td>
</tr>
<tr>
<td>Spray-dried plasma protein, CP 750</td>
<td>20</td>
</tr>
<tr>
<td>Limestone meal</td>
<td>5</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>12</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3</td>
</tr>
<tr>
<td>l-Lysine HCl</td>
<td>1</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin-mineral premix1</td>
<td>10</td>
</tr>
<tr>
<td>Analyzed composition, g/kg</td>
<td></td>
</tr>
<tr>
<td>DE, MJ/kg</td>
<td>14.36</td>
</tr>
<tr>
<td>CP</td>
<td>219.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>14.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.0</td>
</tr>
<tr>
<td>Calcium</td>
<td>9.1</td>
</tr>
<tr>
<td>Total phosphorus</td>
<td>7.2</td>
</tr>
</tbody>
</table>

1Provided per kilogram of diet: vitamin A, 6,000 IU; vitamin E, 50 IU; vitamin D3, 600 IU; vitamin K3, 1.5 mg; biotin, 0.10 mg; riboflavin, 8.0 mg; thiamine, 2.0 mg; niacin, 30 mg; pantothenic acid, 20 mg; pyridoxine, 3.0 mg; folic acid, 0.6 mg; vitamin B12, 0.04 mg; choline, 800 mg; Cu (CuSO4 · 5H2O), 16 mg; Fe (FeSO4), 125 mg; Zn (ZnSO4), 100 mg; Mn (MnSO4 · H2O), 15 mg; Se (Na2SeO3), 0.3 mg; I (KI), 0.2 mg.

2Digestible energy was calculated from data provide by Feed Database in China (2011).

On the day of preweaning (d 0) and d 3, 7, and 14 postweaning, 6 piglets were killed by euthanasia with an intravenous injection of sodium pentobarbital (40 mg/kg BW). Segments of mid-jejunum were harvested immediately after euthanasia and prepared for Ussing chamber studies. Adjacent specimens were fixed in buffered formalin until morphology measurements. Mucosal scrapings from the remaining jejunum were collected, rapidly frozen in liquid nitrogen, and stored at −80°C.
Intestinal Morphological Analyses

The specimens of mid-jejunum were embedded in paraffin, sectioned (5 μm), and stained with hematoxylin-eosin. A total of 10 intact, well-oriented crypt-villus units were selected for each intestinal cross section in triplicate. Villus height and crypt depth were determined using an image processing and analysis system (Version 1, Leica Imaging Systems Ltd., Cambridge, UK).

Ex vivo Ussing Chamber to Measure Intestinal Permeability

The intestinal epithelial tight junction permeability was measured by transepithelial electrical resistance (TER; Ω·cm²) and paracellular flux of fluorescein isothiocyanate (FITC) dextran 4 kDa (FD₄) in Ussing chambers. The Ussing chamber procedures were performed as described previously (Hamard et al., 2010; Overman et al., 2012). Briefly, segments of jejunum were stripped from the seromuscular layer in oxygenated (95% O₂/5% CO₂) Ringer’s solution and then mounted in an EasyMount Ussing chamber system (model VCC MC6, Physiologic Instruments, San Diego, CA). The clamps were connected to Acquire and Analyze software (Physiologic Instruments) for automatic data collection. After a 30-min equilibration period on Ussing chambers, TER was recorded at 15-min intervals over a 2-h period. The probe FD₄ (Sigma-Aldrich, St. Louis, MO) was added to the mucosal side at the final concentration of 0.375 mg/mL. Mucosal-to-serosal flux of FD₄ (ng·cm⁻²·h⁻¹) was performed by sampling 100 μL of solution from the serosal side at 30-min intervals over a 2-h period and replaced with 100 μL of fresh Ringer’s solution to keep the volume constant. The concentrations of FD₄ in the serosal side were measured by fluorescence microplate reader (FLx800, Bio-Tek Instruments Inc., Winooski, VT).

mRNA Expression of Cytokines and Tight Junction Proteins from Jejunal Mucosa by Real-Time PCR

The mRNA expression of cytokines (TNF-α, IL-6, IFN-γ, TGF-β1, and IL-10) and tight junction proteins (occludin, claudin-1, and ZO-1) from jejunal mucosa was determined by quantitative real-time PCR (qRT-PCR) as described by Liu et al. (2008). Briefly, total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I before cDNA synthesis following the manufacturer’s guidelines. The GenBank accession numbers, sequences of forward and reverse primers, and fragment sizes are presented in Table 2. The qRT-PCR was performed on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) using a SYBR Green Master mix (Promega, Madison, WI) according to the kit’s instructions. The housekeeping gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), exhibited no variation across treatment groups. The preweaning (d 0 postweaning) pigs were used as the reference sample. The comparative cycle threshold (Ct) method (2⁻ΔΔCt; Livak and Schmittgen, 2001) was used to analyze the relative expression was used to analyze the relative expression (fold changes), calculated relative to the values from the preweaning (d 0 postweaning) pigs.

Table 2. GenBank accession numbers, sequences of forward and reverse primers, and fragment sizes used for real-time PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>GeneBank number</th>
<th>Primer sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor necrosis factor-α</td>
<td>NM_214022.1</td>
<td>F:5′CATCGCGGTCTCCCTACCA3′ R:5′CCCAGATTCAAGAAAGGTC3′</td>
<td>199</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_001252429.1</td>
<td>F:5′CCTGTCCTACTGGCCACATAAC3′ R:5′CAAGAAACACCTGCTGAAAC3′</td>
<td>253</td>
</tr>
<tr>
<td>Interferon-γ</td>
<td>NM_213948.1</td>
<td>F:5′GAGGTTTTATCTTCTTCTAC3′ R:5′CTAGGTTACACTGGCACAAC3′</td>
<td>140</td>
</tr>
<tr>
<td>Transforming growth factor β1</td>
<td>NM_214015.1</td>
<td>F:5′GAGGTTTTATCTTCTTCTAC3′ R:5′CTAGGTTACACTGGCACAAC3′</td>
<td>133</td>
</tr>
<tr>
<td>IL-10</td>
<td>NM_214041.1</td>
<td>F:5′GAAGGACCAAGATGGGCGACT3′ R:5′CACCTCCTCCAGGCTTT3′</td>
<td>256</td>
</tr>
<tr>
<td>Ocludin</td>
<td>NM_001163647.1</td>
<td>F:5′TCCTGGGTGTGATGTTGCCT3′ R:5′CGTAGAGTCAGTCAAGGCA3′</td>
<td>145</td>
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<tr>
<td>Claudin-1</td>
<td>NM_001244539.1</td>
<td>F:5′GAAGGACCAAGATGGGCGACT3′ R:5′CACCTCCTCCAGGCTTT3′</td>
<td>193</td>
</tr>
<tr>
<td>Zonula occludens-1</td>
<td>XM_003353439.2</td>
<td>F:5′AAGGCCCTTAAGTTCAACTCAAATCT3′ R:5′ATCAAATCTGAGGCGGTC3′</td>
<td>130</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>NM_001206359.1</td>
<td>F:5′ATGGTGAAAGTCGGATGAAAC3′ R:5′GCTGCTCCTGGAAGATGT3′</td>
<td>235</td>
</tr>
</tbody>
</table>
Weaning on intestinal barrier function of pig

**Western Blot Analysis**

The total abundance of each of the 3 primary MAPK (JNK, p38, ERK1/2) and their phosphorylated (p; activated) forms of MAPK (p-JNK, p-p38, p-ERK1/2) in jejunal mucosa were analyzed by Western blot as previously described (Shiflet et al., 2004). Briefly, after electrophoresis the proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA). These primary antibodies were used (Cell Signaling Technology, Inc., Danvers, MA): MAPK antibodies, rabbit IgG; phospho-p38 (Thr180/Tyr182 phosphorylation), rabbit IgG; phospho-ERK 1/2 (Thr202/Tyr204 phosphorylation), rabbit IgG; and phospho-JNK (Thr183/Tyr185 phosphorylation), rabbit IgG. The secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). An enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) was used to detect the positive bands. The values were calculated as the ratios of their phosphorylation levels (p-JNK, p-p38, p-ERK) and the total abundance of MAPK. The values in samples from the preweaning (d 0 postweaning) pigs were used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the values from the preweaning (d 0 postweaning) pigs.

**Statistical Analysis**

Results were expressed as the means ± SEM. Data were subjected to 1-way ANOVA followed by Duncan’s multiple range tests. Differences were considered statistically significant at P < 0.05. Statistical analyses were performed with the SAS software package (SAS Inst. Inc., Cary, NC).

**RESULTS**

**Intestinal Morphology and Barrier Function in Porcine Jejunum after Weaning**

Table 3 shows jejunal morphology and barrier function of piglets at different time points (0, 3, 7, and 14 d postweaning). Compared with the preweaning stage (d 0 postweaning), villus height and the ratio of villus height and crypt depth on d 3 and 7 postweaning were decreased (P < 0.05). However, these variables had no significant effect (P > 0.05) between on d 0 and 14 postweaning. The intestinal barrier function of piglets was reflected by TER and paracellular flux of FD4 measured in Ussing chambers. A significant decrease in jejunal TER and an increase in mucosal-to-serosal flux of paracellular FD4 were observed on d 3, 7, and 14 postweaning, compared with the d 0 values. Although villus height and crypt depth returned to preweaning values on d 14 postweaning, intestinal mucosal barrier was not recovered, indicating the recovery of mucosal barrier is slower than that of morphology.

**Tight Junction Protein Expression**

Table 4 shows mRNA expression of occludin, claudin-1, and ZO-1 in jejunal mucosa during the 2 wk after weaning. Compared with the preweaning stage (d 0 postweaning), mRNA expression of occludin and claudin-1 on d 3, 7, and 14 postweaning and ZO-1 mRNA on d 3 and 7 postweaning was decreased (P < 0.05).

**Proinflammatory Cytokine mRNA**

Table 5 shows cytokine mRNA abundance in jejunal mucosa of piglets during the 2 wk after weaning. For TNF-α and IL-6 mRNA, an increase (P < 0.05) was observed on d 3 and 7 postweaning compared with d 0. An increase (P < 0.05) of IFN-γ mRNA was found on d 3 postweaning compared with d 0. The mRNA expressions of TNF-α and IL-6 on d 14 postweaning and the
mRNA expression of IFN-γ on d 7 and 14 postweaning returned to preweaning values. By contrast, no significant increase of TGF-β1 and IL-10 mRNA during the 2 wk after weaning was observed (P > 0.05).

Mitogen-Activated Protein Kinase Signal Pathways

Table 6 shows the effects of early weaning stress on the 3 MAPK signaling pathways (JNK, p38, ERK). Jejunal mucosa was analyzed by Western blot for the total phosphorylation levels (p-JNK, p-p38, p-ERK) and the total levels of MAPK. The values were calculated as the ratios of their phosphorylated forms and the total ERK (p-ERK/ERK) and p-p38/p38 (p-p38/p38) on d 3 and 7 postweaning were increased (P < 0.05). The ratio of the phosphorylated forms and the total ERK (p-ERK/ERK) on d 3 postweaning was greater (P < 0.05) than that on d 0, then returned to preweaning values on d 7 and 14 postweaning.

**DISCUSSION**

In agreement with earlier reports (Tang et al., 1999; Boudry et al., 2004; Montagne et al., 2007), the present study showed that villus height and the ratio of villus height and crypt depth on d 3 and 7 postweaning were decreased compared with the preweaning stage. The shorter villus and deeper crypt verified the deterioration of intestinal morphology induced by weaning. Additionally, the present study showed that villus height and crypt depth returned to the preweaning value on d 14 postweaning. In contrast to this finding, Boudry et al. (2004) reported that villous height in the jejunum was still significantly lower on d 15 postweaning than d 0.

In the present experiment, the Ussing chamber technique was used to monitor intestinal permeability in terms of TER and flux of FD4. Transepithelial electrical resistance is considered to reflect the opening of the tight junctions between epithelial cells and the paracellular permeability of the intestinal mucosa (Wijtten et al., 2011a). A decreased TER reflects increased paracellular permeability. Marker probe FD4 is added to the solution in the Ussing chamber at the mucosal site. The appearance of this marker probe in the chamber at the serosal site represents its permeability across the epithelium (Wijtten et al., 2011a). The flux of intact FD4 across the intestinal epithelium occurs mainly through paracellular pathways (Hamard et al., 2010; Overman et al., 2012). An increased flux of FD4 reflects increased paracellular permeability and impaired intestinal barrier. A significant decrease in jejunal TER and an increase in mucosal-to-serosal flux of paracellular FD4 on d 3, 7, and 14 postweaning compared with d 0 were observed in the present study. Similarly, using [3H]mannitol and [14C]inulin as marker probes, Moeser et al. (2007) and Smith et al. (2010) reported that the flux of mannotol and inulin over the jejunum was significantly increased and TER was significantly decreased when pigs were weaned at 15 to 21 d of age. However, Montagne et al. (2007) used HRP as a marker probe and found that the HRP flux across the jejunum epithelium declined on d 2 postweaning and stayed at this low level until d 15 postweaning. Wijtten et al. (2011b) used the recovery in urine of orally administered lactulose to assess intestinal permeability and found that lactulose recovery in the urine was greater at d 4, 8, and 12 after weaning compared with the first day after weaning, whereas the HRP flux was not affected over time.

Although villus height and crypt depth returned to preweaning values on d 14 postweaning, intestinal mucosal barrier did not recover, indicating the recovery of intestinal barrier function is slower than that of intestinal mucosal morphology. Similarly, Chang et al. (2005)
investigated the functional and morphological changes of the gut barrier of rats during the restitution process after hemorrhagic shock and found that the restitution of gut barrier function was obviously slower than that of the morphology and there was no direct correlation between them. Early life stress is a predisposing factor for the development of chronic intestinal disorders in adult life (Smith et al., 2010). Epidemiological studies have demonstrated that early life psychological stress is associated with the development of chronic, persistent intestinal disorders in adult life (O’Mahony et al., 2009). Neonatal maternal separation in rodents can induce long-term defects in the intestinal epithelial barrier associated with an exaggerated immune response to an external immune stimulus (Gareau et al., 2007). Smith et al. (2010) demonstrated that early weaning (15- to 21-d weaning age) resulted in sustained impairment in intestinal barrier function at 5 and 9 wk of age. In contrast, Lodemann et al. (2008) reported that the mannitol flux across the mid-jejunum on 7 d postweaning was significantly less than the preweaning value, and TER was not different from preweaning. Wijtten et al. (2011a) indicated that the impairment of the paracellular barrier function of piglets is almost exclusively observed in the first week after weaning and returns to the preweaning level at 2 wk after weaning. These discrepancies might be related to the difference in the weaning process and handling of the pigs.

The intestinal barrier is composed of a layer of columnar epithelium and interepithelial tight junctions. Tight junction proteins, such as claudins, occludins and zona occludens, form the tight junction at the boundary of 2 adjacent cells, working as a rate-limiting step in the paracellular pathway and forming a selectively permeable barrier (Li et al., 2012). To better clarify the molecular mechanism for the impaired mucosal barrier function in weaning piglets, we determined the changes in the mRNA expression of occludin, ZO-1, and claudin-1 after weaning for the first time. The results showed that mRNA expression of occludin and claudin-1 on d 3, 7, and 14 postweaning and ZO-1 mRNA on d 3 and 7 postweaning was decreased compared with d 0. The results indicated that the decreased tight junction protein in early weaned pigs coincided with the increased intestinal epithelial paracellular permeability.

Cytokines play an important role in the immune and inflammatory responses. They also participate in the regulation of the intestinal barrier integrity (Al-Sadi et al., 2009). Most proinflammatory cytokines, such as TNF-α, IFN-γ, and IL-6, increase intestinal epithelial permeability (Youakim and Ahdieh, 1999; Al-Sadi and Thomas, 2007). On the other hand, anti-inflammatory cytokines, like IL-10 and TGF-β, attenuate or protect against intestinal inflammation by preserving the barrier function (Madsen et al., 1997; Howe et al., 2005). Many studies reported that enteric infection and intestinal inflammatory diseases were associated with the alternations in the expression of proinflammatory cytokines in the intestine of humans and animals (Murtugh et al., 1996; McClane and Rombeau, 1999). However, only a few studies investigated the expression of cytokines in the intestine of piglets after weaning. The present study analyzed the gene expressions of proinflammatory and anti-inflammatory cytokines in the intestine of 21-d-old weaned piglets during the 2 wk after weaning. The results showed that mRNA expression of TNF-α and IL-6 was increased on d 3 and 7 postweaning compared with d 0 and returned to preweaning values on d 14 postweaning, and IFN-γ mRNA was increased on d 3 postweaning compared with d 0 and returned to preweaning values on d 7 and 14 postweaning. This study also demonstrated for the first time that no significant alternations of anti-inflammatory cytokines (TGF-β1 and IL-10) mRNA were observed during the 2 wk after weaning. In findings similar to ours, Pié et al. (2004) reported that weaning stress upregulated expression of proinflammatory cytokines in the intestine of 28-d-old weaned piglets. However, there are discrepancies between the 2 studies. Pié et al. (2004) reported that the mRNA expressions of proinflammatory cytokines (TNF-γ, IL-6, and IL-1β) increased between d 0 and 2 and rapidly returned to preweaning values between d 2 and 8. However, the present results showed that mRNA expression of TNF-α and IL-6 was still increased on d 7 postweaning compared with d 0. The discrepancies might be related to the difference in the weaning age (weaned at the age of 21 and 28 d, respectively).

Overproduction of proinflammatory cytokines has a negative influence on gut integrity and epithelial function (Liu et al., 2008). The tight junction barrier disruptive actions of TNF-α and IFN-γ have been well established (Madara and Stafford, 1989; Youakim and Ahdieh, 1999; Ma et al., 2005). It has been reported that IFN-γ-induced reductions in epithelial barrier function are linked to decreases in the expression of tight junction proteins such as occludin and ZO-1 (Blikslager et al., 2007). The cytokine INF-γ can act synergistically with TNF-α to induce impairment in tight junctions (Al-Sadi et al., 2009). Conversely, TGF-β and IL-10 are anti-inflammatory cytokines and can attenuate the defects in tight junction permeability (Madsen et al., 1997; Howe et al., 2005). Reported actions of TGF-β on the repair of the epithelium after injury include enhanced epithelial cell migration, upregulated expression of extracellular matrix proteins and cell surface integrin, and decreases in epithelial paracellular permeability (Blikslager et al., 2007). Exogenous IL-10 administration in total parenteral nutrition (TPN) mice attenuated the TPN-induced reductions in
expression of occludin and ZO-1 and a loss of intestinal barrier function (Sun et al., 2008). The present study showed that early weaning upregulated the expression of proinflammatory cytokines in the intestine of piglets but had no effect on the expression of anti-inflammatory cytokines, which coincided with the decreased expression of tight junction protein and increased intestinal epithelial paracellular permeability. From the nutritional perspective, controlling the early intestinal inflammation and facilitating the restoration of the injured intestinal mucosa may have potential benefits in alleviating intestinal barrier impairment induced by weaning stress.

The MAPK signaling pathways transduce signals from a diverse array of extracellular stimuli and form a highly integrated network to regulate cellular functions, such as differentiation, proliferation, and cell death (Pearson et al., 2001; Shifflott et al., 2004). The 3 primary MAPK signaling pathways are the ERK1/2, p38 and JNK. The MAPK signaling cascades are viewed as parallel pathways, although cross talk may exist (Xiao et al., 2002; Shifflott et al., 2004). Upon phosphorylation and subsequent activation of an MAPK, the MAPK can enter the cell nucleus and activate transcription factors, which ultimately results in expression of target genes and a biological response (Shifflott et al., 2004). To determine whether early weaning influences MAPK signaling pathways, we measured the total levels of each of the 3 primary MAPK (JNK, p38, ERK) and their phosphorylated forms (p-JNK, p-p38, p-ERK). We demonstrated for the first time that the ratios of p-p38/p38 and p-JNK/JNK on d 3 and 7 postweaning and the ratio of p-ERK/ERK on d 3 postweaning were increased compared with d 0, indicating early weaning stress in pigs induced activation of MAPK signaling pathways. Patrick et al. (2006) suggest that downstream effectors of MAPK signaling pathways mediate the TNFα/IFNγ-induced junctional reorganization that modulates Madin-Darby canine kidney cell barrier function. Our hypothesis is that the weaning stress activates MAPK signaling pathways in the intestine, resulting in intestinal dysfunction, which may be an important mechanism of weaning-associated enteric disorders of piglets. However, the exact role of this signaling pathway remains to be elucidated. In a follow-up study, the specific inhibitors of MAPK may be used to investigate whether inhibition of MAPK can block the weaning-induced damage of the intestinal epithelial barrier.

In summary, the shorter villus and deeper crypt verified the deterioration of intestinal morphology induced by early weaning. Weaning resulted in sustained impairment in the intestinal barrier on d 3, 7, and 14 postweaning. Although villus height and crypt depth returned to preweaning values on d 14 postweaning, intestinal mucosal barrier was not recovered, indicating the recovery of the intestinal barrier function is slower than that of

**LITERATURE CITED**


