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Full Length Research Paper

Protease-activated receptor-2 (PAR-2) regulates enterotoxigenic Escherichia coli-induced diarrhea during weaning in piglets

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Protease-activated receptor-2 (PAR-2) is a member of the G-protein-coupled receptor family. The proteases that activate PAR-2 are released during inflammation and injury, with PAR-2 regulating the response to these insults. In the gastrointestinal tract, PAR-2 is known to alter gastrointestinal secretion, motility, inflammation, and pain. In many cases, PAR-2 has been reported as pro-inflammatory and proliferative. Paradoxically, PAR-2 is also suggested to be anti-inflammatory in some instances. Weaning piglet diarrhea is severely detrimental to the porcine industry, being responsible for 11% of total piglet deaths, while those that survive from the disease experience developmental problems and fail to grow to the size of their healthy counterparts. Thus, we sought to determine any correlation between PAR-2 and weaning diarrhea. We hypothesized that PAR-2 might represent a new target in the treatment of weaning diarrhea. The current study measured PAR-2 using immunohistochemistry on sections of piglet gastrointestinal tract mucosa and identified changes in receptor expression during the development and course of weaning. Moreover, the effect of PAR-2 stimulation using lipopolysaccharide (LPS) and heat-labile enterotoxin (LT) on IL-6 and IL-8 production in pig intestinal epithelial cells (IEC) was determined. This study found that PAR-2 is expressed abundantly in the piglet gastrointestinal tract mucosa, and revealed that PAR-2 mRNA and protein expression are both correlated with the severity of diarrhea. The generation of IL-6 and IL-8 by IECs was significantly increased following stimulation with PAR-2 agonists dose-dependently. Thus, we suggest PAR-2 may be involved in the development of diarrhea during weaning in piglets.

Key words: Diarrhea, weaning piglet, gastrointestinal tract, protease activated receptor-2, immunostaining, RT-PCR, IL-6, IL-8.

INTRODUCTION

Protease-activated receptors (PARs) are a family of G-protein-coupled, seven-transmembrane receptors, consisting of four known members, PAR-1 to PAR-4 (Déry et al., 1998; Macfarlane et al., 2001). PARs are activated by proteolytic cleavage of their extracellular NH_2-terminus. Among the four PARs identified to date, PAR-1, PAR-3, and PAR4 are known to be activated by thrombin, while trypsin and mast cell-derived tryptase are shown to activate PAR-2 (Kanke et al., 2005). PAR-2 was first identified in 1944 and reported to be widely expressed in many cell types (Dulon et al., 2005; Saifeddine et al., 1996) such as enterocytes, endothelial cells of the lamina propria and the submucosa, fibroblasts, myenteric neurons, and immunological and inflammatory cells (including lymphocytes, neutrophils, and mast cells) (Kawao et al., 2002; Nystedt et al.,1996).

The physiological and pathological roles of PAR-2 have

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been investigated in animal models *in vivo* using immunohistochemistry, pharmacology (employing synthetic PAR-2 activating peptide), and PAR-2 deficient (PAR2−/−) mice (Kawagoe et al., 2002; Vergnolle, 1999). PAR-2 was found to participate in leukocyte rolling, ad-hesion, and infiltration of neutrophils, as well as inducing nuclear factor kappa B-DNA binding (Kanke et al., 2001). With regard to the role of PAR-2 in the intestine, Hansen et al. (2005) reported PAR 2 to augment enteritis from many kinds of bacteria. PAR-2 can be activated by proteolytic enzymes secreted from bacteria, promoting substance P release, which binds and activates the neurokinin A receptor, which in turn induces tissue edema and effusion. Acute activation of PAR-2 causes colitis characterized by gut wall edema, granulocyte recruitment, increased permeability and the release of pro-inflammatory cytokines such as interleukin-1 and TNF-α (Cenac et al., 2002). Other studies have shown activation of PAR-2 with chronic, systemic administration of a PAR-2 agonist to provide protection against a chronic model of inflammatory bowel disease (IBD). This protection was suggested to be the result of PAR-2-induced gastric mucus secretion and mucosal cytoprotection (Fiorucci et al., 2001; Kawabata et al., 2001).

Cytokines play a central role in the modulation of the intestinal immune system and can stimulate proinflammatory (IL-1, TNF, IL-12, IL-6, IL-8) or anti-inflammatory (IL-4, IL-10, IL-1ra, IL-11) responses. Levels of both proinflammatory and anti-inflammatory cytokines are elevated in patients with IBD; however the ratio between pro- and anti-inflammatory cytokines becomes unbalanced, leading to inflammation. The major source of IL-6 within the gut is from macrophages, although the primary source of IL-6 in epithelial cells remains to be defined (Rogler et al., 1997). IL-6 is elevated in both the serum and mucosa of patients with IBD (Murata et al., 1995).

Furthermore, IL-8 (a powerful neutrophil chemoattractant and activator (Daig et al., 1996) correlates with the level of inflammation in the colon of patients with ulcerative colitis (UC) (Mazzucchelli et al., 1994) in which large numbers of neutrophils are found in crypt abscesses. Our previous reports have demonstrated that direct activation of PAR-1 and PAR-2 in human endothelial cells leads to the production of IL-6, and both LPS and TNF-*a* markedly enhance PAR-induced activation (Chi et al., 2001). PAR-2 activation stimulates the proliferation of endometriotic stromal cells (ESC) and the secretion of IL-6 and IL-8 from the ESC (Hirota et al., 2005).

At weaning, piglets are exposed to marked alterations in living conditions, such as shifting from milk to a cereal-based diet, becoming separated from the sow and mixed with other piglets. One of the consequences of weaning is the high risk of post weaning diarrhea (Wellock et al., 2007). It has been reported that following three days of weaning, piglets become more susceptible to the detrimental effects of Escherichia coli enterotoxin when compared to their unweaned littermates. Further investigations report morbidity and mortality of enterotoxigenic Escherichia coli (ETEC)-induced diarrhea to account for up to 56.2 and 24.7% of total diarrhea-induced morbidity and mortality, respectively (Alexander, 1994; Hampson, 1994; Jin et al., 1998; Stevens et al., 1972). Characteristics of ETEC-induced disease include a rapid development and fast spread of infection resulting in either high mortality or chronically stunted growth. Both outcomes seriously compromise productivity and animal yield for the porcine industry.

Thus, PAR-2 plays a potentially protective or pathogenic role in gastrointestinal tract mucosa when under pathological conditions, possibly activated in response to tissue injury or inflammation. Many studies have found PAR-2 in humans and mice; however, few studies have found PAR-2 in pigs. The current study sought to determine the role of PAR-2 on diarrhea in weaned piglets. The results presented in this report demonstrate that PAR-2 is expressed abundantly in the piglet gastrointestinal tract mucosa, and reveals that PAR-2 mRNA and protein expression are increased with the severity of diarrhea. Direct activation of PAR-2 by both LPS and LT in pig intestinal epithelial cells leads to enhanced production of IL-6 and IL-8. Taken together we suggest that PAR-2 may be involved in the development of diarrhea during weaning in piglets.

MATERIALS AND METHODS

Regents and materials

Tryptone and yeast extract were obtained from Oxoid (Basingstoke, UK). Goat anti-PAR-2 antibody and anti-goat horseradish secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Epithelial cells were cultured in high-glucose formulated Dulbecco's Modified Eagle's Medium (DMEM, Gibco). Various concentrations of epidermal growth factor (EGF; Sigma), insulin (Sigma), fetal bovine serum (Gibco), glutamine (Gibco), penicillin (sigma) and streptomycin (Sigma) were also added to the medium to induce epithelial proliferation. The enzyme-linked immu-nosorbent assay (ELISA) kits for IL-6 were purchased from R and D systems (Minneapolis, MN). PAR-2 agonist peptides -Ser-Leu-Ile-Gly-Arg-Leu-NH_2 (SLIGRL-NH2) - and related hexa-peptides with an altered consensus sequence (LRGILS-NH_2) were synthesized by Biotechnology Corp. (Xian, China). Trypsin, the soy bean trypsin inhibitor, *E. coli* LPS and *E. coli* LT were all obtained from Sigma-Aldrich.

ETEC challenge strains

The ETEC K88 O149 strain (provided by the China Institute of Veterinary Drugs Control, China) was grown in Luria broth (LB) medium comprising: 1% tryptone, 0.5% yeast extract, and 1% NaCl, final pH 7.0. After an overnight incubation at 37 C with shaking, the bacterial cells were then diluted to an optical density at 600 nm (OD600) of 0.1 in fresh LB medium and allowed to grow to an OD600 of ≈1.0. The cell cultures were then centrifuged at 4,000 G

for 10 min at 4°C. The bacterial pellets were resuspended in 20% dextrose and 5% non-fat milk. The challenge dose consisted of an equal amount of each strain and was determined by serial dilution and plating to provide a total of 2×10^{10} CFU/0.5 ml oral dose, twice daily with an 8-h interval for three days.

Animals

Animal care and procedures were in accordance with the National Institutes of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by the appropriate Animal Use Committee of Huazhong Agricultural University. 30 Duroc piglets aged 25 - 28 days and weighing ~7 kg were provided by HuBei Academy of Agricultural Sciences China. 18 piglets were infected with ECET (Enterotoxigenous *E. coli*), and 12 piglets suffering from diarrhea were selected as a diarrhea group. We killed 3 healthy piglets and 3 diarrheal pigs on the first, third, fifth and seventh day, respectively. All piglets were anaesthetized using sodium pentobarbital prior to opening the gut and dissecting the stomach, dodecadactylon jejunum ileum, cecum and colon. All sec-tions were washed 3 times in cold 0.9% NaCl. Half of the sections were fixed by immersion in 10% neutral-buffered formalin, dehydrated, embedded in paraffin wax, and stored at 4 C for immunohistochemistry. Other sections were placed into liquid nitrogen for 3 h and maintained at -70 C for real-time RT-PCR.

Histopathology

A subsection of samples were subject to histological analysis to verify the cellular changes within the gastrointestinal tract by H and E staining.

Immunostaining of PAR-2 in the piglet gastrointestinal tract mucosa

Immunostaining of PAR-2 was performed using a goat polyclonal antibody. Briefly, sections of gut were deparaffinized, rehydrated, immersed in phosphate-buffered saline (PBS) for 15 min at 37°C and blocked in endogenous peroxidase (3% peroxide) for 5 min. After washing in PBS, blocking was performed with a 5% solution of normal rabbit serum for 30 min at 37°C. Primary goat polyclonal antibody, diluted 1:100 in PBS, was applied to each section and incubated for 12 h at 4°C in a humidified chamber. After washing three times, the slides were flooded with biotinylated rabbit anti-goat linking antibody (1:200) for 20 min at 37°C. Sections were then washed twice more in PBS, treated with peroxidase-conjugated avidin for 30 min, rewashed twice in PBS, and then incubated with 3-amino-9-ethylcarbazole for 25 min. Gut sections were stained with Mayer's hematoxylin counterstain for 1 min, dewatered, mounted by neutral balsam, and then analyzed under a microscope (OLYMPUS, IX71, Japan).

SYBR green real-time RT-PCR

PAR-2 mRNA expression was determined by SYBR green I real-time quantitative polymerase chain reaction (RT-qPCR) analysis using an IQ-5 real-time PCR detection system (Bio-Rad, America). Total RNA was extracted from the ileum, cecum and colon mucus using the TRIzol reagent (Invitrogen). After verification of its integrity, RNA was quantified spectrophotometrically with 1 ug processed for complementary DNA (cDNA) synthesis using SuperScript II reverse transcriptase (Toyobo, Japan). Specific primers for the PAR-2 gene were designed using Primer5 software. The sequences of the primers used were: PAR2, sense: 5'-GCA ACA ACT GGG TTT ACG GG -3'; antisense: 5'-GGT GTG ATG TGA AGG GCT GG -3'; GAPDH, used as a housekeeping gene, sense: 5'-GGT GAA GGT CGG AGT GAA CG-3'; antisense: 5'-CTC GCT CCT GGA AGA TGGTG-3'.

The efficacy of the RT-PCR primer pairs was determined by amplifying serial dilutions of cDNA. The RT-PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo). Each cycle consisted of three steps: denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and 30 s of elongation at 72°C. The data acquired from each sample were normalized to those of GAPDH. The specificity of the real-time reverse transcriptase was further confirmed by a regular RT-PCR followed by agarose gel electrophoretic analysis to verify the presence of a single band corresponding to the predicted size of the amplicon. Relative Ct (cycle time) values were obtained using the Bio-Rad iQ5 Optical System Software Data Acquisition Server.

Culture of pig intestinal epithelial cells

Newborn piglets were anaesthetized using sodium pentobarbital and killed. The small intestine was opened and sectioned into 2 - 3 mm lengths. Tissue sections were transferred to a 25 ml flask and washed at least 8 times in 50 ml of fresh Hanks' balanced salt solution (HBSS) with vigorous shaking. Tissue was then placed on a Petri dish and a sharp scalpel blade used to dice the tissue into <1 mm^3 pieces before being returned to a T25 ml flask with 20 ml of collagenase type 1 (Sigma-Aldrich). Tissue was shaken vigorously for 2 h at 37 °C**.** 15 ml of DMEM-S (DMEM + 2.5% FCS +2% sorbitol) was then added to the flask and shaken again. The sedi-ment was allowed to dissociate from the supernatant under gravity for 60 s, and the supernatant carefully removed. This procedure was repeated twice. 10 ml of DMEM-S was added to the supernatant before being vortexed and centrifuged at 200 - 300 rpm for 2 min. The supernatant was carefully removed and the pellet resuspended in 20 ml DMEM-S. This procedure was repeated at least 5 - 6 times until the supernatant became completely clear and the pellet well defined. Finally, the pellet was resuspended in the appropriate growth medium.

Assay of IL-6 and IL-8 production

Intestinal epithelial cells (1×10^5) were added to each well of a 12**-**well microtiter plate and allowed to adhere for 24 h. Following adherence, the medium containing serum was removed and serum-free medium added. Selected concentrations of PAR-2 agonist peptides, trypsin, a soy bean trypsin inhibitor, *E. coli* LPS, *E. coli* LT or medium were added to the monolayers. All incubations were carried out at 37°C in 5% humidified CO_2 for 24 h unless otherwise indicated. After incubation, the supernatant was collected and IL-6 and IL-8 levels were quantified by ELISA according to the manufacturer's protocol.

Statistical analysis

Data are displayed as the mean + SEM. All statistical analyses were performed using SPSS statistical software. The means among different groups were compared by one way analysis of variance (ANOVA).The PAR-2 positive area was calculated in pictures of immunohistochemistry using Image-Pro Plus software (IPP6.0). The data from SYBR Green Real-Time RT-PCR was analyzed using the formula: -[Mean Ct of specific gene of diarrhea group - Mean Ct of house-keeping gene of diarrhea] -2- $^{\Delta\Delta Ct}$ (2 [Mean Ct of specific gene of normal group - Mean Ct house-keeping gene of normal group]).

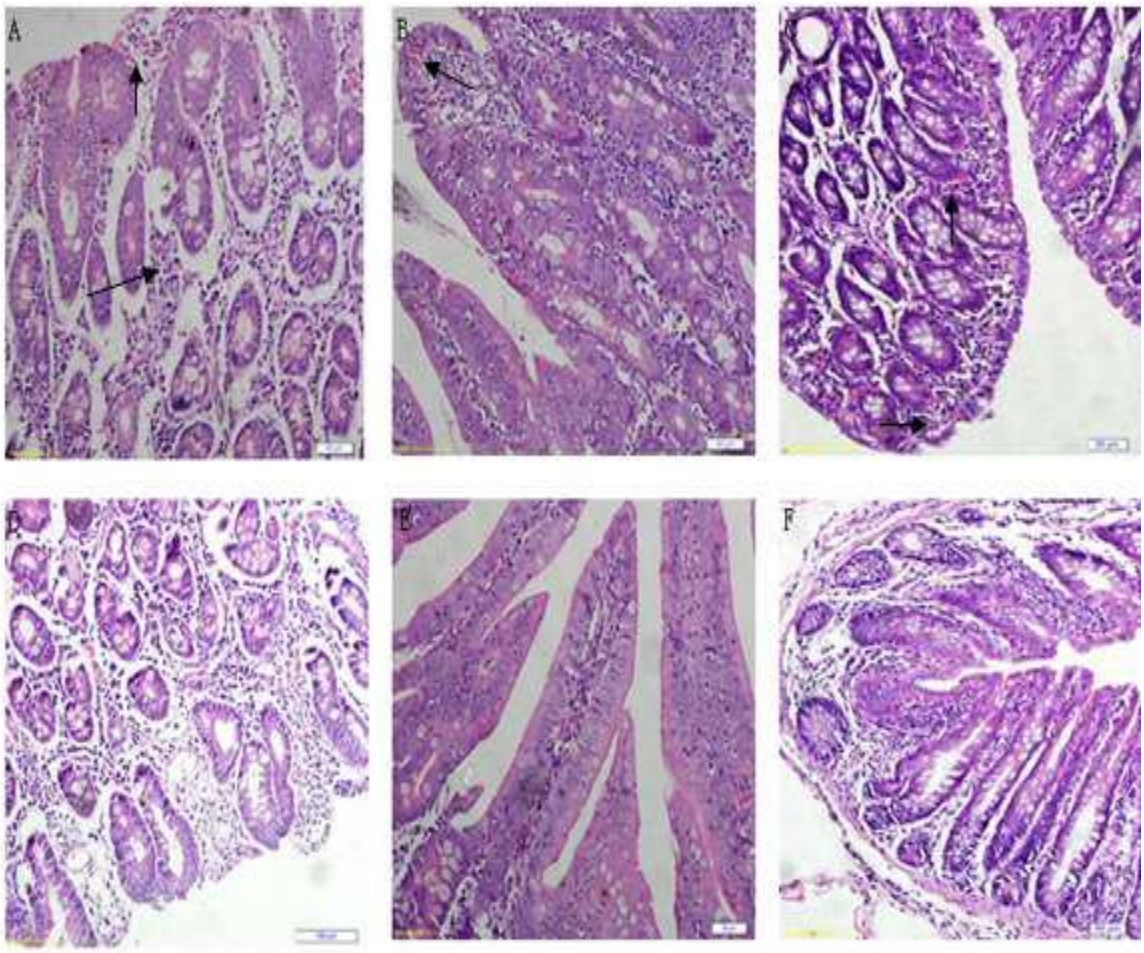

Figure 1. Representative H and E-stained histological sections of stomach, small intestine and large intestine from the diarrhea group (Figure 1A, B and C) with slides D, E and F providing normal comparisons respectively. Bar = 100μm.

RESULTS

Microscopic examination of histopathology

The most striking pathological changes of the diarrhea group was desquamation and denaturation of the epithelium mucosa, smooth muscle thinning of the bowels, infiltrated neutrophils and increased eosinophils exudated from the mucosal layer (Figure 1).

Immunolocalization of PAR-2 in the piglet gastrointestinal tract mucosa

PAR-2 was found expressed throughout the gastrointestinal tract, including in the mucosa of the stomach, duodenum, jejunum, ileum, cecum and colon (Figure 2B1-B6). Further observations revealed elevated staining of PAR-2 within the lamina propria and greater staining in intestinal mucosa of the diarrhea group compared with control (Figure 2A1-A6). Histochemical analysis using the serum from a non-immunized goat displayed no positive immunostaining for PAR-2 in the gastrointestinal tract mucosa (Figure 2C1-C6).

SYBR green real-time RT-PCR

Using the $2^{-\Delta\Delta Ct}$ method to analyze the mean Ct, our results display the ratio of PAR-2 mRNA expression in pathogenic versus normal tissue over the development of diarrhea. During this time the ratio of PAR-2 expression was not found to significantly differ in the stomach mucosa (Figure 3a), although it was significantly elevated in the ileum and colon. Changes in the PAR-2 ratio were apparent from day 3 in the ileum and markedly so in the colon (Figure 3b and c).

Effect of PAR-2 agonist peptides on IL-6 and IL-8 production by naive and LPS+LI-stimulated IEC

To examine the role of PAR-2 on intestinal epithelial cell

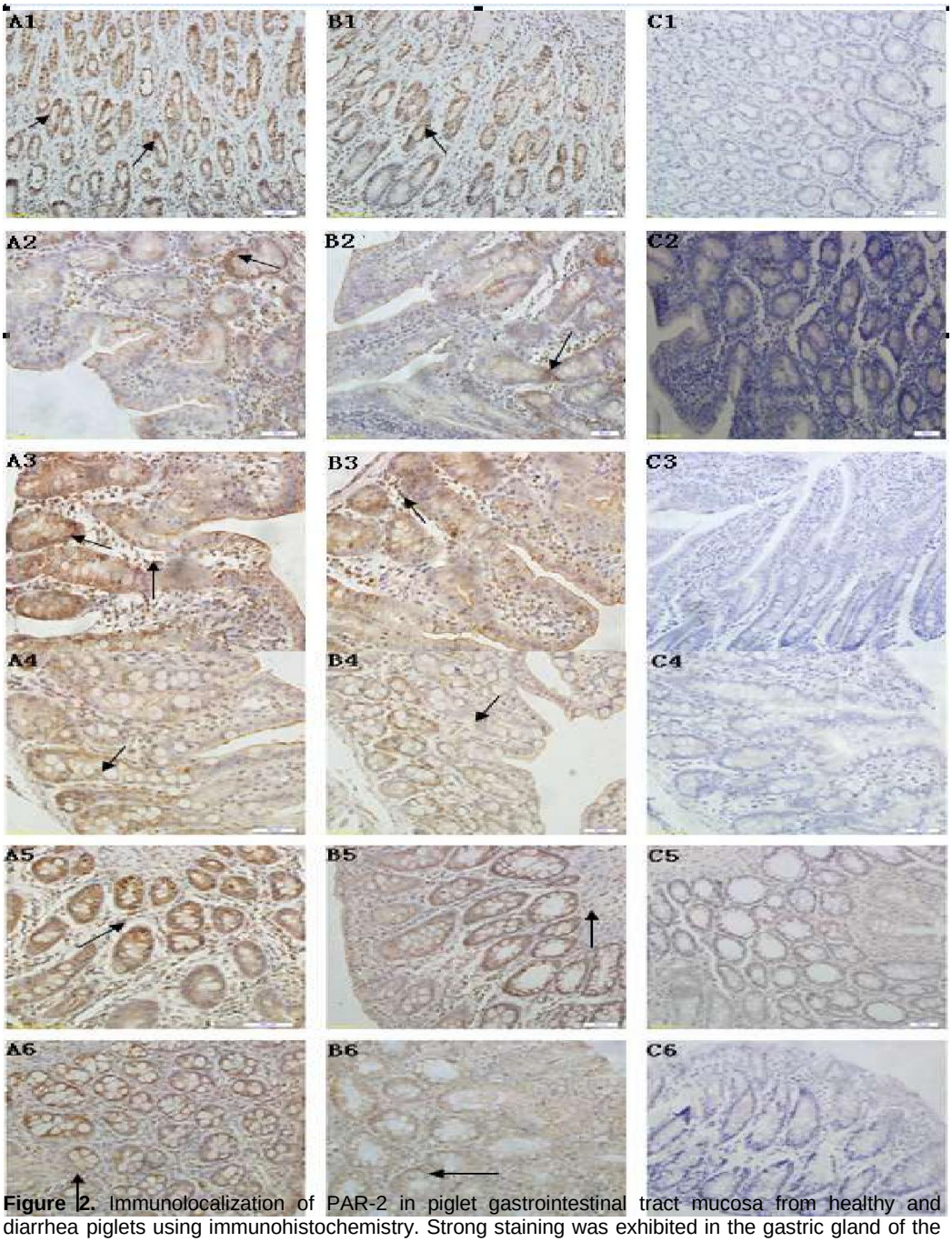

Figure 2. Immunolocalization of PAR-2 in piglet gastrointestinal tract mucosa from healthy and diarrhea piglets using immunohistochemistry. Strong staining was exhibited in the gastric gland of the stomach mucosa (A1), duodenum mucosa (A2), jejunum mucosa (A3), ileum mucosa (A4), cecum mucosa (A5) and colon mucosa (A6) compared to control cells (slides C1-C6 respectively) and healthy cells (B1-B6 respectively). Arrows indicate positive staining for PAR-2, reference bar = 50 m.

production of IL-6 and IL-8, the direct and amplifying effects of PAR-2 agonists on baseline and LPS+LT-activated IEC were studied. The data shown in Figure 4 demonstrate IL-6 and IL-8 production by IECs during 24-h incubation with PAR-2 agonist peptides and the effect of agonists in the presence of LPS+LT. Non-activated IECs produced negligible amounts of IL-6 and IL-8. Incubation of IECs with a PAR-2 agonist

resulted in a low, but significant increase in IL-6 and IL-8 expression compared with controls. To examine the possible synergy between PAR-2 agonists and LPS+LT on IEC activation, IECs were incubated with trypsin and SLIGKV-NH2 in the presence concentrations of LPS+LT Incubation of IECs with the PAR-2 agonists in the presence of LPS+LT resulted in marked potentiation of IL-6 and IL-8 production at all concentrations tested. The

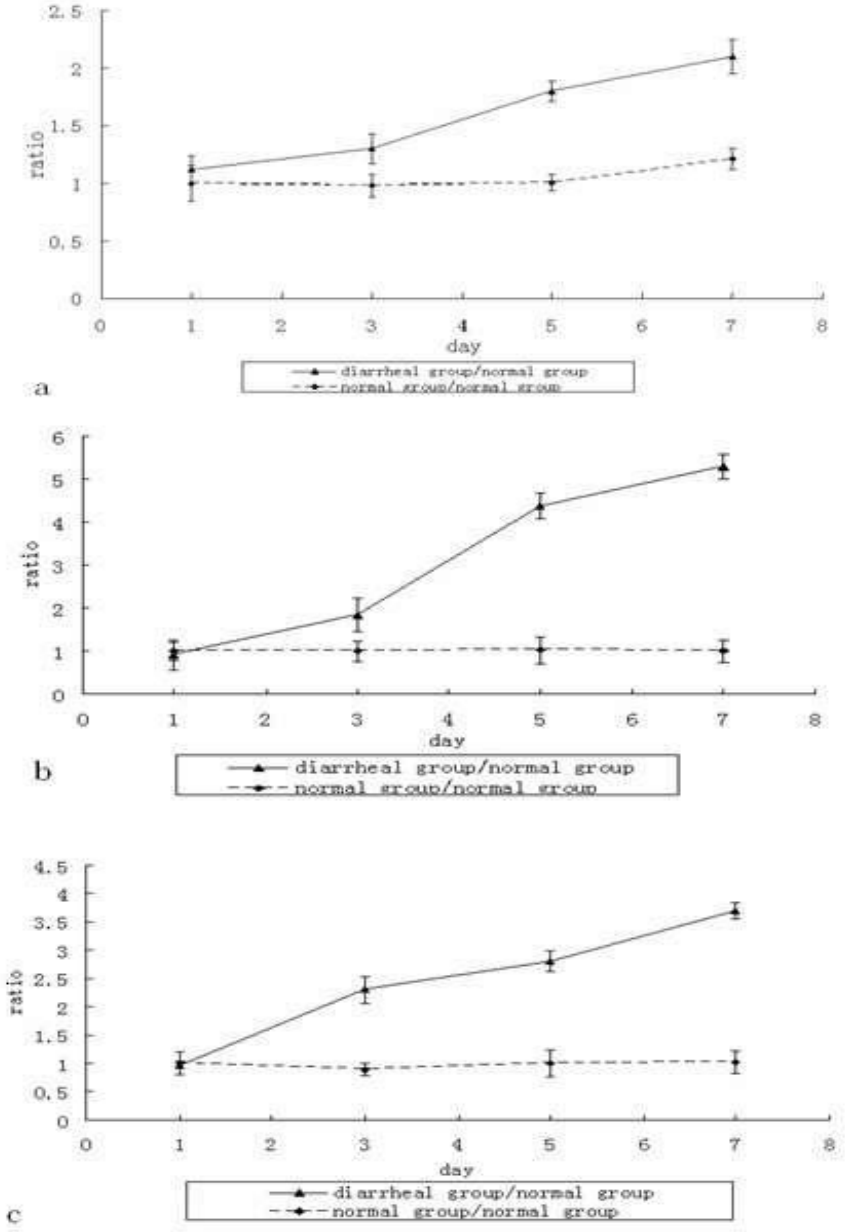

Figure 3. Expression of PAR-2 mRNA in the mucosa of the stomach (b), ileum (c), and colon (d). Data are displayed as the ratio of PAR-2 mRNA in diarrheal tissue versus that of normal tissue using RT-PCR at four time points over a 1-week period.

change in IL-8 expression was more pronounced to that of IL-6.

DISCUSSION

The present study demonstrates that PAR-2 is expressed abundantly in the piglet gastrointestinal tract mucosa, and reveals that both PAR-2 mRNA and protein expression increases with the severity of diarrhea. *In vitro* analysis using piglet intestinal epithelial cells demonstrates that activation of PAR-2 leads to the production of IL-6 and IL-8. The effects of PAR-2 agonists on the IEC were greatly enhanced by concomitant stimulation by LPS and LT. The mechanism of amplification of PAR-mediated IL-6 and IL-8 production when in the presence or absence of LPS and LT is currently unknown. At least two possible mechanisms could explain the observed LPS+LT-enhanced IEC activity through PAR-2. The first is that LPS +LT induce expression of PAR-2 which, in turn, is activated by the agonists. Second, the inflammatory activators prime PAR-2, causing amplification of

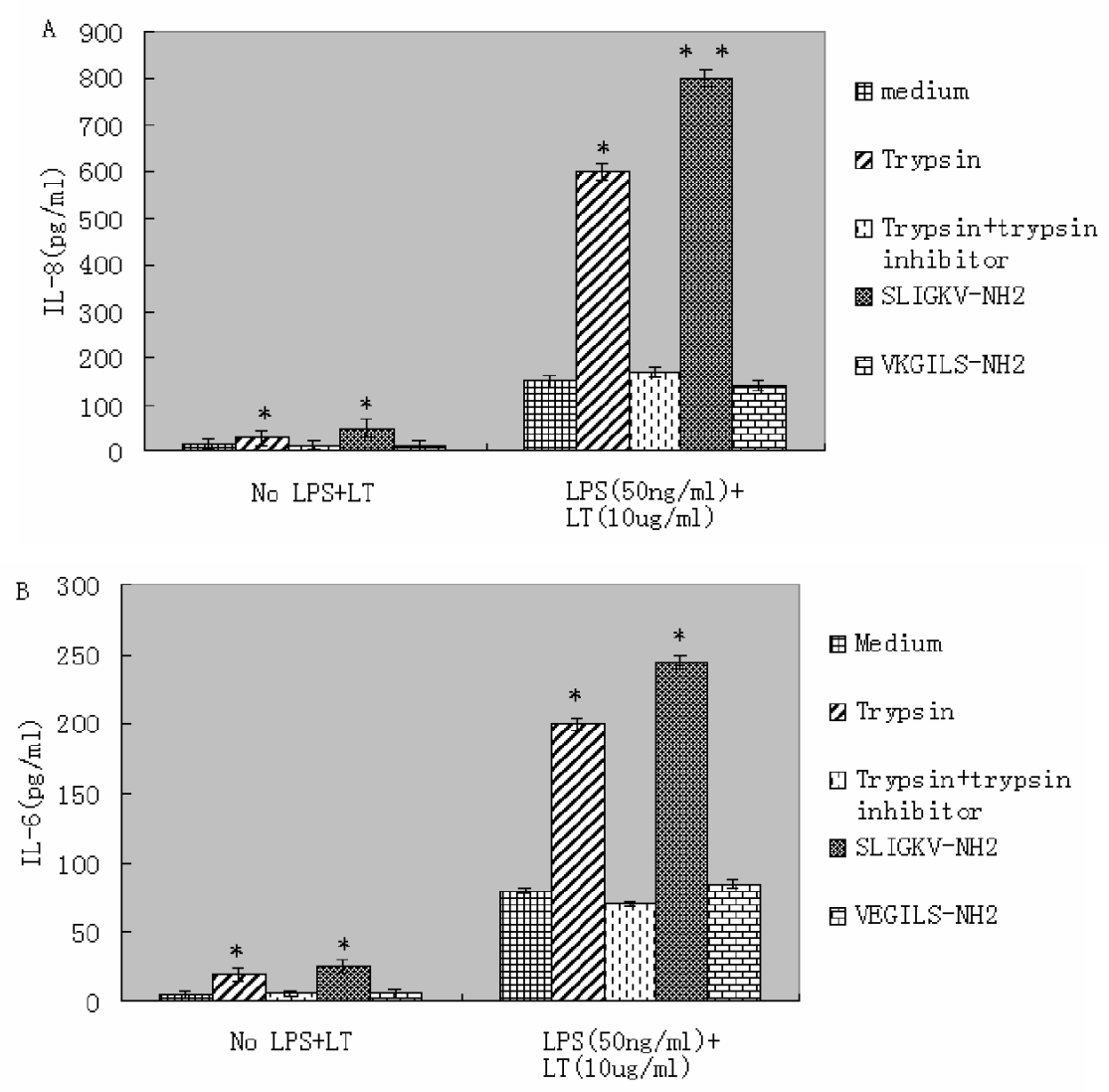

Figure 4. Specificity of PAR-2 agonist on intestinal epithelial cell activation. IEC monolayers (1×10^5/well) were incubated with an agonist for 24 h in the presence or absence of LPS+LT. After incubation, IL-8 and IL-6 expression in the culture medium was measured by ELISA. Values represent the mean ± SEM of quadruplicate determinations. Similar results were obtained in three replicate experiments (*P<0.05, **P<0.01).

independent or common signal transduction pathways leading to increased cytokine gene expression. One report supports the initial hypothesis that LPS independently induced the expression of PAR-2 on endothelial cells without affecting the expression of PAR-1 (Nystedt et al., 1996).

The pathogenic mechanism of ETEC works by invading the intestine of a susceptible host. The pathogens pilus combines with the enterocyte microvillus and is accepted onto the cell surface where it adheres to the enteric mucosa to boycott enterokinesia and stimulate intestinal secretion. ETEC amplifies rapidly within the intestine and releases enterotoxin. The enterotoxin is highly toxic and effective in destroying the regulatory system of gastrointestinal mucous, pathologically remodeling the electrolyte/water balance, severely compromising digestive function, as observed by the current study (Madec et al., 2000; Wellock et al., 2007). Taken together, our results suggest PAR-2 may be a regulatory factor in the pathogenesis of piglet diarrhea during weaning; one potential mechanism being the mediation of inflammatory factor production. PAR-2 is reported to be activated by multiple proteases such as trypsin, mast cell tryptase, and coagulation factors VIIa and Xa. These factors may be activated and/or accessible to mucosal tissues including chief cells and sensory neurons during inflammation or tissue injury (Camerer et al., 2000; Kawabata et al., 1999). Exogenous and endogenous PAR-2 activation has

been shown to significantly reduce rat intestinal mucosal damage, but did not influence leukocyte infiltrates induced by intestinal ischemia/reperfusion injury (Cattaruzza et al., 2006). With regard to the role of PAR-2 in piglet weaning diarrhea, we hypothesize that PAR-2 is activated by endogenous agonists and the production of inflammatory factors will increase further in the present of LPS and LT, mediating the pathologenic processes of piglet diarrhea during weaning. The physiological dual role of PAR-2 in the gastrointestinal tract mucosa remains a topic of debate, with application of a PAR-2 agonist shown to facilitate pepsin secretion, as well as produce dose-dependent cytoprotection at low doses (Kawao et al., 2002). The production of inflammatory factors is necessary for physiological function, and PAR-2 may have many physiological functions despite the patho-physiological mediation of diarrhea observed here; so we tentatively suggest PAR-2 functions as a double-edged sword in the gastrointestinal tract dependent upon the progressive state of diarrhea. For example, when activated PAR-2 initially promotes the release of mucus, providing protection for the mucosa; however, the mechanism regulating PAR-2 activity is not expounded in the present study.

To the authors knowledge, this study is the first to exhibit that both PAR-2 mRNA and protein expression are present throughout the gastrointestinal tract mucosa of weaning piglets. We found that PAR-2 mRNA expression was significantly elevated in the intestine of piglets with weaning diarrhea, and PAR-2 mRNA expression increased with the course of diarrheal development. Direct activation PAR-2 in pig intestinal epithelial cells leads to the production of IL-6 and IL-8, and both LPS and LT markedly enhanced PAR-induced activity. Taken together it is suggested that PAR-2 may be involved in the development of diarrhea during weaning in piglets. Future studies are required in which an exogenous or endo-genous agonist of PAR-2 will be administered into piglets with weaning diarrhea to further our knowledge of the disorder. The current study reveals a potentially novel therapeutic target in combating weaning piglet diarrhea.

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