Effect of Epidermal Growth Factor on Intestinal Sodium-Phosphate Transporter Protein IIb Expression in Lipopolysaccharide-Stimulated Weaned Piglets: Postprint

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Abstract

The present study was designed to investigate the effects of epidermal growth factor (EGF) on the expression of sodium-phosphate transport protein type IIb (NaPi- b) in the intestine of piglets under lipopolysaccharide (LPS)-stimulated stress conditions. The experiment consisted of two parts: 1) Cell experiment: Using porcine intestinal epithelial cells (IPEC-J2) as a model, the experiment was designed with four groups: control group (0 ng/mL EGF, 0 g/mL LPS), EGF group (100 ng/mL EGF, 0 g/mL LPS), LPS group (0 ng/mL EGF, 1.0 g/mL LPS), and EGF+LPS group (100 ng/mL EGF, 1.0 g/mL LPS), with three replicates per group; 2) Animal experiment: Twenty-four 21-day-old weaned castrated male "Landrace × Large White" crossbred piglets with similar body weight and good health status [average body weight (5.76±0.38 kg)] were selected and randomly divided into four groups: control group (basal diet), EGF group (basal diet + 2 mg/kg EGF), LPS group (basal diet + intraperitoneal injection of 100 g/kg BW LPS), and EGF+LPS group (basal diet + 2 mg/kg EGF + intraperitoneal injection of 100 g/kg BW LPS), with six replicates per group and one pig per replicate. The results showed that: 1) In the cell experiment, compared with the control group, NaPi- b mRNA and protein expression in IPEC-J2 cells in the EGF group decreased significantly ($P<0.05$), while NaPi- b mRNA and protein expression in the EGF+LPS group increased significantly ($P<0.05$). The interaction effect between EGF and LPS immune stress had a significant impact on NaPi- b mRNA and protein expression ($P<0.05$). 2) In the animal experiment, there was no significant difference in serum calcium (Ca) content among groups ($P>0.05$). Serum phosphorus (P) content in the LPS group was significantly higher than that in the control, EGF, and EGF+LPS groups ($P<0.05$). Serum alkaline phosphatase (ALP) activity

in the EGF group was significantly higher than that in the LPS group (P<0.05). The interaction effect between EGF and LPS immune stress had a significant impact on serum P content (P<0.05), but had no significant effect on serum Ca content and ALP activity (P>0.05). Compared with the control group, NaPi- b mRNA expression in the jejunum and ileum in the EGF group decreased significantly (P<0.05), while NaPi- b mRNA expression in the jejunum and ileum in the EGF+LPS group increased significantly (P<0.05). The interaction effect between EGF and LPS immune stress had a significant impact on NaPi- b mRNA expression in the jejunum and ileum (P<0.05). The results from both cell and animal experiments indicated that EGF exerted an inhibitory effect on NaPi- b expression, but could promote NaPi- b expression under immune stress conditions, suggesting that EGF can facilitate active intestinal phosphorus transport under stress conditions.

Full Text

Effects of Epidermal Growth Factor on Intestinal Type b Sodium-Phosphate Cotransporter Expression in Weaned Piglets Challenged with Lipopolysaccharide

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Abstract

This study investigated the effects of epidermal growth factor (EGF) on intestinal type b sodium-phosphate cotransporter (NaPi- b) expression in piglets under lipopolysaccharide (LPS)-induced stress conditions. The experiment comprised two parts: (1) a cell culture study using porcine intestinal epithelial cells (IPEC-J2) divided into four groups (n=3 replicates/group): control (0 ng/mL EGF, 0 g/mL LPS), EGF (100 ng/mL EGF, 0 g/mL LPS), LPS (0 ng/mL EGF, 1.0 g/mL LPS), and EGF+LPS (100 ng/mL EGF, 1.0 g/mL LPS); and (2) an animal trial with 24 healthy 21-day-old weaned castrated male piglets (Large White × Landrace) with an average body weight of (5.76±0.38) kg, randomly assigned to four groups (n=6 replicates/group, 1 piglet/replicate): control (basal diet), EGF (basal diet + 2 mg/kg EGF), LPS (basal diet + intraperitoneal injection of 100 g/kg BW LPS), and EGF+LPS (basal diet + 2 mg/kg EGF + intraperitoneal injection of 100 g/kg BW LPS).

In the cell experiment, EGF treatment significantly decreased NaPi- b mRNA and protein expression in IPEC-J2 cells compared with the control group (P<0.05), whereas the EGF+LPS group showed significantly increased expression (P<0.05). A significant interaction between EGF and LPS immunological

stress was observed for both mRNA and protein expression ($P<0.05$). In the animal trial, no significant differences in serum calcium (Ca) content were detected among groups ($P>0.05$). However, serum phosphorus (P) content in the LPS group was significantly higher than in the control, EGF, and EGF+LPS groups ($P<0.05$), and serum alkaline phosphatase (ALP) activity in the EGF group was significantly higher than in the LPS group ($P<0.05$). A significant EGF × LPS interaction was observed for serum P content ($P<0.05$) but not for Ca content or ALP activity ($P>0.05$).

Compared with the control group, EGF supplementation significantly reduced NaPi- b mRNA expression in the jejunum and ileum ($P<0.05$), while the EGF+LPS treatment significantly increased expression in both segments ($P<0.05$). The EGF × LPS interaction effect on jejunal and ileal NaPi- b mRNA expression was significant ($P<0.05$). These findings demonstrate that while EGF generally suppresses NaPi- b expression, it promotes NaPi- b expression under immunological stress conditions, suggesting that EGF enhances intestinal P active transport during stress states.

Keywords: epidermal growth factor; phosphorus; NaPi- b; lipopolysaccharide; porcine intestinal epithelial cells; weaned piglets

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Introduction

Phosphorus (P) is an essential component of biological systems involved in numerous physiological processes, including energy metabolism, cell signaling, nucleotide and phospholipid biosynthesis, and formation of teeth and bones. Previous studies have confirmed that the type b sodium-phosphate cotransporter (NaPi- b) represents the primary pathway mediating intestinal P active transport. NaPi- b expression is regulated by multiple factors, with epidermal growth factor (EGF) being one of the key regulators. EGF is a crucial growth factor widely distributed in bodily fluids including milk, saliva, urine, intestinal fluid, blood, and amniotic fluid, playing vital roles in cell survival, proliferation, differentiation, migration, and apoptosis. Research in human Caco-2 cells and porcine intestinal epithelial cells (IPEC-J2) has demonstrated that EGF inhibits NaPi- b expression, suggesting that under normal culture conditions, EGF may regulate cellular P absorption through alternative pathways. Theoretically, the EGF-mediated repair of intestinal barrier function necessarily involves extensive synthesis of DNA, RNA, and proteins, which requires increased intestinal P absorption. However, the mechanism by which this additional P demand is met remains unreported. Therefore, this study employed both cell culture and animal experiments to investigate the effects of EGF on small intestinal P absorption in weaned piglets under LPS-induced stress conditions, providing novel insights into EGF' s role in P absorption.

Materials and Methods

1.1.1 Reagents EGF was purchased from Peprotech. LPS, Tris, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), Tween-20, acrylamide, bis-acrylamide, and Ponceau S were obtained from Sigma. Fetal bovine serum (FBS), trypsin, and penicillin-streptomycin were from GIBCO. DMEM/F12 medium was from HyClone (GE). CCK-8 kit, BCA protein assay kit, phosphate-buffered saline (PBS), and RIPA lysis buffer were from Solarbio. TRIzol reagent was from Invitrogen. Reverse transcription kit, UltraSYBR Mixture, and DM 2000 Plus DNA Marker were from Beijing Kangwei Century. Super ECL Plus substrate was from Thermo. Primary antibodies against NaPi- b (Cat#: 21773-1-AP) and -actin (Cat#: 60008-1-Ig), and secondary antibody (Goat Anti-Rabbit IgG/HRP) were from Proteintech.

1.1.2 Cell Culture and Experimental Design IPEC-J2 cells were provided by the Institute of Subtropical Agriculture, Chinese Academy of Sciences. Cells were maintained in medium containing 10% FBS and 1% penicillin-streptomycin at 37°C in a 5% CO incubator. Upon reaching 80-90% confluence, cells were digested with 0.25% trypsin, counted under a microscope, and collected for subculture or subsequent experiments. Four treatment groups were established: control (0 ng/mL EGF, 0 g/mL LPS), EGF (100 ng/mL EGF, 0 g/mL LPS), LPS (0 ng/mL EGF, 1.0 g/mL LPS), and EGF+LPS (100 ng/mL EGF, 1.0 g/mL LPS), with three replicates per group. EGF and LPS doses and incubation time were selected based on reference [11]. Cells were seeded at 1×10 cells/well in 6-well plates with 2 mL of complete medium. After 24 h, the medium was removed, cells were washed twice with 37°C PBS, and treatments were added with fresh medium to a final volume of 2 mL for 24 h.

1.1.3 Quantification of NaPi- b mRNA Expression Total RNA was extracted using TRIzol reagent following the manufacturer' s protocol. RNA concentration was measured and integrity was verified by 1% agarose gel electrophoresis. Total RNA was reverse transcribed to cDNA using a reverse transcription kit. Quantitative PCR was performed using a fluorescent qPCR kit. Primers were synthesized by Shanghai Sangon Biotech: NaPi- b forward 5' -GCCCGAGCTTAAGAACACA-3' , reverse 5' -CATGACACCAGCACCATCGTT-3' ; -actin forward 5' -CATCCTGCGTCTGGACCTGG-3', reverse 5'-TAATGTCACGCACGATTTCC-3' . The qPCR protocol followed methods described by Tang [11]. Relative gene expression was calculated using the 2^(-ΔΔCt) method with -actin as the internal reference.

1.1.4 Western Blot Analysis of NaPi- b Protein Expression After treatment, cells were washed once with PBS and lysed on ice for 15 min with 250 L RIPA buffer containing 1 mmol/L protease inhibitor. Lysates were centrifuged at 12,000 rpm for 3-5 min at 4°C, and supernatants were aliquoted and stored at -80°C. Protein concentration was determined using the BCA protein assay

kit (Nanjian Jiancheng) according to the manufacturer' s instructions. Western blot procedures followed methods described by Tang et al. [11].

1.2 Animal Experiment

1.2.1 Experimental Materials EGF was provided by a Changsha company (4,000 mg/kg content). LPS (E. coli serotype O55:B5), paraffin, neutral balsam, and eosin were from Sigma. RT-PCR reagents were identical to those described in section 1.1.3.

1.2.2 Animals and Experimental Design Twenty-four healthy 21-day-old weaned castrated male piglets (Large White × Landrace) with an average body weight of (5.76±0.38) kg were randomly allocated to four groups (n=6 replicates/group, 1 piglet/replicate): control (basal diet), EGF (basal diet + 2 mg/kg EGF), LPS (basal diet + intraperitoneal injection of 100 g/kg BW LPS), and EGF+LPS (basal diet + 2 mg/kg EGF + intraperitoneal injection of 100 g/kg BW LPS). The 14-day trial utilized a basal diet formulated according to NRC (2012) nutrient requirements, with composition and nutrient levels shown in Table 1 . On days 8 and 15, LPS and EGF+LPS groups received LPS injections (100 g/kg BW), while control and EGF groups received equivalent saline injections [12].

1.2.3 Feeding Management The trial was conducted from November 12-29, 2017, at Yiyang Zhaofeng Agriculture and Technology Co., Ltd. Piglets were housed individually with ad libitum access to feed and water. Feed was provided at 08:00, 12:00, 16:00, and 20:00 daily, with orts collected before the next feeding. Ventilation was provided for approximately 15 min at noon daily. Ambient temperature was maintained at 25-28°C with 50-70% relative humidity.

1.2.4 Sample Collection Blood samples (10 mL) were collected from the anterior vena cava 6 h after the final LPS injection on day 15. Serum was harvested after centrifugation at 3,500 r/min for 10 min at 4°C and stored at -20°C. For intestinal mucosa samples, piglets were euthanized 6 h post-injection with 50 mg/kg BW sodium pentobarbital. The abdominal cavity was opened, and approximately 5 cm segments of jejunum and ileum were excised, gently rinsed with ice-cold 1×PBS, and mucosa was scraped on ice, aliquoted into two tubes, snap-frozen in liquid nitrogen, and stored at -80°C.

1.2.5 Serum Calcium and Phosphorus Analysis Serum calcium was measured using a calcium assay kit (microplate method, Cat# C004-2) and serum phosphorus using a phosphorus assay kit (phosphomolybdate method, Cat# C006) from Nanjian Jiancheng Bioengineering Institute, following manufacturer protocols.

1.2.6 Serum Alkaline Phosphatase Activity Serum alkaline phosphatase (ALP) activity was determined using a Mindray BS-200 automatic biochemical analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd.) with reagents purchased from Mindray, following the manufacturer' s instructions.

1.2.7 Intestinal Mucosa NaPi- b mRNA Expression NaPi- b mRNA expression in jejunal and ileal mucosa was quantified by qRT-PCR as described in section 1.1.3.

1.3 Statistical Analysis Data are presented as means ± SD. Two-way ANOVA was performed using SPSS 21.0 GLM procedure with EGF treatment, LPS treatment, and their interaction as main effects. One-way ANOVA with Duncan' s multiple comparison test was used for pairwise comparisons. Significance was declared at $P<0.05$.

Results

2.1 Effects of EGF on NaPi- b mRNA Expression in LPS-Challenged IPEC-J2 Cells As shown in Figure 1 [Figure 1: see original paper], NaPi- b mRNA expression was significantly decreased in the EGF group compared with the control group ($P<0.05$) but significantly increased in the EGF+LPS group ($P<0.05$). The EGF+LPS group also showed significantly higher expression than both the EGF and LPS groups ($P<0.05$). A significant interaction between EGF and LPS immunological stress was observed ($P<0.05$). These results indicate that EGF suppresses NaPi- b mRNA expression under normal conditions but promotes its expression under stress conditions.

Note: Values with different letters differ significantly ($P<0.05$). The same applies to Figures 2 [Figure 2: see original paper] and 3 [Figure 3: see original paper].

2.2 Effects of EGF on NaPi- b Protein Expression in LPS-Challenged IPEC-J2 Cells Western blot analysis revealed that NaPi- b protein expression was significantly reduced in the EGF group compared with the control group ($P<0.05$) but markedly elevated in the EGF+LPS group ($P<0.05$) (Figure 2 [Figure 2: see original paper]). The EGF+LPS group exhibited significantly higher protein levels than both the EGF and LPS groups ($P<0.05$). The EGF × LPS interaction effect was significant ($P<0.05$). These findings confirm that EGF inhibits NaPi- b protein expression under basal conditions but enhances it under immunological stress.

A: Western blot image; B: NaPi- b protein expression level.

2.3 Effects of EGF on Serum Calcium, Phosphorus, and Alkaline Phosphatase in LPS-Challenged Piglets Table 2 shows that serum cal-

cium content did not differ among groups (P>0.05) and was not affected by the EGF × LPS interaction (P>0.05). Serum phosphorus content in the LPS group was significantly higher than in all other groups (P<0.05), with a significant EGF × LPS interaction (P<0.05). Serum ALP activity was significantly higher in the EGF group than in the LPS group (P<0.05) but did not differ from control or EGF+LPS groups (P>0.05), and the interaction effect was not significant (P>0.05).

Values with different superscripts within a row differ significantly (P<0.05).

2.4 Effects of EGF on NaPi- b mRNA Expression in Small Intestinal Mucosa of LPS-Challenged Piglets As illustrated in Figure 3 [Figure 3: see original paper], EGF supplementation significantly decreased NaPi- b mRNA expression in both jejunum (Figure 3A) and ileum (Figure 3B) compared with the control group (P<0.05). Conversely, the EGF+LPS treatment significantly increased expression in both intestinal segments (P<0.05). Compared with the LPS group, the EGF group showed significantly reduced expression while the EGF+LPS group showed significantly increased expression in both jejunum and ileum (P<0.05). The EGF × LPS interaction effect was significant for both segments (P<0.05). These animal trial results align with cell culture findings, demonstrating that EGF suppresses NaPi- b mRNA expression under normal conditions but promotes expression during immunological stress.

A: Relative NaPi- b mRNA expression in jejunum; B: Relative NaPi- b mRNA expression in ileum.

Discussion

Phosphorus is an essential mineral element critical for animal growth, bone formation, energy metabolism, nucleic acid synthesis, cell signal transduction, and blood acid-base balance. Serum alkaline phosphase serves as a biochemical indicator of calcium-phosphorus metabolism, with increased release occurring during Ca/P deficiency. The current study demonstrated that EGF did not significantly affect serum calcium content in LPS-challenged piglets but significantly influenced serum phosphorus levels. The elevated ALP activity in the EGF group compared with the LPS group, combined with the lack of significant interaction effect on ALP activity, suggests distinct regulatory mechanisms. The significantly higher serum phosphorus in the LPS group likely reflects LPS-induced kidney injury, as LPS is a major toxin released from Gram-negative bacterial cell walls that can cause acute renal damage and impair phosphorus reabsorption, leading to abnormal serum phosphorus elevation.

Intestinal phosphorus absorption occurs via passive diffusion and active transport, with NaPi- b serving as the primary carrier for active transport, mediating 70-90% of active phosphorus transport. NaPi- b expression is regulated by multiple factors including phosphorus status, vitamin D3, estradiol, neuropeptide

Y, calcitonin gene-related peptide, and EGF. EGF, a 53-amino-acid peptide, exerts its biological functions by binding to the epidermal growth factor receptor (EGFR), which is abundantly expressed on both apical and basolateral surfaces of intestinal epithelial cells. EGF-EGFR binding induces dimerization, activates receptor tyrosine kinase (RTK) activity, and triggers downstream signaling pathways including Ras/MAPK, PI3K/AKT, and PLC- /PKC, thereby influencing cell survival, proliferation, differentiation, migration, and apoptosis.

Previous studies in human Caco-2 cells demonstrated that EGF suppresses NaPi- b transcriptional activity by modifying c-myb protein through PKC/PKA and MAPK signaling pathways. Our research group similarly observed EGF inhibition of NaPi- b expression in IPEC-J2 cells, identifying the responsive element at the -1,092 to -1,085 bp region (5' -TCCAGTTG-3') of the NaPi- b promoter. We further demonstrated that EGF downregulates NaPi- b expression through activation of EGFR, PKA, PKC, P38, ERK, and JNK signaling molecules. Interestingly, Tang et al. reported that EGF promotes IPEC-J2 cell proliferation, a process requiring substantial phosphorus for RNA and DNA synthesis, suggesting that EGF enhances phosphorus absorption through pathways other than NaPi- b-mediated active transport.

Stress is ubiquitous in animal production, causing intestinal damage and impairing performance. LPS exerts toxic effects on intestinal epithelial cells, stimulating secretion of pro-inflammatory cytokines including IL-1 , IL-6, and TNF- , ultimately triggering inflammation. Tang et al. demonstrated that LPS induces oxidative stress and apoptosis in IPEC-J2 cells, while EGF protects against LPS-induced injury by alleviating oxidative stress and reducing apoptosis. Theoretically, EGF-mediated repair of damaged intestinal cells requires extensive DNA, RNA, and protein synthesis, necessitating increased intestinal phosphorus absorption. Whether EGF relieves its inhibitory effect on NaPi- b expression to promote active phosphorus transport during this process remained unknown.

Our combined cell and animal experiments revealed that EGF suppresses NaPi- b mRNA and protein expression in IPEC-J2 cells and reduces NaPi- b mRNA expression in jejunal and ileal mucosa under basal conditions, consistent with previous reports. However, under LPS-induced stress, EGF enhanced NaPi- b mRNA and protein expression in IPEC-J2 cells and increased NaPi- b mRNA expression in intestinal mucosa. These findings indicate that while EGF inhibits NaPi- b-mediated active phosphorus transport under normal conditions, it can relieve this inhibition during stress states, thereby regulating NaPi- b-mediated active phosphorus absorption to meet physiological demands and accelerate intestinal repair. Previous studies showing EGF' s growth-promoting effects in weaned piglets further support its role in enhancing phosphorus absorption through alternative pathways.

Systemic phosphorus homeostasis is maintained through intestinal absorption and renal reabsorption. Besides NaPi- b-mediated active transport, type sodium-dependent transporters (PiT1 and PiT2) also mediate partial active phosphorus absorption. Renal reabsorption is primarily mediated by NaPi- a

and NaPi- c proteins, with enhanced renal reabsorption compensating for insufficient intestinal absorption. Therefore, EGF may enhance phosphorus absorption through increased passive diffusion, upregulation of PiT1/PiT2-mediated active transport, or enhanced renal reabsorption. The precise mechanisms underlying EGF's relief of NaPi- b inhibition under stress conditions require further investigation.

Conclusion

EGF inhibits intestinal NaPi- b expression under normal conditions but promotes NaPi- b expression during immunological stress. The specific regulatory mechanisms warrant further investigation.

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