Cellular Signaling Pathways Regulating Sodium-Dependent Phosphate Transporter Type IIb Expression by Epidermal Growth Factor in Porcine Intestinal Epithelial Cells: A Post-Print Study

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Abstract

This study aimed to investigate the molecular mechanism by which epidermal growth factor (EGF) regulates the expression of sodium-dependent phosphate transporter type IIb (NaPi-IIb) in porcine small intestinal epithelial cell line IPEC-J2. IPEC-J2 cells were co-treated with EGF and various inhibitors including EGF receptor tyrosine kinase inhibitor (tyrphostin AG 1478), protein kinase A (PKA) inhibitor (H89), protein kinase C (PKC) inhibitor (k4393), p38 inhibitor (SB 203580), extracellular signal-regulated kinase (ERK) inhibitor (PD98059), and c-Jun N-terminal kinase (JNK) inhibitor (anisomycin). Western blot analysis was employed to detect the expression levels of relevant pathway proteins and the target protein (NaPi-IIb). The results showed that compared with the control group, EGF treatment significantly decreased NaPi-IIb expression level ($P<0.05$); compared with the inhibitor-untreated group, treatment of IPEC-J2 cells with specific inhibitors of EGF receptor, PKA, PKC, mitogen-activated protein kinase (MAPK)/p38, MAPK/ERK1/2, and MAPK/JNK significantly increased NaPi-IIb expression level ($P<0.05$). Notably, addition of the MAPK/ERK1/2-specific inhibitor significantly reduced the phosphorylation level of MAPK/ERK1/2 at the Tyr204 site ($P<0.05$), while addition of the MAPK/JNK-specific inhibitor significantly reduced the phosphorylation level of MAPK/JNK1/2/3 at Thr183 and Tyr185 sites ($P<0.05$), indicating that the inhibitory effects of these two groups of inhibitors on their respective pathways were achieved by reducing phosphorylation levels at the aforementioned sites. The results of this study demonstrate that EGF receptor, PKA, PKC, p38, ERK, and JNK all mediate EGF regulation of NaPi-IIb expression in IPEC-J2 cells.

Full Text

Identifying Signaling Pathways that Mediate Epidermal Growth Factor Regulation of Sodium-Dependent Phosphate Cotransporter Type IIb Expression in Porcine Intestinal Epithelial Cells

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Abstract

This study investigated the molecular mechanisms through which epidermal growth factor (EGF) regulates the expression of the sodium-dependent phosphate cotransporter type IIb (NaPi-IIb) in porcine intestinal epithelial cells (IPEC-J2). IPEC-J2 cells were treated with EGF in combination with specific inhibitors of the EGF receptor tyrosine kinase (tyrphostin AG 1478), protein kinase A (PKA) (H89), protein kinase C (PKC) (k4393), p38 (SB 203580), extracellular signal-regulated kinase (ERK) (PD98059), and c-Jun N-terminal kinase (JNK) (anisomycin). Western blot analysis was used to detect the expression levels of proteins in the relevant signaling pathways and the target protein NaPi-IIb.

The results demonstrated that EGF treatment significantly reduced NaPi-IIb expression levels compared to the control group ($P<0.05$). In contrast, specific inhibitors of the EGF receptor, PKA, PKC, mitogen-activated protein kinase (MAPK)/p38, MAPK/ERK1/2, and MAPK/JNK significantly increased NaPi-IIb expression levels compared to the EGF-only treatment group ($P<0.05$). Notably, the MAPK/ERK1/2 inhibitor significantly decreased the phosphorylation level of MAPK/ERK1/2 at the Tyr204 site ($P<0.05$), while the MAPK/JNK inhibitor significantly reduced the phosphorylation level of MAPK/JNK1/2/3 at the Thr183 and Tyr185 sites ($P<0.05$). These findings indicate that the inhibitory effects of these two inhibitors on their respective pathways were achieved by reducing phosphorylation at these specific sites. In summary, the EGF receptor, PKA, PKC, p38, ERK, and JNK all mediate the regulation of NaPi-IIb expression by EGF in IPEC-J2 cells.

Keywords: epidermal growth factor; phosphate; NaPi-IIb; signal pathway; porcine intestinal epithelial cells

Introduction

Phosphorus is an essential mineral element in animals that plays crucial roles in growth and development, bone formation, energy metabolism, nucleic acid synthesis, cell signal transduction, and maintenance of blood acid-base balance [1-2]. Transcellular active phosphate absorption mediated by the sodium-dependent phosphate cotransporter type IIb (NaPi-IIb) represents the primary mechanism of intestinal phosphate absorption [3]. The expression of intestinal NaPi-IIb is regulated by numerous factors, with epidermal growth factor (EGF) being one of the important regulators [4-6]. EGF is a growth-promoting factor secreted by the lactating mammary gland, submandibular gland, kidney, duodenal Brunner' s glands, pancreas, and placenta. It possesses multiple biological functions including promoting cell growth, migration, proliferation, wound healing, bone healing, and nutrient transport [2,7-8]. EGF regulates various physiological functions in cells by binding to EGF receptors on the cell surface [8]. Upon binding, EGF induces autophosphorylation of tyrosine residues on the EGF receptor, which provides docking sites for various signaling molecules and subsequently regulates the expression of target genes mediated by intracellular signaling molecules such as protein kinase A (PKA), protein kinase C (PKC), mitogen-activated protein kinase (MAPK)/p38, MAPK/extracellular regulated protein kinases (ERK) 1/2, and MAPK/c-Jun N-terminal kinase (JNK) [9].

Although Xu et al. [4-5] reported that EGF inhibits the transcriptional activity of NaPi-IIb in human intestinal Caco-2 cells by modifying the affinity of c-Myb protein for the NaPi-IIb gene and regulating downstream promoter function through PKC/PKA and MAPK signaling pathways, and our previous research also demonstrated that EGF inhibits NaPi-IIb gene expression in porcine intestinal epithelial cells and identified the EGF-responsive element on the NaPi-IIb gene promoter (the c-Myb binding site at -1,092 to -1,085 bp, 5' -TCCAGTTG-3') [6], the specific signaling pathways through which EGF regulates NaPi-IIb expression in porcine intestinal epithelial cells remain unclear. Therefore, this study employed specific inhibitors of key signaling molecules, including the EGF receptor tyrosine kinase inhibitor (tyrphostin AG 1478), PKA inhibitor (H89), PKC inhibitor (k4393), p38 inhibitor (SB 203580), ERK inhibitor (PD98059), and JNK inhibitor (anisomycin), in combination with EGF treatment of porcine intestinal epithelial cells IPEC-J2 to examine NaPi-IIb expression at the protein level and elucidate the precise pathways mediating EGF regulation of NaPi-IIb expression.

Materials and Methods

1.1 Reagents and Antibodies

Porcine EGF was purchased from Wuhan Sanying. DMEM/F12 medium and sodium dodecyl sulfate (SDS) were obtained from Sigma (USA). 0.25% trypsin-EDTA was from Invitrogen (USA). 30% acrylamide solution was from

Nanjing Jiancheng. Fetal bovine serum, calf serum, tetramethylethylenediamine (TEMED), developer powder, fixer powder, SDS-polyacrylamide gel electrophoresis (PAGE) gel preparation kit were from Changsha Aijia Biological Company. Penicillin-streptomycin was from Huazhong Agricultural University Veterinary Pharmaceutical Factory. Skim milk powder was from Yili. Prestained protein marker was from Fermentas (Canada). Protein marker and BCA protein concentration assay kit were from Beyotime Biotechnology. Ammonium persulfate was from Shanghai Sangon. Nitrocellulose (NC) membrane was from Millipore (USA). Filter paper was from Whatman (USA). Film was from Kodak (USA). Enhanced chemiluminescence (ECL) kit was from Pierce (USA).

The following inhibitors were used: EGF receptor tyrosine kinase inhibitor (T4182-5MG, Sigma, USA), PKA inhibitor (S1643, Beyotime), PKC inhibitor (K4393-10UG, Sigma, USA), p38 inhibitor (SML0543-25MG, Sigma, USA), ERK inhibitor (SCP0214, Sigma, USA), and JNK inhibitor (S5567-10MG, Sigma, USA). Primary antibodies included: NaPi-IIb (21295-1-AP, Proteintech, USA), PKA catalytic subunit (ab187515, Abcam, UK), PKA regulatory subunit I (ab94613, Abcam, UK), PKA / / (ab211265, Abcam, UK), PKC (ab32376, Abcam, UK), PKC (ab4132, Abcam, UK), PKC (ab71558, Abcam, UK), phospho-p38 MAPK (Tyr323) (bs-5477R, Bioss, USA), phospho-p38 MAPK (Thr180) (bs-5476R, Bioss, USA), phospho-ERK1/2 (Thr202+Tyr204) (bs-3016R, Bioss, USA), phospho-ERK1 (Thr197+Thr202) (bs-3292R, Bioss, USA), JNK1+JNK2+JNK3 (bs-2592R, Bioss, USA), phospho-JNK1+JNK2+JNK3 (Thr183+Tyr185) (bs-1640R, Bioss, USA), and GAPDH (ab8245, Abcam, UK). Secondary antibodies included HRP-labeled antibodies from Santa Cruz Biotechnology (USA), anti-mouse IgG-HRP (1:1,000) from Beijing Solarbio Biological Company, and anti-rabbit IgG-HRP (1:500) from Beijing Solarbio Biological Company.

1.2 Cell Culture and Treatment

1.2.1 Cell Culture Porcine intestinal epithelial cells IPEC-J2 were kindly provided by the Institute of Subtropical Agriculture, Chinese Academy of Sciences. IPEC-J2 cells were cultured in DMEM/F12 complete medium (containing 10% fetal bovine serum and 1% penicillin/streptomycin) with medium changed every 24 hours. Cells were passaged with 0.25% trypsin-EDTA when they reached 80% confluence and maintained in a cell culture incubator at 37°C with 5% CO2.

1.2.2 Effect of EGF Receptor Inhibitor on NaPi-IIb Expression Logarithmic growth phase cells were digested with 0.25% trypsin-EDTA and seeded into 6-well plates (1×10^5 cells/well) in DMEM/F12 complete medium. When cells reached 80% confluence under microscopic observation, the medium was replaced with DMEM/F12 incomplete medium (serum-free, hormone-free, containing penicillin/streptomycin) for 16 hours of transition culture. The experi-

mental group was then treated with 100 ng/mL EGF [6] plus 0, 1, 2, 5, 10, or 20 mol/L EGF receptor inhibitor, while the control group received the same volume of medium without EGF or inhibitor. Cells were cultured for an additional 8 hours, with six replicates per treatment. After incubation, cells were collected for Western blot analysis.

1.2.3 Effect of PKA, PKC, ERK, JNK, and p38 Inhibitors on NaPi-IIb Expression Logarithmic growth phase cells were digested with 0.25% trypsin-EDTA and seeded into 6-well plates (1×10^5 cells/well) in DMEM/F12 complete medium. When cells reached 80% confluence, the medium was replaced with DMEM/F12 incomplete medium for 16 hours. The control group was treated with 100 ng/mL EGF [6], while experimental groups received 100 ng/mL EGF plus PKA, PKC, p38, ERK, or JNK inhibitors. Cells were cultured for 8 hours with six replicates per treatment, after which cells were collected for Western blot analysis.

1.3 Western Blot Analysis

1.3.1 Cell Protein Sample Preparation After culture, the medium was removed and cells were washed 2-3 times with pre-warmed phosphate-buffered saline (PBS) at 37°C. An appropriate amount of ice-cold protein lysis buffer was added and cells were incubated on ice for 10-20 minutes. Cells were scraped off, collected in EP tubes, and sonicated (100-200 W) for 3 seconds. Samples were centrifuged at 12,000×g for 5 minutes. Protein concentrations were determined using the BCA protein assay kit. All protein samples were adjusted to equal concentrations, mixed thoroughly, and either loaded directly with sample buffer or stored at -70°C in 1× sample buffer.

1.3.2 Western Blot Detection Western blot procedures were performed as described by Xiong et al. [10]. Based on the molecular weight of target proteins, 8%, 10%, 12%, and 15% separating gels and 5% stacking gels were used for protein separation. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes at constant current for 90 minutes at room temperature. Membranes were blocked with 5% skim milk in PBST buffer for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies: NaPi-IIb (1:500), PKA catalytic subunit (1:500), PKA regulatory subunit I (1:1,000), PKA / / (1:500), PKC (1:1,000), PKC (1:1,000), PKC (1:1,000), phospho-p38 MAPK (Tyr323) (1:500), phospho-p38 MAPK (Thr180) (1:500), phospho-ERK1/2 (Thr202+Tyr204) (1:500), phospho-ERK1 (Thr197+Thr202) (1:500), JNK1+JNK2+JNK3 (1:500), phospho-JNK1+JNK2+JNK3 (Thr183+Tyr185) (1:500), and GAPDH (1:800). After washing three times with PBST, membranes were incubated with anti-mouse IgG-HRP (1:8,000) or anti-rabbit IgG-HRP (1:4,000) for 1 hour at room temperature. Following secondary antibody incubation and three PBST washes, proteins were visualized using the HRP-ECL method. ECL reagents A and B were mixed according to the manufacturer' s instructions, applied to PVDF membranes, and scanned using a Western blot

imaging system. Results were analyzed using Image J gel analysis software with GAPDH as the internal reference.

1.4 Statistical Analysis

The EGF receptor inhibitor experiment was analyzed using one-way ANOVA with SAS 9.2 software, followed by Duncan' s multiple comparison test. Experiments with PKA, PKC, p38, ERK, and JNK inhibitors were analyzed using t-tests with SAS 9.2 software. Data are presented as means ± standard error. $P<0.05$ was considered statistically significant, and $P<0.01$ was considered highly significant.

Results and Analysis

2.1 Effect of EGF Receptor Tyrosine Kinase Inhibitor on NaPi-IIb Expression Levels

To confirm that the EGF receptor mediates EGF regulation of NaPi-IIb expression, we treated IPEC-J2 cells with 100 ng/mL EGF in combination with varying concentrations of EGF receptor inhibitor (0-20 mol/L). Western blot analysis revealed that NaPi-IIb expression levels increased progressively with increasing concentrations of the EGF receptor inhibitor [Figure 1: see original paper]. Compared to the control group, EGF treatment without inhibitor significantly reduced NaPi-IIb expression in IPEC-J2 cells ($P<0.05$). However, compared to EGF treatment alone, addition of 5, 10, or 20 mol/L EGF receptor inhibitor significantly increased NaPi-IIb expression ($P<0.05$), while 1 or 2 mol/L had no significant effect ($P>0.05$). These results demonstrate that the EGF receptor mediates EGF regulation of NaPi-IIb expression levels.

In the column diagrams, * indicates significant difference compared to the control group ($P<0.05$), # indicates significant difference compared to the EGF treatment group ($P<0.05$), and ## indicates highly significant difference compared to the EGF treatment group ($P<0.01$). The same notation applies to all subsequent figures.

2.2 Effect of PKA Signaling Pathway on EGF Regulation of NaPi-IIb Expression

To determine whether PKA mediates EGF regulation of NaPi-IIb expression, we treated IPEC-J2 cells with 100 ng/mL EGF plus a specific PKA inhibitor. Western blot analysis showed that the PKA inhibitor did not alter the expression levels of PKA catalytic subunit , PKA regulatory subunit I , or PKA / / [Figure 2: see original paper], indicating that PKA inhibition was not achieved by affecting PKA expression but through other mechanisms. The addition of PKA inhibitor significantly increased NaPi-IIb expression in IPEC-J2 cells ($P<0.05$),

demonstrating that PKA mediates EGF regulation of NaPi-IIb expression levels.

2.3 Effect of PKC Signaling Pathway on EGF Regulation of NaPi-IIb Expression

As shown in [Figure 3: see original paper], the specific PKC inhibitor did not change the expression levels of PKC , PKC , or PKC , indicating that PKC inhibition was not mediated through effects on PKC expression. However, the PKC inhibitor highly significantly blocked EGF-induced downregulation of NaPi-IIb expression ($P<0.01$), establishing PKC as a key signaling molecule in EGF regulation of NaPi-IIb expression levels.

2.4 Effect of MAPK/p38 Signaling Pathway on EGF Regulation of NaPi-IIb Expression

As shown in [Figure 4: see original paper], the specific MAPK/p38 inhibitor did not alter the phosphorylation levels of MAPK/p38 at Tyr323 and Thr180 sites, indicating that MAPK/p38 inhibition was not achieved by affecting its expression or phosphorylation status. Nevertheless, the MAPK/p38 inhibitor significantly blocked EGF-induced downregulation of NaPi-IIb expression ($P<0.05$), demonstrating that the MAPK/p38 signaling pathway participates in EGF regulation of NaPi-IIb expression levels.

2.5 Effect of MAPK/ERK1/2 Signaling Pathway on EGF Regulation of NaPi-IIb Expression

As shown in [Figure 5: see original paper], the specific MAPK/ERK1/2 inhibitor did not change the phosphorylation level of ERK1 at Thr197 and Thr202 sites but significantly reduced the phosphorylation level of MAPK/ERK1/2 at the Tyr204 site ($P<0.05$). This indicates that MAPK/ERK1/2 inhibition primarily occurs through reduced phosphorylation at the Tyr204 site. The addition of MAPK/ERK1/2 inhibitor significantly blocked EGF-induced downregulation of NaPi-IIb expression ($P<0.05$), confirming that MAPK/ERK1/2 signaling molecules participate in EGF regulation of NaPi-IIb expression levels.

2.6 Effect of MAPK/JNK Signaling Pathway on EGF Regulation of NaPi-IIb Expression

As shown in [Figure 6: see original paper], the specific MAPK/JNK inhibitor did not alter the expression level of MAPK/JNK1/2/3 but significantly reduced the phosphorylation levels at Thr183 and Tyr185 sites ($P<0.05$). This indicates that MAPK/JNK1/2/3 inhibition was achieved primarily through reduced phosphorylation at these sites rather than decreased expression. The MAPK/JNK inhibitor significantly reversed EGF-induced downregulation of NaPi-IIb expression ($P<0.05$), demonstrating that the MAPK/JNK1/2/3 signaling pathway participates in EGF regulation of NaPi-IIb expression levels.

Discussion

EGF is a small peptide with a molecular mass of 6 kDa, containing 53 amino acid residues and three disulfide bonds. It exhibits multiple biological functions including promoting cell growth, migration, proliferation, wound healing, bone healing, and nutrient transport [7-8]. Numerous studies have shown that EGF exerts its biological functions by binding to and activating cell surface EGF receptors [9,11]. The EGF receptor is a 170 kDa transmembrane glycoprotein composed of extracellular, transmembrane, and intracellular domains [7,9]. Upon EGF binding, the receptor dimerizes and activates its intrinsic tyrosine kinase activity, recruiting various intracellular signaling molecules to regulate signal transduction. These signals can modulate multiple transcription factors in the nucleus, inducing or inhibiting gene transcription and thereby exerting complex physiological functions [12-14].

Phosphorus is a non-renewable resource with very low biological utilization efficiency in animals. Improving phosphorus utilization efficiency, enhancing economic benefits in animal production, and reducing environmental pollution from excessive phosphorus excretion are critical challenges in animal husbandry and animal nutrition science. Xu et al. [4-5] reported that EGF inhibits NaPi-IIb gene expression in Caco-2 cells, and Xing et al. [6] demonstrated that EGF inhibits NaPi-IIb gene expression in IPEC-J2 cells. Our current results similarly show that EGF treatment decreases NaPi-IIb expression in IPEC-J2 cells. These findings collectively indicate that EGF plays an important regulatory role in intestinal phosphate absorption. Elucidating the molecular mechanisms and signaling pathways through which EGF regulates porcine intestinal NaPi-IIb expression is therefore significant for improving phosphorus utilization efficiency and reducing phosphorus excretion. To this end, we treated IPEC-J2 cells with EGF in combination with specific inhibitors of the EGF receptor tyrosine kinase, PKA, PKC, p38, ERK, and JNK to examine NaPi-IIb protein expression and identify the precise pathways mediating EGF regulation.

Treatment with 5, 10, or 20 mol/L EGF receptor inhibitor increased NaPi-IIb expression in IPEC-J2 cells, indicating that EGF could not suppress NaPi-IIb expression when receptor function was blocked. This demonstrates that EGF inhibition of NaPi-IIb expression in IPEC-J2 cells depends on binding to the EGF receptor.

EGF binding to its receptor activates multiple intracellular signaling pathways, including phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), AMP-activated protein kinase (AMPK), MAPK, PKC, and PKA [15-16]. In mouse hepatocytes, EGF activates PKA through direct or indirect mechanisms, which enhances EGF-induced ERK2 activation, and PKA signaling can in turn influence EGF/EGF receptor signaling [17]. EGF activation of PKC may occur through regulation of phospholipase C (PLC) , and activated PKC can

phosphorylate Src to induce PI3K phosphorylation, subsequently activating ERK1/2. PKC also regulates the cleavage and release of EGF precursors including transforming growth factor (TGF) , neuregulin 1 , and heparin-binding EGF. Additionally, PKC activation prevents EGF receptor phosphorylation at Thr654 [18,19]. These findings indicate that EGF can activate PKC, which in turn regulates EGF secretion and EGF receptor activity. EGF promotes nestin expression in mouse reactive astrocytes through the Ras-Raf-ERK signaling pathway. In the EGF receptor-ERK signaling cascade, Ras is the only membrane-anchored component, and its localization determines downstream intracellular signals [20,21]. EGF also regulates JNK and p38 MAPK activity. Cdc42, a Ras-related GTP-binding protein, has been shown to bind the subunit of coat protein complex I (COP), which is crucial for Cdc42 regulation of cell phenotype switching. Further research revealed that Cdc42 binding to COP induces EGF receptor accumulation, enabling EGF to activate ERK1/2, JNK, and PI3K, thereby promoting cell division [22].

In human intestinal Caco-2 cells, EGF primarily regulates NaPi-IIb expression through modulation of MAPK, PKC, and PKA [5]. To determine whether these pathways mediate EGF regulation of NaPi-IIb in porcine intestinal epithelial cells, we employed specific inhibitors of PKA, PKC, p38, ERK, and JNK. Our results showed that all these inhibitors blocked EGF-induced downregulation of NaPi-IIb expression, indicating that these signaling molecules all mediate this effect and that EGF downregulates NaPi-IIb expression through multiple signaling pathways. Notably, PKC inhibition caused a highly significant increase in NaPi-IIb expression with a greater magnitude than inhibition of other signaling molecules, suggesting that the PKC-mediated signaling pathway plays a predominant role in EGF downregulation of NaPi-IIb expression.

While this study demonstrates that MAPK (p38, ERK, and JNK), PKC, and PKA mediate EGF regulation of NaPi-IIb expression in porcine intestinal epithelial cells, these three signaling pathways are highly complex and interconnected. The downstream signaling molecules through which they ultimately regulate NaPi-IIb expression require further investigation.

In conclusion, our findings indicate that EGF regulation of NaPi-IIb expression in IPEC-J2 cells is mediated by the EGF receptor, PKA, PKC, and MAPK (p38, ERK, JNK) signaling pathways.

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