NLRX1 mediates the disruption of intestinal mucosal function caused by porcine astrovirus infection via the ERK/MLCK pathway

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Case Report

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Abstract

Considering the possible zoonotic nature of Porcine astrovirus (PAstV) and its frequent co-infection with porcine epidemic diarrhoea virus (PEDV), we investigated the impact of NLRX1 on the dysfunction of the intestinal mucosal barrier induced by PAstV infection. The study revealed a significant up-regulation of NLRX1 and LC3 II proteins in Caco-2 cells infected with PAstV. The reduction in PAstV titer occurred with the silencing of NLRX1 and the treatment of the autophagy inhibitor 3-MA. However, the use of 3-MA did not impact the expression of NLRX1. Additionally, PAstV infection triggered the activation of the ERK/MLC pathway and led to the down-regulation of tight junction proteins (Occludin and ZO-1) as well as MUC-2 expression. Silencing the expression of NLRX1 or treating it with 3-MA demonstrated the ability to inhibit MLC phosphorylation and elevate the levels of Occludin and ZO-1 proteins. Moreover, the inhibition of MLC phosphorylation was observed by treating the ERK inhibitor PD98059. Furthermore, adding the MLC inhibitor ML-7 mitigated the down-regulation of mucosa-related protein expression induced by PAstV infection. Nevertheless, the administration of PD98059 and ML-7 did not affect the expression of NLRX1. In summary, the data from this study confirm that NLRX1 plays a role in the disruption of intestinal mucosal function triggered by PAstV infection via the ERK/MLCK pathway. This information contributes to a better understanding of the pathogenesis of PAstV.

Introduction

The diarrhoeal disease ranks as the second-leading cause of death among children under 5 years of age, accounting for approximately 2 billion cases worldwide annually. In China, diarrhoeal disease in children is reported to be 1.9 times per person per year (1). Human astrovirus (HAstV) is recognised as the second leading cause of gastroenteric diarrhoea in humans (2). Meanwhile, Porcine astrovirus (PAstV) has been identified as a potential emerging zoonotic infectious agent capable of cross-species transmission (3). Currently, PAstV is prevalent in China, inducing piglet symptoms resembling those caused by the Porcine epidemic diarrhoea virus (PEDV). The positive rate of PAstV ranges from 7.5–46.3% (4, 5). PAstVs frequently coexist with other diarrhoea-inducing diseases, including viruses, such as coronaviruses, caliciviruses, and sapelovirus (6). PAstV exhibits diverse clinical symptoms due to the presence of different genotypes characterised by significant genetic variations (7). Additionally, recombination events between PAstV and HAstV have been identified. Hence, it is crucial to focus on research regarding the pathogenesis of PAstV, aiming to identify potential antiviral drug targets. This effort could offer new strategies for treating both human and animal gastroenteritis.

The intestinal mucosal barrier serves as the primary defense mechanism against foreign pathogens in the body. When this barrier is compromised, it leads to increased intestinal permeability, disrupting the intestinal barrier (8). Ultimately, the compromise of the intestinal mucosal barrier initiates an inflammatory response, contributing to the onset of intestinal diseases. It has been reported that granulosomal dysfunction can impair intestinal epithelial cell function integrity, culminating in gastroenteritis disease (9). Our previous research revealed that PAstV infection in PK15 cells induces mitochondrial damage. Among the only differential proteins, NLRX1 was identified as the sole protein...
localised in mitochondria. This suggests a potentially significant role for NLRX1 in the context of PAstV infection (10). Hence, in this study, we examined the impact of NLRX1 on PAstV replication and further investigated its regulatory role in disrupting intestinal mucosal barrier function induced by PAstV infection. The findings indicated that NLRX1 can enhance the replication of PAstV, and the suppression of NLRX1 alleviated the down-regulation of tight junction protein in intestinal epithelial cells through the ERK/MLCK pathway.

Materials and Methods

Cells, viruses, and antibodies

The human colon adenocarcinoma cell line Caco-2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) within a humidified atmosphere containing 5% CO₂ at 37 °C. The Porcine astrovirus type 4 stain PAstV/SH/2022/CM1, identified in our laboratory, was propagated in Caco-2 cells with 15 µg/ml Pancreatin. The viral titer was determined by measuring the 50% tissue culture infective dose (TCID₅₀). The mouse monoclonal antibody against PAstV was obtained from LanDu Biotechnology (China). Additionally, the anti-NLRX1 polyclonal antibody was received from Proteintech (China), and the anti-HA polyclonal antibody, anti-β-actin antibody (Abcam), HRP-conjugated anti-mouse IgG, and FITC-conjugated anti-mouse IgG (Abcam) were utilised following the instructions of the manufacturers.

Transfection and silencing of the NLRX1 gene using siRNA

For siRNA knockdown experiments, Caco-2 cells were seeded in 6-well plates and transfected twice with 25 nM of the designated siRNAs over 48 h using Lipofectamine 3000. The siRNA employed were as follows: siRNA/NLRX1-1, 5'-UUGUCAAUCUGCUGCGCAA-3'; siRNA/NLRX1-2, 5'-GUGCUGGGCUUGCGGAAGA-3'; siRNA/NLRX1-3, 5'-GCAUGUCCUUCCGCCGGGAU-3'; Negative control siRNA, 5'-UUCUCCGAACGUGUCACGUU-3'.

TCID₅₀ for PAstV

PAstV titers were assessed through an indirect fluorescence assay using the Reed-Muench method. Caco-2 cells were seeded in 96-well plates, and eight replicates of serial 10-fold dilutions of PAstV were inoculated. After 96 h, the indirect fluorescence assay was conducted to calculate the TCID₅₀ using PAstV specific antibody (11).

Quantitative real-time PCR (qPCR)

At the specified time points, total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, reverse transcription was carried out utilising the PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Following reverse transcription, quantitative PCR (qPCR) was conducted using SYBR Green Real-Time PCR Master Mix (Takara Bio, Shiga, Japan) and the ABI7500
system (ABI, Madison, USA). The amplification conditions included an initial denaturation at 95 °C for 30 sec, followed by 40 cycles of denaturation at 95 °C for 5 sec, annealing at 60 °C for 30 sec, and extension at 95 °C for 15 sec. The β-actin gene served as an internal standard, and the results were calculated utilising the 2^(-△△Ct) method (12). The primer sequences employed for amplification were as follows: Occludin (F: 5’-TCCAACGGGAAAGTGAACGA-3’; R: 5’-GTGGATATTCCCTGATCCAGTCTT-3’), ZO-1 (F: 5’-AAGGTAAGTCTGCTGAGGCTG AA-3”; R: 5’-GACACTGAATTACCTTCCGCG-3’), MUC-2 (F: 5’-GTCGAGTACATCCTGCT
GACG-3”; R: 5’-GAGTCCTCTCTGTTTCCACACG-3’), NLRX1(F:5’-CAGACCCTCACAAG CATCTA; R: 5’-CACGGACATCCTCTCCAGA-3’), β-actin (F: 5’-TGGGTCAGAAGGACTCATG-3”; R: 5’-TGGGTCAGAAGGACTCT 3’).  

**Western blotting analysis**

Cells cultured in 60 mm dishes were prepared by adding 200 µL of 2×lysis buffer A (65 mM Tris-HCl, 4% SDS, 3% DL-dithiothreitol, and 40% glycerol) and incubated for 30 min on ice. Subsequently, the samples were mixed with 5×SDS buffer and boiled for 10 min. The proteins were separated by 12% SDS-PAGE and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA) using eBlot L1 (GenScript™, China). After blocking for 1 hour at room temperature with Tris-buffered saline containing 5% skim milk, the membranes were incubated overnight at 4 °C with the specified primary antibodies. This was followed by incubation with HRP-conjugated secondary antibodies at room temperature for 1 h. Finally, the proteins were visualised by staining using enhanced chemiluminescence detection kits (Thermo Fisher Scientific, Waltham, MA, USA).

**Statistical analysis**

The values are presented as mean ± standard deviation (SD). Data were analysed using the student’s t-test and processed using SPSS. P values less than 0.05 were considered statistically significant.

**Results**

**Down-regulation of tight junction proteins in Caco-2 cells following PAstV-4 infection**

To investigate the impact of PAstV infection on the mucosal barrier function of intestinal epithelial cells, the susceptibility of PAstV-4 on human colon cancer epithelial cells (Caco-2) was initially assessed. The results confirmed the successful proliferation of the PAstV/SH/2022/CM1 strain on Caco-2 cells, with a
viable titer of $10^{5.23}$TCID$_{50}$/0.1 mL (Fig. 1A). Subsequently, the effect of PAstV infection on the epithelial mucosal barrier function was assessed. The results demonstrated a down-regulation in the transcription and expression of the MUC-2 protein and the tight junction proteins of Occludin and ZO-1 upon PAstV-4 infection (Fig. 1B). Furthermore, the degree of inhibition increased with the increasing infection dose of PAstV-4 (Fig. 1C).

**Upregulation of NLRX1 expression induced by PAstV infection**

Building upon our prior findings indicating that PAstV/SH/2022/CM1 infection upregulated the expression of NLRX1 in PK15 cells, we sought to confirm this result in the intestinal epithelial cells during PAstV infection. To achieve this, caco-2 cells were infected with PAstV-4 at an MOI of 1. Real-time PCR analysis confirmed a significant up-regulation of NLRX1 expression at 24 h post-infection (Fig. 2A). Notably, UV-inactivated PAstV-4 failed to induce NLRX1 mRNA expression, suggesting that the up-regulation of NLRX1 depends on viral replication (Fig. 2A). Furthermore, the results revealed that PAstV-4 upregulated NLRX1 dose-dependently (Fig. 2B).

**PAstV-4 replication augmented by NLRX1 knockdown in Caco-2 cells**

To investigate the impact of the endogenous NLRX1 on PAstV-4 replication, three pairs of siRNA duplexes targeting NRLX1 were separately transfected into Caco-2 cells. The knockdown efficiency of these siRNAs was assessed through real-time PCR, revealing that siRNA/NLRX1-2 achieved the highest efficiency (Fig. 2C). Western blot analysis confirmed that siRNA/NLRX1-2 significantly reduced the level of NLRX1 protein by approximately 89% compared to control cells transfected with NC siRNA (Fig. 2D). Subsequently, siRNA/NLRX1-2 was transfected into Caco-2 cells, followed by PAstV-4 infection, which decreased PAstV-4 replication within Caco-2 cells (Fig. 2E). This suggests that the endogenous NLRX1 can potentially promote the replication of PAstV-4 in Caco-2 cells. PAstV-4 infection also resulted in the up-regulation of LC3II proteins, and this effect was inhibited by treatment with siRNA/NLRX1 (Fig. 3A). Additionally, treatment with 20 µM 3-MA (autophagy inhibitor) decreased the viral titer of PAstV (Fig. 3B) while not affecting NRLX1 expression (Figs. 3C and 3D). We speculate that PAstV-4 infection activates mitochondrial autophagy to resist host innate immunity through the up-regulation of NLRX1, thereby promoting its replication.

**Disruption of Caco-2 mucosal barrier through ERK/MLCK pathway activation by NLRX1**

The ERK/MLCK pathway is crucially associated with the intestinal mucosal barrier function. Following infection with PAstV-4 (1 MOI) for 24 h, increased p38, p-ERK, and p-MLC levels were observed through western blot analysis. Moreover, the knockdown of NLRX1 hindered the expression of p-ERK and p-MLC
induced by PAstV-4 infection (Fig. 4A). Moreover, the treatment with 20 µM PD98059 (ERK inhibitor) decreased the expression of p-MLC. In contrast, NLRX1 expression remained unaffected (Fig. 4B). Subsequently, the impact of mitophagy on the ERK/MLC pathway was assessed. Results indicated that treatment with 3-MA down-regulates ERK and MLC phosphorylation and upregulated the expression of Occludin and ZO-1. This suggests that NLRX1 is implicated in regulating the activation of the ERK/MLCK pathway through mitophagy.

To investigate the role of NLRX1 in regulating the intestinal mucosal barrier function, siRNA/NLRX1-2 was transfected into Caco-2 cells, followed by PAstV-4 infection. Subsequently, the expression of MUC-2, Occludin, and ZO-1 were assessed using western blot analysis. The results demonstrated that the downregulation of the three proteins induced by PAstV-4 infection was alleviated (Fig. 5A). Furthermore, treatment with 25 µM ML-7 (MLCK inhibitor) exhibited this phenomenon while not affecting NLRX1 expression (Figs. 5B and 5C). This suggests NRLX1 may regulate the mucosal barrier function through the ERK/MLCK pathway during PAstV-4 infection.

**Discussion**

AstV is recognised as a potential zoonotic pathogen, with reported infections in thirty-one species of mammals and six poultry species, leading to symptoms such as diarrhoea and neurological issues (13). In particular, Porcine astrovirus primarily induces symptoms like diarrhoea, vomiting, and anorexia in piglets. Diarrheal pig faeces are characterised by a mushy consistency or yellow water, exhibiting clinical symptoms similar to those caused by the porcine epidemic diarrhoea virus (PEDV) (6, 14). Furthermore, synergistic effects with PEDV may exist, emphasising the need for careful attention to such co-infections. Our previous study discovered that the NLRX1 protein, predominantly in mitochondria, exhibited a significant upregulation following PAstV-4 infection. This upregulation was associated with the mitochondrial autophagy pathway, indicating a potentially crucial role for NLRX1 in the context of PAstV infection. Hence, this study explored understanding the impact and regulation of the NLRX1 protein on PAstV infection. In the examination of the effects of PAstV on the intestinal mucosal barrier, Caco-2 cells were infected with PAstV. Notably, PAstV-4 demonstrated robust proliferation on Caco-2 cells in the presence of 15 µg/ml Pancreatin, providing a basis for further exploration of the underlying pathogenic mechanisms. Indeed, the impairment of tight junctions and adhesive junction structures has been established as a contributing factor in the pathogenesis of PEDV and various other pathogens (15–17). Fang et al. reported that being infected with PAstV-GX1 in seven-day-old suckling piglets led to mild diarrhoea, growth retardation, and damage to the intestinal mucosal villi. Notably, the damage was observed to recover within 7–10 days post-inoculation (18). Our investigation observed that PAstV-4 infection led to a down-regulation in the transcription and expression of MUC-2, Occludin, and ZO-1 proteins. Notably, silencing NRLX1 alleviated this phenomenon and concurrently reduced the viral titer of PAstV-4. These findings suggest that the endogenous NRLX1 may enhance PAstV-4 replication and contribute to intestinal mucosal barrier function impairment caused by PAstV-4 infection. Studies have reported that HAstV can enhance self-replication by activating the TGF-β pathway, and the TGF-β/Rho kinase pathway increases the permeability of the endothelial cell barrier through the activation of MLCK
phosphorylation (19, 20). In our study, we observed that PAstV-4 impaired the mucosal barrier function of Caco-2 cells by activating the ERK/MLCK pathway, potentially upregulating NLRX1 protein. However, the mechanism by which PAstV-4 infection upregulates NLRX1 remains unclear, and this aspect will be investigated in further research. In summary, this study represents the first exploration into the impact of PAstV-4 infection on the intestinal mucosal barrier function. These findings contribute to a better understanding of the clinical pathogenesis of PAstV infection and provide a foundation for future research on antiviral drug development.

**Declarations**

**Authors’ contributions**

Conceptualisation: HLL, JT and YS; methodology: JHC and YS; validation: BQL and PT; resources: HLL, BQL and JT; data curation: JJJ; writing and editing: HLL, JT and JHC; project administration: JT and HLL; All authors have read and agreed to the published version of the manuscript.

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**Competing interests**

The authors do not have any conflict of interest.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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**References**


Figures

Figure 1

Down-regulation of tight junctions in Caco-2 cells upon PAstV infection. (A) PAstV propagation on Caco-2 cells with 15 μg/ml Pancreatin resulted in specific red fluorescence detected in the cytoplasm, as demonstrated by indirect immunofluorescence assay using a PAstV monoclonal antibody. (B) Following inoculation of Caco-2 cells with 1MOI of PAstV, the expression of tight junction proteins was detected 24 h later through western blotting. (C) The influence of PAstV infection at various MOIs (0.1, 0.5, and 1.0) on
the transcription of MUC-2, Occludin, and ZO-1 was determined by relative fluorescence quantification PCR, utilising β-actin as the intrinsic reference protein. Fold changes were calculated using the $2^{-\Delta \Delta Ct}$ method.

**Figure 2**

Reduction of PAstV replication through NLRX1 knockdown in Caco-2 cells. (A) Caco-2 cells were infected at an MOI of 1.0 with either PAstV or UV-inactivated PAstV for 24 h. Real-time PCR was employed to determine the relative NLRX1 levels, normalised to β-actin. Asterisks (*) denote significant differences from uninfected cells ($p < 0.01$), while "ns" indicates no significant difference. (B) Cell lysates were collected at 24 h and 48 h, respectively, following infection, and NLRX1 expression was assessed through western blotting using the indicated antibodies. (C) and (D) Caco-2 cells were transfected with negative control siRNA or three different siRNA duplexes targeting NLRX1 for 24 h. Subsequently, the relative...
NLRX1 and expression levels were detected, with β-actin as an internal control. (E) Cells were transfected with negative control siRNA or siRNA/NLRX1-2 for 24 h and then infected with 1.0 MOI PAstV. Virus titers were determined through a TCID<sub>50</sub> assay at 60 h post-infection.

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**Figure 3**

Induction of mitophagy by PAstV infection via up-regulation of NLRX1 protein. (A) Caco-2 cells were transfected with negative control siRNA or siRNA/NLRX1-2 for 24 h, followed by infection with 1MOI PAstV. Cell lysates were blotted with anti-LC3I/II, anti-NLRX1, and anti-β-actin antibodies. (B) Cells treated with the mitophagy inhibitor 3-MA for 6 h were subsequently infected with 1.0MOI PAstV. Virus titers were determined through a TCID<sub>50</sub> assay 60 h post-infection. (C) and (D) Cells subjected to treatment were infected with 1.0MOI PAstV, and the relative and expression levels of NLRX1 were assessed 24 h later.
Figure 4

Reduction of tight junction protein expression by PAstV infection via the ERK/MLC pathway. (A) Caco-2 cells were transfected with negative control siRNA or siRNA/NLRX1-2 for 24 h, followed by PAstV infection at an MOI of 1.0. After 24 h, cell lysates were blotted with the indicated antibodies. (B) Cells were treated with 20 μM PD98059 (ERK inhibitor) for 6 h, followed by infection with 1MOI PAstV. Subsequent western blotting using the indicated antibodies was conducted as described above. (C) Cells were treated with 20 μM 3-MA (mitophagy inhibitor), and the subsequent steps were the same as in (B).
Figure 5

Essential role of NLRX1 in impaired intestinal barrier function induced by PAsTv infection. (A) Caco-2 cells were transfected with negative control siRNA or siRNA/NLRX1-2 for 24 h, followed by PAsTv infection at an MOI of 1.0. Cell lysates were then blotted with the indicated antibodies 24 h later. Further, cells were treated with 25 μM ML-7 (MLCK inhibitor) for 6 h and infected with 1MOI PAsTv. (B) The relative MUC-2, Occludin, ZO-1, and NLRX1 levels were detected after treatment with ML-7 (MLCK inhibitor), using β-actin as an internal control.
as an internal reference. (C) MUC-2, Occludin, ZO-1, and NLRX1 expression levels were assessed after treatment with ML-7 (MLCK inhibitor), with β-actin as an internal control.

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