Ziyuglycoside II Inhibits Rotavirus Induced Diarrhea Possibly via TLR4/NF-κB Pathways

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INTRODUCTION

Rotavirus (RV) induced diarrhea has been a major reason affecting children healthy under 5 years old especially in developing countries. Although specific vaccines have preventive effects, antiviral therapy is essential for the diarrhea patients. Ziyuglycoside II is a traditional Chinese herb which has been proven to possess anti-virus effects. This study aimed to investigate the roles of Ziyuglycoside II in rotavirus-induced diarrhea and the underlying molecular mechanism. We found that normal MA104 cells treated with RV became swollen and gather together. However, Ziyuglycoside II treatment inhibited cell growth in a dose- and time dependent manner and suppressed RV replication. Moreover, Ziyuglycoside II reversed RV-induced downregulation of anti-inflammatory cytokine interleukin (IL)-10 and upregulation of pro-inflammatory factors, such as interferon-γ (IFN-γ), IL-1β, IL-6, and tumor necrosis factor (TNF-α). Moreover, Ziyuglycoside II administration and ribavirin blocked toll-like receptor 4 (TLR4)/nuclear factor kappa-B (NF-κB) signaling pathway both in mRNA and protein level, which was paralleled with immunohistochemical assay. Additionally, Ziyuglycoside II administration improved diarrhea symptoms and decreased diarrhea scores. Ziyuglycoside II and ribavirin inhibited the apoptosis of small intestine epithelial cells induced by RV. Taken together, RV treatment induced diarrhea. Ziyuglycoside II administration inhibited TLR4/NF-κB pathway and inflammatory response and improved RV-induced diarrhea. The inhibitory effects of Ziyuglycoside II on RV-induced diarrhea predicted Ziyuglycoside II may be a promising drug for diarrhea.

Key words Ziyuglycoside II; diarrhea; rotavirus; toll-like receptor 4; nuclear factor kappa-B; inflammation

Toll-like receptors (TLRs), as transmembrane receptors, positively participate in mucosal innate immune regulation. TLR4, a member of the Toll-like families, localizes to both the cell membrane and the cytoplasm and serves as a pattern recognition receptor for lipopolysaccharide (LPS). Interaction of LPS with TLR4 and triggers the activation of the downstream nuclear factor kappa-B (NF-κB) signaling pathway and eventually results in inflammatory response. Increasing studies have focused on blocking TLR4/NF-κB pathways in the therapy of gastrointestinal diseases. TLR4 induces the secretion of cytokines, chemokines, and growth factors involved in inflammation and the combination of TLR4-dependent claudin-1 internalization and secretagogue-mediated chloride secretion contribute to diarrhea. Therefore, the potential roles of Ziyuglycoside II in TLR4/NF-κB signaling in the treatment of diarrhea are of vital importance.

In the present study, we detected whether Ziyuglycoside II exhibited anti-virus effects against the MA104 and diarrhea mouse induced by rotavirus, and the related signaling pathway participating in the anti-rotavirus progression. This may provide a novel therapy for rotavirus induced diarrhea.

MATERIALS AND METHODS

Cells, Virus and Reagents Rhesus monkey kidney cell line MA104 was purchased from ATCC (U.S.A.). Cells were incubated in EMDM medium (Nissui Pharmaceutical, Japan) supplemented with 10% fetal bovine serum (FBS, Equitech-Bio, U.S.A.) at 37°C under 5% CO2. Simian rotavirus (RV)
SA11 strain was bought from H. Malherbe (University of Texas Health Science Center, U.S.A.). Cells were incubated with RV strain SA11 till 2 freeze-thaw cycles. Ziyaglycoside II was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and prepared as a stock solution in dimethyl sulfoxide (DMSO) at −20°C. The concentration was adjusted to (0, 2, 4, 8, 16, 32, 64 μmol/mL) for the following experiment.

Then the infected cells were divided into five groups treated with Ziyaglycoside II at different concentrations at 12 h post-infection: Rotavirus control (RV, without treatment), low group (RV + L, 3 μM treatment), medium group (RV + M, 6 μM treatment), high group (RV + H, 9 μM treatment), and Ribavirin group (RV + Ribavirin, Ribavirin group, 100 mg treatment). To observe the morphological change after rotavirus infection, the cells were observed under an inverted microscope (Nikon, Japan).

*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay* Cells were plated in 96-well plate (2 × 104 cells/well). The cells were then incubated for another 48 h. Subsequently, the culture medium was removed and 100 μL of DMSO was added. The absorbance of each well was measured on the microplate absorbance reader (BIORAD, CA, U.S.A.) at the wavelength of 490 nm.

*Rotavirus Quantity* Rotavirus RNA was extracted from the MA104 by TRIZol (Invitrogen, CA, U.S.A.), purified by ribonuclease (RNase)-free deoxyriboonuclease (DNase) digestion (Qiagen, U.S.A.) and determined with NanoDrop 2000 (Thermo Scientific, CA, U.S.A.). Quantitative qPCR was used to calculate the copied RNA. The virus were denatured for 95°C denaturation for 3 min, 40 cycles of 95°C denaturation for 12 s and 60°C annealing and elongation for 40 s. The primer sequences for were designed as 5'-TTCTTACCAAGACGCGAAGAG3' (forward) and 5'-ATT CGGGCCTGAGATCAGTG3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as loading control.

*Animals and RV-Induced Diarrhea* Specific pregnant BALB/c male mice (aged 3–4 d, n = 90) were obtained from Model Animal Research Center of Nanjing University and kept at 25°C under a 12 h light/dark cycle. This study was supervised by the Committee for Animal Ethics Experiments. At 3 d of mouse age, mice were administrated with 25°C under a 12 h light/dark cycle. This study was conducted at Model Animal Research Center of Nanjing University and BALB/c male mice (aged 3–4 d, n = 15). Each experiment was performed in triplicate.

**Detection of Rotavirus RNA in Fecal Specimens** Fecal samples were harvested and homogenized. Total RNA was separated with TRizol reagent (Life Technologies, Westminister, SC, U.S.A.) and determined with reverse transcription kit (Toyobo, Japan). GAPDH were used as loading control.

**Diarrhea Score** Diarrhea score was performed to evaluate the severity of diarrheal illness. Briefly, stool was collected, observed and given a score. The rules are as follows: Normal feces were scored 1, loose feces 2, loose yellow-green feces 3, and watery feces 4. ≥2 was deemed as diarrhea. Mice with no stool were considered as no diarrhea. The average score was calculated. It was performed every day.

**Enzyme-Linked Immunosorbent Assay (ELISA) for the Inflammatory Factors** The serum levels of cytokines were determined with ELISA kits (R&D Systems Inc., U.S.A.). Finally, the volume of the cytokines was determined.

**Q-RT-PCR Assay** To examine the signaling pathway, qRT-PCR was performed using small intestinal segments. For each mouse, total RNA was reversely transcribed into cDNA with SuperScript II Reverse Transcriptase (Invitrogen) and analyzed with the QuantStudio 6 Flex Realtime PCR system (Applied Biosystems, CA, U.S.A.). The expression level was determined with 2−ΔΔCq method.

**Western Blot** Protein was collected with Bio-Rad Protein Assay (Bio-Rad), separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and moved onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies against TLR4 (ab13556, 1:500, Abcam, U.S.A.), NF-κB (ab32536, 1:5000, Abcam) overnight at 4°C. On the next day, the membrane were incubated with secondary antibodies (ab6721, 1:5000, Abcam). The protein level were determined with ECL Plus (Thermo Scientific) and analyzed with ImageJ 1.49 software (National Institutes of Health). GAPDH served as loading control.

**Histological Examination** Mouse small intestine was dissected after 7 d of treatment, fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. The slices (5 μm) were cut, deparaffinized by being immersed in xylene and rehydrated in graded ethanol solutions. The slices were stained with hematoxylin–eosin (H&E) and observed under light microscope. Histopathological evaluation was performed in reference to the following system. a) Inflammation severity: none = 0; slight = 1; moderate = 2; severe = 3; b) Crypt damage: none = 0; basal one-third damaged = 1; basal two-thirds damaged = 2; only surface epithelium intact = 3; entire crypt and epithelium lost = 4; d) Percentage of area involved: none = 0; 1–25% = 1; 25–50% = 2; 51–75% = 3; 76–100% = 4. The final scores are the averages of all individual scores of 10 pieces per sample.

**Immunohistochemical Staining** Mouse small intestine was fixed with formalin, embedded with Paraffin. The tissue sections were cut into 4-μm-thick. The slice was incubated with hydrogen peroxide (3%) and blocked with 5% non-fat milk. Sections were incubated with anti-TLR4 (ab13556, 1:500, Abcam) and anti-NF-κB (ab32536, 1:5000, Abcam). The expression of TLR4/NF-κB was analyzed with fluorescent microscope (Olympus BX51, Japan) at magnification, ×400.

**Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nickel-End Labeling (TUNEL) Assay** After the fixation in formalin, the small intestine of each mouse was embedded in paraffin. Then the slice was supplemented with Fresh TUNEL mix (50 μL). After washed with phosphate buffered saline (PBS) for 3 times, the slices (five fields) dehydrated, mounted and analyzed with an optical microscope (Olympus, Japan).

**Statistical Analysis** All data were presented as mean ± standard deviation (S.D.) and analyzed with GraphPad 5 statistical software. Student t test was applied to analyze the difference between two groups and one-way ANOVA followed
Fig. 1. The Effects of Ziyuglycoside II on Proliferation

(A) The cells in RV group became swollen and gather together. (B) The quantification of A. (C) The growth of MA104 cell was inhibited by Ziyuglycoside II in a dose-dependent manner. Each experiment was performed in triplicate. *p < 0.05, **p < 0.05 vs. RV group at 24 h.

Fig. 2. The Effects of Ziyuglycoside II on TLR4/NF-κB Pathways

(A) qRT-PCR was performed to detect the mRNA expression in TLR4/NF-κB signaling pathway. (B) Western blot was performed to detect the protein level of TLR4/NF-κB signaling pathway. (C) Immunohistochemistry was performed to detect the mRNA expression in TLR4/NF-κB signaling pathway. Each experiment was performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. RV group.

Fig. 3. The Therapeutical Effects of Ziyuglycoside II on Rotavirus Induced Diarrhea Mouse

(A) The diarrhea score for rate administered different concentration of Ziyuglycoside II and Ribavirin. Score of ≥2 was considered diarrhea, whereas ≤2 was considered normal. Values are mean ± standard deviation. (B) Viral antigens shedding of neonatal mouse administrated different concentrations of Ziyuglycoside II after induction of rotavirus diarrhea. Each experiment was performed in triplicate. *p < 0.05, **p < 0.01, ***p < 0.001 vs. RV group.
RESULTS

The Effects of Ziyuglycoside II on Cell Proliferation of MA104 To evaluate the effects of Rotavirus and Ziyuglycoside II on cell morphology, after Rotavirus infection and Ziyuglycoside II treatment, the MA104 cells were observed under an inverted microscope. As Figs. 1A and B showed, the normal cells were distributed evenly and had clear boundaries, 24h after infection, the cells became swollen and gather together. After 48h, the cell morphology change was aggravated. The growth of MA104 cell was inhibited by Ziyuglycoside II in a dose-dependent manner. The IC\textsubscript{50} of Ziyuglycoside II on MA104 were 30 and 32\,\mu M at 24 and 48h, respectively (Fig. 1C).

Effects of Ziyuglycoside II on TLR4/NF-κB Signaling Pathway As showed in Figs. 2A and B, in the diarrhea mouse, the TLR4/NF-κB signaling related mRNA and protein were increased compared with the normal group, indicated that the signaling was activated after rotavirus induced diarrhea. After Ziyuglycoside II and ribavirin treatment, the level of TLR4 and NF-κB were down-regulated in a dose-dependent manner. As Fig. 2C showed, the level of TLR4/NF-κB in small intestine was up-regulated in diarrhea mouse, while Ziyuglycoside II and ribavirin could decrease the expression of TLR4/NF-κB in a dose-dependent manner.

Effects of Ziyuglycoside II on Rotavirus Induced Diarrhea Mice To investigate the in vivo effects of Ziyuglycoside II, Rotavirus induced diarrhea mice were inoculated by oral gavages. Ribavirin, as an inhibitor of rotavirus replication, plays a crucial role in suppressing the RNA duplication of rotavirus. As showed in Fig. 3A, the diarrhea score was decreased in Ziyuglycoside II group, which suggested that RV-induced diarrhea was alleviated by Ziyuglycoside II. Moreover, there was no significant changes in rotavirus shedding in rotavirus group. Ziyuglycoside II significantly decreased the RNA copy of rotavirus in a dose- and time-dependent manner (Fig. 3B).

Effects of Ziyuglycoside II on Cytokine in Serum ELISA was performed to detect the cytokines in the serum. As shown in the Fig. 4, pro-inflammatory factors of interleukin (IL)-1β, TNF-α, IL-6 and interferon-γ (IFN-γ) were significantly upregulated after rotavirus infection, anti-inflammatory factor IL-10 was downregulated. Thereby, after oral gavages with Ziyuglycoside II, the serum level of pro-inflammatory factors was decreased in a dose-dependent manner, while IL-10 has the opposite change.

Effects of Ziyuglycoside II on Small Intestine Histopathological Changes and Small Intestine Apoptosis H&E staining was conducted to investigate the role of Ziyuglycoside II in small intestine at days 3 and 7. Rotavirus induced swollen villus tips. Moreover, virus-inoculated mice showed a thickening of the lamina propria and substantial mononuclear cells infiltrate. Ziyuglycoside II improved the lesion changes in a dose-dependent manner compared with RV group (Fig. 5A). Mice given Ribavirin dramatically exhibited better improvement of lesion changes. Moreover, the increase of histological score induced by RV was mediated by Ziyuglycoside II and Ribavirin.

TUNEL was performed to detect the apoptosis of rotavirus infected small intestine cell after Ziyuglycoside II treatment, as shown in Fig. 5B, in the rotavirus induced diarrhea mouse, small intestine cell apoptosis was increased compared with the healthy one, while after Oral gavage with Ziyuglycoside II, the apoptosis was improved and the higher Ziyuglycoside II concentration, the lower apoptosis rate. As the Ribavirin has the same anti-apoptosis effect, suggested that Ziyuglycoside II treatment inhibits the apoptosis of small intestine epithelial cells in RV-treated mouse (Fig. 5B).
DISCUSSION

Rotavirus strains play an important role in host-immune response. Rotavirus infection occurs in a great number of infants. MA104 cells are widely used for the growth and characterization of animal culture-adapted RV strains. Previously study found that Ziyuglycoside II possesses antiviral effects. However, there is no evidence to prove that Ziyuglycoside II could inhibit rotavirus replication. In our study, rotavirus induced MA104 swollen cells. However, Ziyuglycoside II inhibited the growth of MA104 infected with rotavirus. Moreover, Ziyuglycoside II may inhibit rotavirus copy. Nevertheless, the potential mechanisms is still unknown. In the previous study, the expression of TLR4/NF-κB was upregulated in diarrhea mouse. Interestingly, downregulated TLR4/NF-κB improve the symptoms of diarrhea. In He et al. study, TLR4/NF-κB protein expression levels are elevated in diarrhea model. Thence TLR4/NF-κB signaling may play a crucial role in the progression of diarrhea. Therefore, we predicted that Ziyuglycoside II may inhibit the progression of RV-induced diarrhea via regulating TLR4/NF-κB signaling pathways. In this study, Ziyuglycoside II downregulated the mRNA and protein level of TLR4/NF-κB, which was further proved in immunohistochemical staining. However, the underlying mechanisms were still unclear. Growing evidence proves rotavirus infection upregulated the level of inflammatory factors. For instance, TNF-α is involved in inflammatory responses, which is upregulated in rotavirus infection. Previous evidence has demonstrated that TLR4 activated NF-κB may induce the expression of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β. In this study, rotavirus increased the expression of IL-1β, TNF-α, IL-6, IFN-γ and decreased IL-10, which was reversed by the treatment with Ziyuglycoside II. Ribavirin plays an inhibitory role in TLR4 pathways. The combination of Ribavirin and Ziyuglycoside II were more potent in inactivating TLR4/NF-κB signaling pathways. Taken together, Ziyuglycoside II may inhibit rotavirus induced diarrhea via regulating TLR4/NF-κB pathways. Ziyuglycoside II could inhibit Rotavirus replication in vitro. Then the mice were used to evaluate the effect of Ziyuglycoside II on rotavirus in vivo. Diarrhea score results suggested that Ziyuglycoside II could improve diarrhea symptoms in rotavirus induced mouse. Moreover, Ziyuglycoside II induced improvement in vacuolar degeneration of MA104 infected with rotavirus, which suggested that Ziyuglycoside II may play a protective role in Rotavirus induced diarrhea. In conclusion, infections with rotavirus may induce diarrhea. Ziyuglycoside II inhibited the RNA copy of rotavirus, and the progression of MA104 infected with rotavirus. Ziyuglycoside II suppressed rotavirus induced diarrhea via downregulating TLR4/NF-κB, which may provide a rationale for the treatment of rotavirus induced diarrhea.

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Conflict of Interest The authors declare no conflict of interest.

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