GTS-21 Promotes α7 nAChR to Alleviate Intestinal Ischemia-Reperfusion-Induced Apoptosis and Inflammation of Enterocytes

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Background: Intestinal ischemia-reperfusion injury is a serious intestinal disease, with main symptoms of inflammatory reaction and severe oxidative damage. In addition, GTS-21-induced α7 nAChR has been shown to exert anti-inflammatory effects and anti-oxidation effects in various organs. However, whether α7 nAChR can alleviate ischemia-reperfusion-induced intestinal injury is unclear.

Material/Methods: We used intestinal epithelial cells (IEC-6) to perform the experiments. Oxygen glucose deprivation/re-oxygenation (OGD/R) was used to simulate the physiological environment of ischemia-reperfusion. First, the expression of α7 nAChR was determined in these cells which was cultured under OGD/R conditions. After that, the GTS-21 was used to treat these cells and the levels of inflammatory factors (TNF-α, IL-1β, IL-6, and IL-10) were assessed by ELISA. Next, the levels of ROS, SOD, and MDA were determined in IEC-6 cells. Finally, the apoptosis rates of IEC-6 cells were measured by flow cytometry.

Results: Results showed that the expression of TNF-α, IL-1β, and IL-6 was enhanced when the IEC-6 cells were cultured under OGD/R conditions. However, after treatment with GTS-21, the levels of pro-inflammatory factors were suppressed. In addition, the levels of ROS and MDA were also inhibited and the expression of SOD was promoted after GTS-21 treatment. We also found that the ratios of apoptotic cells declined after GTS-21 treatment.

Conclusions: GTS-21-induced α7 nAChR decreased the OGD/R-induced inflammatory response, oxidative damage, and apoptosis of intestinal epithelial cells.

MeSH Keywords: Inflammation • Intestinal Diseases • Reperfusion Injury

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**Background**

Intestinal injury induced by ischemia-reperfusion is a severe intestinal disease with high morbidity and mortality. Ischemia-reperfusion also damages distal organs and affects the normal physiological functions of various organs [1]. Ischemia-reperfusion injury is mainly caused by the oxidative damage due to free radicals. The synthesis of antioxidant enzymes in ischemic tissues is often impaired, so reactive oxygen species cannot be cleared quickly after blood supply is restored [2]. Furthermore, the intestinal mucosa is composed of simple epithelial cells that form the crucial barrier protecting the intestinal tract from multiple types of damage [3,4]. However, intestinal ischemia-reperfusion can lead to intestinal mucosal injury, which induces more severe intestinal injury, which is the main cause of deaths induced by intestinal ischemia-reperfusion injury [3,5,6]. Therefore, it is urgent to develop new therapies for intestinal ischemia-reperfusion injury.

The nicotinic acetylcholine receptor (nAChR) was originally found in the nervous system. The role of nAChR is modulating the release of neurotransmitters [7–9]. The α7 nAChR is the crucial subunit of these nicotinic acetylcholine receptors [10]. Furthermore, the α7 nAChR could play an anti-inflammatory role [11], and GTS-21 is a selective agonist of α7 nAChR. Research also revealed that higher concentrations of GTS-21 in the plasma are associated with lower levels of TNF-α, IL-1β, and IL-6 [12]. Other research revealed that the higher levels of α7 nAChR that are induced by GTS-21 inhibit the expression of TNF-α in splenic Ly6C+ monocytes [13], and other studies revealed that ischemia-reperfusion can induce the inflammatory response in various organs [14–16], but whether the GTS-21-induced α7 nAChR can relieve ischemia-reperfusion-induced intestinal injury is unclear.

In the present study, we cultured intestinal epithelial cells under oxygen glucose deprivation/reperfusion (OGD/R). Then, we assessed the expression of α7 nAChR and cell viability of these cells. After that, GTS-21 was used to treat these cells. α-Bgt was used as the antagonist for the suppression of α7 nAChR. Then, the levels of α7 nAChR and ROS in these cells were assessed. Finally, the apoptosis rates and the expression of inflammatory factors (TNF-α, IL-1β, IL-6, and IL-10) were determined. Our experiments revealed the effect of GTS-21-induced α7 nAChR on intestinal ischemia-reperfusion.

**Material and Methods**

**Cells culture and treatment**

The intestinal epithelial cell line IEC-6 was obtained from the Chinese Academy of Sciences (Shanghai, China) and cultured with Dulbecco’s modified Eagle’s medium (DMEM, Gibco USA) supplemented with 10% fetal bovine serum (Gibco, USA). These cells were cultured with GTS-21 in glucose-free balanced salt solution in an environment with 95% N2, 5% CO2, and 37°C for 2 h. After that, the cells were cultured with medium supplemented with fetal bovine serum for 2, 4, 6, and 8 h to mimic the physiological status of reperfusion after ischemia. The GTS-21 (10 µM, 100 µM, and 200 µM) and α-Bgt (100 µM) were used to treat the IEC-6 cells.

**CCK-8 assay**

The IEC-6 cells were plated into 96-well plates, and then were cultured under OGD/R conditions for various lengths of time. After that, the CCK-8 (Dojindo, Japan) was diluted in the culture medium and added into the 96-well plates. Then, these cells were incubated in the incubator for 1.5 h, after which the absorbance was measured with a spectrophotometer (Thermo Fisher Scientific, USA).

**Western blotting**

Total proteins were collected with RIPA buffer (Beyotime, China). The concentration of proteins was measured with the BCA methods. Then, these proteins were separated using 10% SDS-PAGE gel (Beyotime, China), and then were transferred to PVDF membranes (Millipore, USA). Then, the membrane was blocked with 5% skim milk powder solution, followed by incubation with primary antibodies at 4°C overnight. The primary antibodies used were α7 nAChR (Abcam, ab10096), caspase3 (CST, #9662), Cleaved caspase3 (CST, #9664), Bcl-2 (CST, #3498), Bax (CST, #5023), caspase9 (CST, #9502), Cleaved caspase9 (CST, #20750), and GAPDH (CST, #5174). On the second day, the membranes were washed with PBST 3 times and incubated with the secondary antibody for 1.5 h and then were washed with PBST again. The bands emerged after treatment with enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

**Detection of SOD and MDA**

The levels of SOD and MDA in IEC-6 cells were determined using commercial kits. All operations in these experiments were performed according to the manufacturer’s instructions.

**ELISA assays**

The levels of TNF-α, IL-6, IL-1β, and IL-10 in the supernatant of cultured cells was detected with ELISA. The ELISA kits used were TNF-α (Abcam, ab181421), IL-6 (Abcam, ab178013), IL-1β (Sigma-Aldrich, RAB0273), and IL-10 (Sigma-Aldrich, SRP3312). The cell culture supernatants were collected and saved in sterilized tubes. The experiments were carried out according to the manufacturer’s instructions.
**ROS assays**

The IEC-6 cells in different groups were prepared into the single-cell suspension by trypsin (Beyotime, China). Next, these cells were washed with PBS 3 times and were then incubated with H2DCFDA (Beyotime, China) for 40 min. During the process, these cells were shaken every 5 min to confirm the mix of probe and cells. ROS levels were detected with flow cytometry.

**Apoptosis assays**

Apoptosis rates were measured using a commercial kit (Beyotime, China). PBS was used to wash the cells 3 times. After that, Annexin-V and PI were incubated with the cells for 30 min. The cells were shaken every 5 min to make sure they were fully mixed with the dye. The ratio of apoptotic cells was determined with flow cytometry.

**Statistical analysis**

Data analysis was performed with GraphPad Prism 7.0. The comparison of different groups was evaluated by the t test. All data are presented as mean±SD. All the experiments were repeated 3 times. P values less than 0.05 were considered to indicate statistically significant differences between groups.

**Results**

**OGD/R caused downregulation of α7 nAChR in IEC-6 cells**

CCK-8 assay was performed to assess viability of IEC-6 cells cultured under OGD/R conditions. As shown in Figure 1A, with the prolongation of reperfusion time after ischemia and hypoxia, the cell viability gradually decreased. Next, the expression of α7 nAChR in IEC-6 cells was determined by Western blotting, showing that the expression of α7 nAChR also gradually decreased with the extension of reperfusion time (Figure 1B).

**GTS-21-induced α7 nAChR reduced the inflammatory response of IEC-6 cells caused by OGD/R**

GTS-21 is the agonist of α7 nAChR, and a-Bgt is the antagonist of α7 nAChR. Therefore, GTS-21 and a-Bgt were used to treat the IEC-6 cells to study the effect of α7 nAChR on enterocytes cultured under OGD/R conditions. First, the IEC-6 cells were cultured with various concentrations of GTS-21. Then, CCK-8 assays were carried out to determine the cell viability. The results (Figure 2A) showed that there was no difference in cell viability of IEC-6 cells cultured with various concentrations of GTS-21. Inflammatory response is a common type of injury induced by ischemia-reperfusion. Therefore, the levels of inflammatory-related factors (TNF-α, IL-6, IL-1β, and IL-10) were assessed by ELISA. As shown in Figure 2B, expression of TNF-α, IL-1β, and IL-6 was inhibited and the level of IL-10 increased after treatment with GTS-21. However, after the application of a-Bgt, the levels of TNF-α, IL-1β, and IL-6 were increased and the level of IL-10 decreased.

**GTS-21-induced α7 nAChR alleviated the OGD/R-induced oxidative damage of IEC-6 cells**

Oxidative damage another kind of damage induced by ischemia-reperfusion [17]. Therefore, we measured the levels of ROS by flow cytometry. As shown in Figure 3A, the levels of ROS decreased after treatment with GTS-21. However, the application of a-Bgt increased the levels of ROS. Levels of superoxide dismutase (SOD) and malondialdehyde (MDA) are also

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**Figure 1.** The expression of α7 nAChR gradually declined with the extension of reperfusion time after ischemia. (A) CCK-8 was used to determine the cell viability of IEC-6 cells cultured under OGD/R conditions (n=3, ±SD). (B) The protein and mRNA levels of α7 nAChR in IEC-6 cells were detected by Western blotting and RT-PCR, respectively. (n=3, ±SD). * p<0.05; ** p<0.01; *** p<0.001.
indicators of oxidative damage [18]. Therefore, the levels of SOD and MDA in IEC-6 cells was determined used the test kits. The results (Figure 3B) showed that the expression of SOD increased while the level of MDA was inhibited after treatment with GTS-21. Moreover, the level of SOD decreased and the expression of MDA was increased after the application of a-Bgt.

GTS-21-induced α7 nAChR relieved the OGD/R-induced apoptosis of IEC-6 cells

Some studies revealed that the ischemia-reperfusion can induce apoptosis of multiple types of cells [19–21]. In our study, the apoptosis rates of IEC-6 cells were determined by flow cytometry. The results (Figure 4A) showed that the apoptosis rates decreased after treatment with GTS-21. However, the ratios of apoptotic cells were reversed after the stimulation of a-Bgt. Next, the levels of apoptosis-related proteins were determined by Western blotting. As shown in Figure 4B, the expressions of Bax, Cleaved caspase3, and Cleaved caspase9 were inhibited and the levels of Bcl-2 were increased after treatment with GTS-21. Nevertheless, the levels of Bax, Cleaved caspase3, and Cleaved caspase9 were rescued and the expression of Bcl-2 was suppressed after the application of a-Bgt.
Figure 3. GTS-21-induced α7 nAChR alleviated the OGD/R-induced oxidative damage of IEC-6 cells. (A) Flow cytometry was performed to detect the ROS levels of GTS-21-treated IEC-6 cells (n=3, ±SD). (B, C) The expression of SOD and MDA in IEC-6 cells was measured after treatment with GTS-21. (n=3, ±SD). * p<0.05; ** p<0.01; *** p<0.001.

Discussion

Intestinal ischemia-reperfusion injury is an important obstacle in the course of surgery and other treatments, and it can also cause dysfunction and failure of diverse organs [1]. Intestinal ischemia-reperfusion injury is mainly caused by intestinal epithelial cell apoptosis. However, intestinal mucosa epithelial tissue injury weakens its protective effect on the intestinal tract, which can lead to bacterial infection and other symptoms [22]. Furthermore, during the process of intestinal ischemia and reperfusion, the expression of proinflammatory factors (IL-1β and IL-18) is significantly increased, which can lead to local or systemic inflammatory response, eventually damaging the liver, lungs, and other organs [23]. α7 nAChR is a key protein in the nervous system, and a study revealed that it can suppress the expression of proinflammatory factors induced by LPS [24]. Research also indicates that α7 nAChR regulates the inflammation-related pathway by repressing the expression of NF-κB in mice [25]. Subsequent studies have shown that GTS-21, as an activator of α7 nAChR, also plays a critical role in the anti-inflammatory process. Therefore, GTS-21 can also act as an anti-inflammatory factor [26,27]. In our study, we found that the expression of α7 nAChR in enterocytes was gradually inhibited when these cells were cultured under OGD/R conditions. After treatment with GTS-21, the expression of proinflammatory factors (TNF-α, IL-1β, and IL-6) was suppressed in intestinal epithelial cells. Moreover, the application of a-Bgt, which is the antagonist of α7 nAChR, increased the levels of TNF-α, IL-1β, and IL-6. These results indicated that the GTS-21-induced α7 nAChR can alleviate the intestinal ischemia-reperfusion-induced inflammatory response.

Oxidative damage is the other kind of injury induced by the ischemia-reperfusion [2]. A study suggested that activation of α7 nAChR protects the nervous system from oxidative damage [28]. Research also revealed that α7 nAChR alleviated the neurovirulence induced by higher levels of ROS [29]. In this study, we found that the GTS-21-induced α7 nAChR suppressed the production of ROS, and the activation of α7 nAChR also enhanced the expression of SOD, which can protect tissue from oxidative damage. We also found that the levels of MDA, which is the metabolite of lipid peroxidation, decreased. These results suggest that GTS-induced α7 nAChR protected the intestinal epithelial cells from oxidative damage.

As detailed above, the apoptosis of intestinal epithelial cells resulted in the reduced protective effect of intestinal mucosa on the intestinal tract. A study revealed that higher levels of α7 nAChR relieved the ischemic stroke-induced apoptosis of neurons [30]. Research also found that α7 nAChR relieved the β-amyloid peptide-induced apoptosis of neuroblastoma cells [31]. In this study, we revealed that the apoptosis rates of intestinal epithelial cells decreased after treatment with GTS-21, and the expression of Bax, Cleaved caspase3, and Cleaved caspase9 was also repressed. These results indicate that the GTS-21-induced α7 nAChR reduced the OGD/R-induced apoptosis of intestinal epithelial cells.
**Figure 4.** (A-C) GTS-21-induced α7 nAChR relieved the apoptosis of IEC-6 cells which was caused by OGD/R. The apoptosis rates of IEC-6 cells were measured with flow cytometry (n=3, ±SD). The expression of apoptosis-related proteins (Bax, Bcl-2, caspase3, Cleaved caspase3, caspase9, and Cleaved caspase9) in IEC-6 cells was determined with Western blotting. (n=3, ±SD). * p<0.05; **p<0.01; ***p<0.001.
Conclusions:

We revealed that α7 nAChR can alleviate the OGD/R-induced inflammatory response, oxidative damage, and apoptosis of intestinal epithelial cells. Treatment with GTS-21, which is the agonist of α7 nAChR, can further promote this protective effect in intestinal epithelial cells. The results from this study also provide a new strategy for the treatment of intestinal tract injury caused by ischemia-reperfusion.

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Conflict of interest

None.