Original Article
Salvianolic acid B ameliorates hydrogen peroxide-induced barrier disruption in monolayer Caco-2 cells

Chi Ma1*, Liangang Shi1*, Hongshuo Zhang2*, Yujie Zhao2, Zhenlong Yu3, Jingbo Yu1, Changkai Sun3,4, Dong Wang1

1Dalian Central Hospital Affiliated to Dalian Medical University, Dalian, China; 2Department of Biochemistry, Institute of Glycobiology, Dalian Medical University, Dalia, China; 3School of Pharmacy, Dalian Medical University, Dalian, China; 4Research Institute of Integrated Traditional and Western Medicine, Dalian Medical University, Dalian, China. *Equal contributors.

Received March 8, 2016; Accepted July 19, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Disruption of intestinal epithelial barrier is crucial for the pathogenesis of a variety of intestinal and systemic disorders. Salvianolic acid B (Sal B), a natural bioactive component from Salvia miltiorrhiza, has been reported to exert protection in various types of cells. In the present study, we investigated the protective effects of Sal B on the barrier function of Caco-2 cell monolayers exposed to hydrogen peroxide (H2O2). Cell metabolic activity and barrier integrity were assessed by measuring lactate dehydrogenase (LDH) release, cellular morphology change, transepithelial electrical resistance (TEER), and the expressional levels of the tight junction proteins including Claudin-1, Occludin and zonula occludens-1 (ZO-1). Administration of Sal B prevented H2O2-induced changes in cell morphology, LDH release, production of glutathione (GSH), superoxide dismutase (SOD), TEER and the localization of Claudin-1, Occludin and ZO-1 proteins. These results suggest that Sal B protects the barrier of intestinal epithelia from oxidative stress-induced damage. In this study, we have demonstrated for the first time that Sal B was protective against H2O2-induced barrier disruption in Caco-2 cell monolayer model. We determined the functional roles of Sal B from multiple levels, including cellular morphology, ultrastructure, and metabolisms, which were all collectively support the hypothesis that Sal B ameliorates oxidative stress-induced barrier disruption in intestinal epithelia.

Keywords: Salvianolic acid B, hydrogen peroxide, intestine, epithelial barrier, Caco-2 cells, tight junctions

Introduction

Intestinal epithelial barrier is a highly selective permeability barrier restricting the passage of harmful pro-inflammatory and toxic molecules into the mucosa and systemic circulation. The integrity of intestinal epithelial barrier is maintained by intracellular junctional complexes, including tight junction proteins (TJs), adherent junctions, and desmosomes [1-5]. TJs, a multifunctional complex, consists of at least three types of transmembrane proteins (claudin, occluding, and junctional adhesion molecule), forming the molecular basis of tight junctions in epithelial cells. These proteins interacts with cytoplasmic proteins zonula occludens (ZO), F-actin filaments, and myosin II to attach the tight junction strands to the cytoskeleton [6-8]. Disruption of TJs and elevated permeability to luminal toxins, allergens, and pathogens play a critical role in the pathogenesis of a number of gastrointestinal diseases such as inflammatory bowel disease, celiac disease, and alcohol liver disease [9-11]. Pro-inflammatory factors such as reactive oxygen species [12-14], cytokines [15, 16], and toxins [17] disrupt the TJs and the barrier function of the intestinal epithelium. Hydrogen peroxide (H2O2) disrupts TJs in the Caco-2 cell monolayers by the mechanisms involving activation of phosphatidylinositol 3-kinase and c-Src [14], and phosphorylation of TJs proteins [18]. Preventing the inflammation-mediated disruption of TJs and barrier function may provide potential therapeutic approaches for the treatment of many gastrointestinal diseases.
Salvianolic acid B (Sal B), a pure bioactive compound extracted from Salvia miltiorrhiza, is known for its broad pharmacological potential, including neuro- and cardio-protective properties by inhibiting lipid peroxidation and superoxide anion production [19, 20]. Sal B suppresses platelet aggregation, inhibits tumor necrosis factor-induced matrix metallo-proteinase-2 up-regulation, and improves coronary microcirculation and cerebral blood flow, as well as inhibiting myocardial ischemia [20-23] via modulation of Mtor-4EBP1, MKK3/6-p38, MAPK-ATF2 and ERK1/2 signaling pathways [24]. However, the functional role of Sal B on H$_2$O$_2$-induced intestinal epithelial barrier disruption still remains unclear.

Caco-2 cell, a human intestinal epithelial cell line, is a well-known monolayer model for investigating the intestinal epithelial barrier integrity and function in vitro [25-27]. In the present study, we determined the effects of Sal B against H$_2$O$_2$-induced Caco-2 cell injury and barrier disruption. To our knowledge, this is the first report showing that Sal B acts directly on intestinal epithelial cells to protect the barrier function against oxidative stress.

Materials and methods

Materials

Sal B was obtained from Dr. Xiaochi Ma. Caco-2 cells were provided by Dr. Zhonggui He. Millicell transwell inserts were purchased from Millipore (Minneapolis, USA). Cell culture reagents were from Gibco (Grand Island, NY, USA).

Caco-2 cell culture

Caco-2 cells were cultured as described previously [18] with minor modifications. Briefly, Caco-2 cells were routinely maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acid solution, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. All cell cultures were maintained in a humidified incubator at 37°C with an atmosphere of 5% carbon dioxide in air. Culture medium was changed every other day and cells were plated on 24-well transwell inserts at a density of 5×10$^5$ cells/well when the cells reached confluence. The cells were treated on day 21 after seeding onto the transwells.

Assessment of cell damage

Caco-2 cells were pre-treated with Sal B (1, 10, 100 µmol/L) or PBS (control) for 12 h, followed by H$_2$O$_2$ (800 µmol/L) treatment for 12 h. Epithelial monolayers were then photographed with a Nikon camera under an inverted phase contrast microscope (Leica, Germany).

Determination of epithelial cell viability

The cell viability was determined using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg/ml) reduction assay. Caco-2 cells were seeded in 96-wells plates at a concentration of 1×10$^4$ cells/well in DMEM culture medium and cultured at 37°C for 24 h. Subsequently, the cells were treated with Sal B (1, 10, 100 µmol/L) for 12 h, followed by H$_2$O$_2$ treatment for 24 h (800 µmol/L). PBS buffer was employed as a negative control. After 24 h of incubation at 37°C, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, 0.5 mg/ml) was added to the cells and incubated for another 4 h. The media was discarded and 100 µL of DMSO was added to dissolve the formazan crystals formed in intact cells. The absorbance of formazan was measured with a microplate reader at 570 nm. Experiments were repeated independently three times. The results are expressed as percentage of cell viability.

Measurement of lactate dehydrogenase (LDH) activity

Caco-2 cells were pretreated with Sal B (1, 10, 100 µmol/L) or PBS for 12 h prior to the exposure of H$_2$O$_2$ (800 µmol/L, 12 h). LDH in the cell culture media, an indicator of cell injury, was detected by chromatometry with an assay kit according to the manufacturer’s instruction (Nanjing Jiancheng Biochemical Reagent Co., Nanjing, China).

Measurement of glutathione (GSH) content and superoxide dismutase (SOD) activity

Caco-2 cells were pre-treated with Sal B (1, 10, 100 µmol/L) or PBS for 12 h, followed by H$_2$O$_2$ exposure (800 µmol/L) for 12 h. The GSH levels and SOD activity in Caco-2 cells were deter-
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

Measurement of transepithelial electrical resistance (TEER)

Monolayers of Caco-2 cells were grown on the membrane of transwells (0.4 μm pore size; 0.6 cm² effective membrane area). At 100% confluence, TEER was measured using Millicell-ERS (Millipore Corporation, Bedford, MA, USA) when the resistance was stable (>240 Ωcm²). The resistance was calculated as follows: 

\[ \text{TEER} (\Omega \text{cm}^2) = (\text{Total resistance} - \text{Blank resistance}) \times \text{Area} (\text{cm}^2) \] (13).

Transmission electron microscopy

Monolayers of Caco-2 cells grown on transwell membranes were fixed with 2.5% glutaraldehyde for 2 h at 4°C. After washing in 0.2 M Na cacodylate/HCL (pH 7.4) for three times, cells were fixed with 1% \( \text{OsO}_4 \)-0.15 M Na cacodyl-

Figure 1. A. Effects of Sal B on \( \text{H}_2\text{O}_2 \)-induced cell injury. Caco-2 cells were treated with \( \text{H}_2\text{O}_2 \) (800 μmol/L) for 12 h with or without pretreatment of Sal B (100 μmol/mL). Epithelial monolayers were then photographed with a Nikon camera under an inverted phase contrast microscope (200×). Data are representative and are from three independent experiments. B. Effects of Sal B on \( \text{H}_2\text{O}_2 \)-induced loss of cell viability. Caco-2 cells were exposed to 800 μmol/L \( \text{H}_2\text{O}_2 \) for 12 h and cell viability was measured by MTT assay. Data are expressed as mean ± S.D. monolayers n=3. 

#P<0.05 vs. control, **P<0.01 vs. \( \text{H}_2\text{O}_2 \), ***P<0.001 vs. \( \text{H}_2\text{O}_2 \).
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

ate/HCL (pH 7.4) for 30 min. The cells were then dehydrated in graded ethanol and embedding in Araldite epoxy resin. Thin sections (1 μm) were cut and stained with 1% toluidine blue in 1% sodium borate. Areas selected for ultrastructural observations were cut into ultra-thin sections (80 nm in thickness) and stained with saturated uranyl acetate and Reynold’s lead citrate. Images were taken with an H7000 (Hitachi, Japan) transmission electron microscope. At least three images from each sample were analyzed by three people in a blinded fashion [22].

*Immunocytochemical study of TJs protein expression in Caco-2 cells*

Caco-2 cell monolayers on transwell membranes were permeated with 0.5% NP-40, and blocked with 5% normal goat serum (Gibco BRL Co. Ltd., USA). Cells were fixed with 3.5% paraformaldehyde in PBS and incubated with a specific primary antibody (Santa Cruz Biotechnology, USA) for ZO-1, Occludin, or claudin-1 for 30 min at room temperature, washed, and then incubated with HRP-conjugated goat anti-rabbit IgG antibody. Primary antibodies (rabbit monoclonal anti-human ZO-1, Occludin, and Claudin-1, Santa Cruz Biotechnology, USA) were diluted 1:20 to 1:100 in 2% bovine serum albumin-PBS. Monolayers were washed four times with saline and then color developed using diaminobenzidine solution and stained with hematoxylin. The transwell membranes were mounted onto the slides using DPX (BDH Laboratories; Poole, UK).

*Immunoblot study of TJs protein expression in Caco-2 cells*

Caco-2 cells, and Caco-2 cells which were pre-treated with Sal B (100 µmol/L) or PBS for 12 h, followed by H$_2$O$_2$ exposure (800 µmol/L) for 12 h, were prepared for western blot analysis.
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

Standard Western blotting was performed using a rabbit antibody against human ZO-1 (1:1000), Occludin (1:1000), and Claudin-1 (1:1000), and then incubated with HRP-conjugated goat anti-rabbit IgG antibody. Equal protein sample loading was monitored by probing the same membrane filter with an anti-GAPDH antibody (Santa Cruz Biotechnology, USA).

Statistical analysis

Statistical analysis was performed with SPSS 13.0 software. Results are shown as means ± SD. The data were statistically evaluated using one-way ANOVA followed by Student’s t test when only two value sets were compared, and Dunnett’s test when the data involved three or more groups. P<0.05 was considered to be statistically significant.

Results

Sal B inhibits cell morphology and cell viability change induced by H₂O₂

As shown in Figure 1A, most Caco-2 cells are round in shape with decreased attaching ability to the culture surface after exposed to H₂O₂ for 12 h. Pretreatment with Sal B significantly prevented the morphology change of Caco-2 cells induced by H₂O₂. After exposed to H₂O₂, Caco-2 cells viability dramatically decreased as compared to control group (P<0.05) (Figure 1B). Sal B significantly increased cell viability in a dose-dependent manner (P<0.05). At the concentration of 100 µmol/mL, Sal B rescued the cell viability close to the levels of control.

Sal B inhibits H₂O₂-induced LDH release, changes of GSH levels, and regulates SOD activity

To further investigate the protective effects of Sal B on H₂O₂-induced cell damage, the levels of LDH in cultured media of Caco-2 cells were determined. As shown in Figure 2A, there was significantly increase in LDH levels after the exposure to H₂O₂ for 12 h. Pre-incubation of CaCo-2 cells with Sal B attenuated H₂O₂-induced LDH release in a dose-dependent manner. H₂O₂-induced cell damage was usually accompanied with the increase of lipid peroxides. As shown in Figure 5, the levels of GSH in Caco-2 cells were significantly decreased after the stimulation with 800 µmol/L H₂O₂ for 12 h. However, H₂O₂-induced reduction of GSH was remarkably inhibited by Sal B in a dose-dependent manner (Figure 2B). The activity of SOD, an important antioxidant enzyme preventing cellular damage caused by ROS, significantly

Figure 3. A. Effects of Sal B on H₂O₂-induced alteration of TEER in Caco-2 cells. Caco-2 monolayers were treated with H₂O₂ (800 µmol/L) for 3 h, 6 h, 9 h, and 12 h with or without pretreatment with Sal B (1, 10, 100 µmol/L). Data are expressed as mean ± S.D. of monolayers n=3. ###P<0.01, ####P<0.001 compared with H₂O₂ treatment. B. Effects of Sal B on the changes of ultrastructure of TJs induced by H₂O₂. Caco-2 cell monolayers were treated with H₂O₂ (800 µmol/L) for 12 h, with or without pretreatment of Sal B (100 µmol/L). Images were taken with an H7000 transmission electron microscope (15000×). Data are representative of 3 independent experiments. (B1: control; B2: H₂O₂ treatment; B3 & B4: Sal B & H₂O₂ treatment).
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

9023

Int J Clin Exp Pathol 2016;9(9):9018-9027

decreased when the Caco-2 cells were exposed to H₂O₂ (800 µmol/L) for 12 h. Pretreatment with Sal B inhibited the decline of SOD activity in the setting of H₂O₂ exposure (Figure 2C).

Sal B attenuates H₂O₂-induced decrease of TEER and morphology disruption of TJs

To investigate the protective effects of Sal B on intestinal barrier function, we determined the values of TEER in monolayer Caco-2 cells, an indicator of epithelial paracellular permeability to ions. H₂O₂ (800 µmol/L) treatment of monolayer of Caco-2 cells caused significantly decrease in TEER at 9 and 12 h after stimulation (Figure 3A). Pretreatment of the Caco-2 cells with Sal B (10 µmol/L, and 100 µmol/L) significantly inhibited the H₂O₂-induced reduction of TEER. H₂O₂ exposure resulted in the disruption of TJs morphology of Caco-2 monolayers. After stimulation with H₂O₂, TJs complexes in Caco-2 cells were reduced and TJs became markedly “open” with less electron-dense material, although there is no obvious disruption of desmosomes (Figure 3B). Sal B treatment significantly prevented the disruption of TJs in the setting of H₂O₂ exposure.

Sal B prevents H₂O₂-induced redistribution of Claudin-1, Occludin and ZO-1 proteins

Previous studies have shown that intestinal barrier dysfunction induced by ROS is associated with the morphological tight junction disruption and the relocalization of tight junction proteins. To determine the effects of Sal B on the localization of TJs proteins, immunohistochemistry method was used to investigate the expression of ZO-1, occludin and claudin-1. As shown in Figure 4, ZO-1, occludin, and claudin-1 were expressed at intercellular tight junctions under control conditions. Administration of Sal B alone did not alter the localization of these TJs proteins in Caco-2 cells (data was not shown). After exposed to H₂O₂, the distribution of TJs became irregular and discontinuous. However, pretreatment of Sal B significantly prevented there distribution of ZO-1, occludin.
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

and claudin-1 induced by H₂O₂ in Caco-2 cells. And to determine the effects of Sal B on the expression of TJs proteins, we used western blot to investigate the expression of TJs at proteins level, as shown in Figure 5.

Discussion

Intestinal epithelial cell layers are the first line of defense against the entry of luminal antigens. The importance of maintaining the integ-

Figure 5. A. Effects of Sal B on H₂O₂-induced expression of TJs proteins. Caco-2 cells were pretreated with PBS (control) or Sal B (100 µmol/L) for 12 h, followed by H₂O₂ (800 µmol/L) treatment for 12 h. The expression of TJs proteins (Claudin-1, Occludin, ZO-1) were detected by western blot. B. Relative intensity analysis. Data are expressed as mean ± S.D. n=3. #P<0.05 vs. control, ##P<0.01 vs. control, ###P<0.001 vs. control, *P<0.05 vs. H₂O₂, **P<0.01 vs. H₂O₂, ***P<0.001 vs. H₂O₂.
Salvianolic acid B and hydrogen peroxide-induced barrier disruption

Int J Clin Pathol 2016;9(9):9018-9027

...rity of TJs is underscored by large body of studies indicating that increased intestinal permeability contributes to the pathogenesis of several intestinal disorders, including inflammatory bowel disease [3, 28, 29]. Preventing oxidative stress-induced disruption of the integrity of intestinal barrier will provide new therapeutic alternatives in multiple gastrointestinal diseases. In the present study we demonstrated that Sal B plays a critical role in preventing \(H_2O_2\)-induced cell damage, alteration of TEER, morphology change of TJs, and relocation of Claudin-1, Occludin and ZO-1 proteins in Caco-2 monolayer model.

Human colon cancer-derived Caco-2 monolayer model is a well-established in vitro model applied to determine the alteration of cell permeability and distribution of TJs proteins [30]. Oxidative stress is one of the major insults inducing intestinal barrier disruption. Previous study showed that \(H_2O_2\) causes a robust decrease in TEER in Caco-2 cells monolayers [31], which is consistent with our present study showing that \(H_2O_2\) treatment reduced TEER in a time-dependent manner. \(H_2O_2\) disrupts the TJs and increases paracellular permeability [26, 31, 32] by enhancing tyrosine phosphorylation of TJs proteins in Caco-2 cells monolayers [32]. Some studies also demonstrated that p38 MAP kinase [33-35] and ERK1/2 [36] mediate \(H_2O_2\)-induced TJ barrier disruption. In addition, \(H_2O_2\) also induces the re-distribution of TJs proteins, such as Claudin-1, Occludin and ZO-1 leading to the disruption of barrier, which is consistent with our current study.

Preventing oxidative stress-induced intestinal barrier disruption will provide more effective treatment regimens for these oxidative inflammatory disorders. Previous studies have shown that epidermal growth factor (EGF) protects intestinal barrier integrity and microtubule cytoskeleton against oxidant [37-39]. Nitric oxide also has been shown to attenuate \(H_2O_2\)-induced barrier disruption via preventing tyrosine phosphorylation of TJs proteins [40]. Salvianolic acid is one of the bioactive compounds of Salvia miltiorrhiza. Among salvianolic acids, Sal A and Sal B are the most abundant components. The therapeutic potential of salvianolic acids on hepatic protection [41], neural protection [42], cancer treatment [43, 44], and cardiovascular protection [45] have been proposed in recent years. However, Sal B was thought to have much more commercial value for the food and medicine purposes due to the containment of the highest amounts in Salvia miltiorrhiza [46]. Sal B has been shown to exhibit strong antioxidant activity by scavenging reactive oxygen species including HO., O2.-, DPPH radicals, and \(H_2O_2\) [47]. Our present study demonstrates that Sal B prevented \(H_2O_2\)-induced decrease in GSH levels and SOD activity and inhibited cellular LDH leaking as a result of lipid peroxidation. In addition, Sal B has been reported to inhibit cardiovascular inflammation via regulating MMP2/9 and ERK1/2 [48, 49], which could be one of the potential mechanisms accounting for the protective effects of Sal B on Caco-2 cells permeability under the challenge of \(H_2O_2\). In the present study, we observed that Sal B prevented \(H_2O_2\)-induced morphology change of TJs and the relocation of TJs proteins, including ZO-1, occludin and claudin-1. The protective effects of Sal B could arise from the inhibition of protein phosphorylation of TJs [18], which needs to be further investigated in future.

To our knowledge, this is the first report demonstrating that Sal B protects the integrity of intestinal epithelial barrier in the setting of \(H_2O_2\) stimulation. Further studies are required to elucidate the mechanisms employed by Sal B, in vitro and in vivo, to protect the intestinal epithelial barrier function against oxidative stress. The protective effects of Sal B on intestinal barrier function will provide a new therapeutic approach in the treatment of inflammatory bowel diseases.

Disclosure of conflict of interest

None.

Address correspondence to: Dong Wang, Dalian Central Hospital Affiliated to Dalian Medical University, Dalian, China. E-mail: wangdong_2016@126.com

References

Salvianolic acid B and hydrogen peroxide-induced barrier disruption


Salvianolic acid B and hydrogen peroxide-induced barrier disruption


