Berberine reduces enteroglial-derived S100B production to alleviate gut vascular barrier damage

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Abstract

Gut vascular barrier (GVB) controls the systemic dissemination of bacteria. Enteric glial cells (EGCs) in mucosa as a component of gut vascular unit can amplify inflammation via S100B. Berberine (BBR) is an alkaloid that can alleviate gut inflammation and regulate EGCs activity. In this study, we aimed to unravel the mechanism by which BBR inhibited EGCs-derived S100B generation to protect GVB. In vivo BBR was given via oral gavage to mice who were subjected to 40% total body surface area burns. In vitro BBR, S100B and caspase-8 inhibitor Z-IETD-FMK were used to treat mouse intestinal microvascular endothelial cells (MIMECs). The results showed that (1) BBR lowered serum S100B concentration and decreased S100B accumulation in colonic mucosa; (2) BBR and S100B monoclonal antibody (S100BmAb) lowered burn-induced GVB hyperpermeability; (3) BBR antagonized the effect of S100B enema on GVB permeability; (4) S100B and Z-IETD-FMK increased the permeability of MIMECs and reduced the expression of caspase-8, β-catenin, occludin and VE-cadherin in MIMECs, while these effects of S100B were effectively antagonized by BBR treatment; (5) Z-IETD-FMK inhibited β-catenin expression in MIMECs. Collectively, our findings showed that S100B accumulation in gut mucosa damaged GVB, and BBR treatment alleviated GVB damage via modulation of caspase-8/β-catenin signaling pathways.

Introduction

GVB is an anatomical barrier controlling the systemic dissemination of bacteria \(^1\). EGCs in mucosa are the component of gut vascular unit (GVN). EGCs are a large population in enteric nervous system and classified into the activated, reactive and gliopathy phenotypes \(^2\). Activated phenotype is defined as a response to the physiological stimuli while reactive phenotype is to the pathological stimuli with a pathophysiological perturbation of any severity \(^2\). Reactive phenotype may be beneficial via neurotrophins such as GNSO and GDNF \(^3,4\). However, reactive phenotype are often deleterious due to its pro-inflammatory functions via proinflammatory cytokines at the expense of homeostatic functions \(^2\).

In human intestine, among S100 proteins, only S100B is specifically expressed by EGCs \(^5\). S100B protein is a small and easily diffusible molecule which plays a role in regulating intracellular levels of calcium. S100B protein exerts either the trophic or detrimental effect depending on its extracellular level. High concentration (µM) of S100B is proinflammatory. In a pathological condition, reactive EGCs can trigger, support and amplify gut inflammation via S100B protein \(^5-8\). Many studies have supported S100B may induce gut flora dysbiosis \(^7,9\), epithelial barrier disruption \(^5,9\) and blood-brain barrier (BBB) impairment \(^10,11\).

BBR is an alkaloid that inhibits gut inflammation and bacteria dissemination \(^12-14\) via the modulation of microbial \(^14\), epithelial \(^12,15\) and vascular \(^16,17\) barriers. Accumulating evidence shows that berberine can attenuate ulcerative colitis and NSAIDs-induced intestinal mucosal injury through regulation of EGCs \(^18,19\). In this study, we probed into the effect of berberine on EGCs-derived S100B production and intestinal microvascular endothelial barrier in vivo and in vitro. The results showed that berberine
alleviated GVB impairment via reducing S100B overexpression in gut mucosa and modulating caspase-8/β-catenin pathways.

Results

Berberine lowered S100B concentration in burn serum

Some studies have reported that burn serum is rich in proinflammatory molecules. We measured TNF-α, IL-β and S100B concentration in sham and burn serum. Data showed that these cytokines were low in sham serum while significantly high in burn serum. Berberine effectively lowered serum TNF-α, IL-1β and S100B concentration in burned mice (Fig. 1).

Berberine reduced S100B accumulation in colonic mucosa

The accumulation of S100B in colonic mucosa was detected by ELISA. Data showed that S100B content in mucosa rapidly elevated in 0-24 h, and kept a steady state in 24-36 h, and then slowly declined in 36-48 h postburn. Berberine treatment in a dose-dependent manner decreased S100B accumulation (Fig. 2).

Berberine and S100BmAb lowered gut-vascular barrier permeability in burned mice

The effect of increased S100B in mucosa on GVB permeability was assessed. The results showed burns increased GVB permeability, as demonstrated by a significant rise in serum FITC-dextran 70 kDa. S100BmAb dose-dependently lowered burn-elicited GVB permeability. Berberine also in a dose-dependently manner lowered GVB permeability (Fig. 3).

Berberine lowered S100B-increased gut-vascular barrier permeability

We evaluated the effect of S100B enema on GVB permeability. Data showed S100B enema increased GVB permeability of control mice. S100B enema also enhanced the effect of burns on GVB permeability. Berberine treatment abolished the upregulatory effect of S100B enema or in combination with burns leading to a reduction of GVB permeability (Fig. 4).

Berberine lowered S100B-increased endothelial permeability of MIMECs

Above in vivo experiments confirmed S100B and S100BmAb influenced GVB permeability. The effect of S100B on the permeability of MIMECs in vitro was also assessed. The results revealed the permeability of MIMECs was raised by S100B protein. By contrast, berberine abolished the effect of S100B leading to a reduction of MIMECs permeability (Fig. 5).

Berberine increased S100B-decreased occludin and VE-cadherin in MIMECs

Endothelial barriers are characterized by the presence of junctional complexes that include tight junction (TJ) and adherens junction (AJ). Occludin and VE-cadherin are the members of TJ and AJ protein. Our experimental data showed that S100B protein reduced occludin and VE-cadherin, while berberine
treatment reversed the effects of S100B protein resulting in the upregulation of occludin and VE-cadherin in MIMECs (Fig. 6).

**Berberine increased S100B-decreased caspase-8 and β-catenin in MIMECs**

Caspase-8 is a pro-survival factor in gut endothelial cells, and knockdown of it leads to GVB hyperpermeability. β-catenin plays a key role in GVB integrity. We found *in vitro* S100B decreased caspase-8 and β-catenin in MIMECs, while this effect of S100B was antagonized by berberine (Fig. 6). Z-IETD-FMK increased the permeability of MIMECs (Fig. 5) and the immunoblot analysis showed it was able to decrease β-catenin protein in MIMECs (Fig. 7).

**Discussion**

GVB has recently identified as the second barrier, just beneath the epithelial barrier, in intestinal mucosa in human and mice. GVB controls the systemic dissemination of bacteria and unwanted molecules. Disruption of GVB is closely associated with some diseases. Therefore, the maintenance of GVB integrity plays an important role in homeostasis. Besides regulating the function of neurons and muscle contraction, EGCs are involved in modulating gut microbial, immune and epithelial barrier. According to the criteria, EGCs in response to burns are classified into the reactive phenotype. Reactive EGCs may be beneficial in the early period of pathological conditions. However, reactive EGCs are often deleterious because enteroglial-derived S100B (µM) can amplify inflammation and injury epithelial barrier.

Berberine is an alkaloid that is hardly absorbed so that it can be mostly retained to affect the digestive tract. Berberine has the strong anti-bacterial and anti-inflammatory properties. In addition, berberine has been recently reported to protect gut barrier via modulation of EGCs. In this experiment, we found that severe burns increased serum S100B, TNF-α and IL-1β concentration and induced a significant S100B accumulation in colonic mucosa. By contrast, berberine treatment not only lowered serum S100B, TNF-α and IL-1β levels, but also reduced S100B accumulation in gut mucosa.

Many similarities have been found between blood-brain barrier (BBB) and GVB. Severe burns may lead to BBB open, tight junctional structure breakdown and hyperpermeability. High concentration of S100B plays a key role in BBB disruption. To confirm the hypothesis that high S100B would compromise GVB, we treated burned mice with different doses of S100BmAb. Data showed that burns induced GVB hyperpermeability and S100BmAb in a dose-dependent manner lowered GVB permeability. Subsequently, we injected S100B protein (µM) via rectal enemas to colonic lumen. The results demonstrated that S100B enema raised GVB permeability in sham and burned mice. Berberine concentration-dependently reduced burn-induced GVB permeability, which was in line with previous reports. In the model of S100B enema, berberine also lowered GVB permeability of S100B-treated and burns/S100B-cotreated mice. *In vitro* study, S100B concentration-dependently increased the permeability of MIMECs, while this effect of S100B was alleviated by berberine treatment.
The increase in GVB permeability of mice in vivo and in endothelial permeability of MIMECs in vitro reflected the dysfunction of endothelial barrier. The endothelial barriers are characterized by the presence of junctional complexes that include the tight junction (TJ) and adherens junction (AJ), which control paracellular trafficking of solutes and fluids. To study the change of GVB characteristics, we analyzed the composition of TJ and AJ in MIMECs. In vivo experiments MIMECs were exposed to S100B protein (µM level). The experimental data demonstrated S100B protein effectively decreased the expression of TJ protein occludin and AJ protein VE-cadherin in MIMECs. Accordingly, high concentration of S100B could impair the endothelial barrier structure.

Caspase-8 is a unique member of caspases with a dual role in cell death and survival. Caspase-8 can activate caspase-3 to induce cell apoptosis. However, it also represents the molecular switch that controls apoptosis, necroptosis and pyroptosis. Caspase-8 has been identified to be a pro-survival factor for gut epithelial cells and mature gut endothelial cells. Caspase-8 is required to actively maintain vascular homeostasis in small bowel. Thus, loss of caspase-8 in gut endothelial cells leads to gut hemorrhages, inflammation, and GVB hyper-permeability to FITC-dextran 70 kDa. Caspase-8 deficiency also causes embryonic lethality in mice. In this experiment, Z-IETD-FMK, a caspase-8 inhibitor, was found to aggravate S100B-increased endothelial permeability of MIMECs. Subsequent experiments revealed that Z-IETD-FMK decreased expression of TJ protein occludin and AJ protein VE-cadherin in MIMECs, suggesting caspase-8 played a beneficial effect on gut endothelial integrity. S100B protein reduced the protein production of caspase-8, while this inhibitory effect of S100B was reversed by berberine in MIMECs.

Wnt/β-catenin signaling pathway is of importance for the maintenance of GVB integrity. The association between caspase-8 and Wnt/β-catenin signaling has been documented in previous studies. Alkaloid berberine has been found to reduce/inhibit caspase-8 or increase/activate caspase-8. Thus, in this experiment we assessed the effect of S100B on caspase-8 and β-catenin in MIMECs and found S100B (µM) reduced caspase-8 and β-catenin protein expression. Inhibition of caspase-8 pathway by Z-IETD-FMK decreased β-catenin, suggesting that caspase-8 regulated β-catenin signaling in MIMECs. Berberine treatment increased β-catenin in S100B-treated MIMECs, indicating that it antagonized the effect of S100B on Wnt/β-catenin signaling.

In conclusion, our findings showed that aberrant S100B accumulation in colonic mucosa was harmful to GVB integrity. Berberine treatment reduced S100B accumulation to protect GVB through modulation of caspase-8/β-catenin signaling pathways.

Materials And Methods

Experimental animals

Male C57BL/6J mice (6-week-old, 22-26 g) were purchased from the Laboratory Animal Center of Southern Medical University, China. Mice were housed in a 21-22 °C temperature and humidity...
environment with a 12-h/12-h cycle and free access to chow diet and water. This study was approved by the Animal Care and Use Committee of Southern Medical University. The experiments were performed in accordance with the guidelines and regulations of the Laboratory Animal Center of Southern Medical University. All researchers comply with the ARRIVE Guidelines 2.0.

Scald burns induction

The protocols producing full-thickness cutaneous burns covering 40% of total body surface area (TBSA) in mice were previously described. In brief, mice were deeply anesthetized with 3% isoflurane. 40% TBSA burn injury was generated by applying brass probes (2 × 3 cm with 3 mm thickness) that were preheated to 100 °C in boiling water to the animal's sides and back for 5 s. Sham animals were subjected to a room temperature brass probes. All mice were resuscitated with 2-mL saline and returned to their cages and monitored the complications.

S100BmAb, recombinant S100B and berberine treatment

Mouse S100B monoclonal antibody (S100BmAb) (Invitrogen™, MA1-25005), dissolved in sterile saline, was i.v. injected (5, 10, 15 μg/kg, respectively) into burned mice. S100B protein (Absin®, abs04491) was dissolved in saline. 200-μl solution containing recombinant S100B (1 and 5 μM, respectively) was administrated via rectal enemas. Berberine chloride (BBB, > 98% purity, MedChemExpress, CAS: 633-65-8) dissolved in saline was administrated via oral gavage at the dose of 25 and 50 mg/kg/d, respectively, for 3 d prior to burns.

Gut vascular barrier permeability

A laparotomy was performed on the anesthetized mice. Two ligatures were placed in cecum to make a gut loop. 100-μl of FITC-dextran 70 kDa (1 mg/ml) was injected into loops to assess GVB. The blood was collected by a cardiac puncture. Blood samples were centrifuged (8000 rpm, 10min, 4°C) to obtain serum. FITC-dextran in serum was assayed by the spectro-photometer (Infinite M200 PRO, Tecan, Germany) at 480nm excitation and 520nm emission.

S100B content in serum and colonic mucosa

Gut mucosal scrapings were obtained as previously described. S100B content in serum and colonic mucosa was detected at indicated time-points by using ELISA kits according to the manufacturer's instructions.

Cell culture and treatment in vitro

MIMECs (Cat#: C1476) were purchased from ChenXue BioTech Co. Guangzhou. MIMECs were cultured in DMEM containing 15% fetal bovine serum, 100 U/mL of penicillin and streptomycin and 0.584 g/L of glutamine in a 37°C incubator under 5% CO₂. Cells were treated with 0.25% trypsin and 0.02% EDTA and
passaged twice. The subcultured cells were allowed to grow to 70% - 80% confluence before experiments.

**Endothelial permeability assays in vitro**

6.5-mm transwell inserts were used for endothelial permeability experiments. MIMECS were treated with different concentrations of S100B protein (LSBio, LS-G40136) or Z-IETD-FMK. 100-μl of HBSS containing 0.5 mg/ml of FITC-dextran 70 kDa was added into confluent MIMECs. 2-h late, FITC-dextran clearance across the filter to lower chamber was assayed by a spectrophotometer (Infinite M200 PRO, Tecan, Germany) at $\lambda_{em/ex} = 530/485$ nm. Data were expressed as arbitrary units.

**Western blotting**

Cells were harvested, washed twice with ice-cold PBS buffer and centrifuged at 180 × g for 10 min at 4 °C. Cell pellet was suspended in 100-μL ice cold lysis buffer. To lyse the cells, the suspension was rapidly passed through a syringe needle 5-6 times prior to centrifugation for 15 min at 13,000 × g to obtain cytoplasmic fraction. The cytosolic fraction proteins were mixed with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 2 mg bromophenol/mL) at a 1:1 ratio, boiled for 3 min and centrifuged at 10,000 × g for 10 min. Protein concentration was determined by a Bradford assay. 50 µg of each sample were electrophoresed on a 12% discontinuous polyacrylamide gel (PAG). Proteins were transferred onto the nitrocellulose membrane. The membrane was blocked with 5% milk/TBST (0.1% Tween), followed by incubating with following primary antibodies including occludin (1:1000, Santa Cruz), VE-cadherin (1:1000, Santa Cruz), β-catenin (1:500, Santa Cruz) and β-actin (1:1000, Santa Cruz) antibodies. Then, the membranes were incubated with secondary antibodies (1:2000, Santa Cruz). The immune complexes were revealed by the enhanced chemiluminescence detection reagents. The immunoblots were analyzed by a scanning densitometry ImageQuant LAS 4000 (GE Healthcare Life sciences).

**Statistical analysis**

All statistical analyses were performed by using the SPSS software (version 19.0; SPSS Inc., Chicago, IL). The results from tests of normality showed the data were normally distributed. Data were expressed as mean ± standard deviation (SD) or error (SEM). Significance among groups was tested by the analysis of Student's t-test or one-way variance (ANOVA) followed by Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

**Declarations**

**Data availability**

The datasets generated and analyzed in the present study are available from the corresponding author upon reasonable request.

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Author contributions

Y. H. and M.M analyzed the data, and wrote the paper. A. F. conceived the study, organized all data and revised the paper. Y. H. and Y. K. performed the animal experiments and collected the samples. Y. K. and J. Q performed and analyzed the laboratory experiments. J. Q. and J. Z. performed Western-blotting assays. All authors reviewed the manuscript.

Conflict of interest

All authors declare that they have no competing interests.

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Additional information

Correspondence and requests for materials and methods should be addressed to A. F.

References


Figures
**Figure 1**

**BBR lowered serum TNF-α, IL-1β and S100B concentration in mice with burns**

Mice were treated with sham, burns, and burns plus BBR, respectively. Blood samples were obtained from mice (n=18/group; 3 mice were killed at 2, 6, 12, 24, 36, and 48 h postburn, respectively). Serum TNF-α, IL-1β and S100B concentration was assayed by ELISA. Data were expressed as mean ± SD. **P < 0.01 vs. Sham; ##P < 0.01 vs. Burns.

**Figure 2**

**Mucosal S100B content (ng/mg)**
BBR reduced S100B accumulation in colonic mucosa in mice with burns

Colonic mucosal scrapings were obtained from mice treated with sham, burns, and burns plus BBR, respectively, at indicated time-points. S100B content in mucosal scrapings was assayed by ELISA. Data were expressed as mean ± SD. **P < 0.01 vs. Sham; ##P < 0.01 vs. Burns.

Figure 3

BBR and S100BmAb reduced the permeability of GVB in mice with burns

FITC-dextran 70 kDa was injected into colonic loops of mice treated with sham, burns, burns plus BBR, and burns plus S100BmAb, respectively. 48-h postburn, serum FITC-dextran 70 kDa was measured by a spectrophotometer. Data were expressed as mean ± SD. **P < 0.01 vs. Sham; ##P < 0.01 vs. Burns.
Figure 4

**S100B enema increased while berberine reduced the permeability of GVB**

Following S100B protein administration *via* rectal enema, colonic loops were made. FITC-dextran 70 kDa was injected into loops. 12-h after S100B enema, serum FITC-dextran 70 kDa was measured. Data were expressed as mean ± SD. **P < 0.01 vs. saline (control) or sham; ## P < 0.01 vs. S100B (5 μM) or burns; && P < 0.01 vs. burns+S100B (1 μM).**
S100B and Z-IETD-FMK increased while BBR decreased the transendothelial permeability of MIMECs

MIMECs were treated with saline (control), S100B, S100B plus Z-IETD-FMK, and S100B plus BBR, respectively. FITC-dextran 70 kDa was applied to evaluate the transendothelial permeability of MIMECs. The experiments were performed in triplicate. Data were expressed as means ± SEM. **P < 0.01 vs. control; # P < 0.01 vs. S100B (5 μM).
**Fig. 6**

**A**

- β-catenin
- occludin
- VE-cadherin
- caspase-8
- β-actin

MIMECs were treated with saline (control), S100B, and S100B plus BBR, respectively. Occludin, VE-cadherin, β-catenin and caspase-8 protein expression was assayed by Western blot. Representative immunoblots were shown in the left panel and statistical results in the right panel. The experiments were performed in triplicate. Data were expressed as means ± SEM. **P < 0.01 vs control; #P < 0.05 vs. S100B, ##P < 0.01 vs. S100B.

**B**

Relative density to β-actin

**Figure 6**

**S100B reduced while BBR increased occludin, VE-cadherin, beta-catenin and caspase-8 expression in RIMECs**

MIMECs were treated with saline (control), S100B, and S100B plus BBR, respectively. Occludin, VE-cadherin, β-catenin and caspase-8 protein expression was assayed by Western blot. Representative immunoblots were shown in the left panel and statistical results in the right panel. The experiments were performed in triplicate. Data were expressed as means ± SEM. **P < 0.01 vs control; #P < 0.05 vs. S100B, ##P < 0.01 vs. S100B.
**Fig. 7**

**A**

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**B**

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<tr>
<td>Relative density of β-catenin to β-actin</td>
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**Figure 7**

Z-IETD-FMK decreased β-catenin protein expression in MIMECs

MIMECs were treated with saline (control) and Z-IETD-FMK, respectively. Beta-catenin protein was determined by Western blot. Representative immunoblots were shown in the left panel and statistical results in the right panel. The experiments were performed in triplicate. Data were expressed as means ± SEM. *P < 0.05, **P < 0.01 vs. control.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Fulllengthandcroppedgelblots.pdf