Original Article
Adenosine A2B receptor modulates intestinal barrier function under hypoxic and ischemia/reperfusion conditions

Yang Yang[1*], Yuan Qiu[1*], Wensheng Wang[1], Weidong Xiao[1], Hongyin Liang[1], Chaojun Zhang[1], Hanwenbo Yang[3], Daniel H Teitelbaum[2], Li-Hua Sun[1], Hua Yang[1*]

[1]Department of General Surgery, Xinqiao Hospital, Third Military Medical University, Chongqing, China; [2]Department of Surgery, University of Michigan, MI, USA; [3]University of Michigan, Ann Arbor, MI, USA. *Equal contributors.

Abstract: Background: Intestinal barrier function failure from ischemia/reperfusion (I/R) and acute hypoxia has been implicated as a critical determinant in the predisposition to intestinal inflammation and a number of inflammatory disorders. Here, we identified the role of Adenosine A2B receptor (A2BAR) in the regulation of intestinal barrier function under I/R and acute hypoxic conditions. Methods: C57BL/6J mice were used, and were randomized into three groups: Sham, I/R, IR+PSB1115 (a specific A2BAR antagonist) groups. After surgery, the small bowel was harvested for immunohistochemical staining, RNA and protein content, and intestinal permeability analyses. Using an epithelial cell culture model, we investigated the influence of hypoxia on the epithelial function, and the role of A2BAR in the expressions of tight junction and epithelial permeability. The expressions of Claudin-1, occludin and ZO-1 were detected by RT-PCR and Western-Blot. Epithelial barrier function was assessed with transepithelial resistance (TER). Results and conclusions: The A2BAR antagonist, PSB1115, significantly increased tight junction protein expression after intestinal I/R or acute hypoxia conditions. PSB1115 also attenuated the disrupted distribution of TJ proteins. Furthermore, inhibition of A2BAR attenuated the decrease in TER induced by I/R or acute hypoxic conditions, and maintained intestinal barrier function. Antagonism of A2BAR activity improves intestinal epithelial structure and barrier function in a mouse model of intestinal I/R and a cell model of acute hypoxia. These findings support a potentially destructive role for A2BAR under intestinal I/R and acute hypoxic conditions.

Keywords: Ischemia/reperfusion, hypoxia, adenosine A2B receptor, tight junction, claudin-1, occludin, ZO-1, TER

Introduction

Acute intestinal ischemia/reperfusion (I/R) is a critical problem resulting in multiple organ failure [1-4]. Acute intestinal I/R are caused by clinical processes such as strangulation-obstruction of the intestine, sepsis, vascular surgery, hemorrhagic shock, small bowel transplantation, cardiopulmonary bypass, and abdominal aortic surgery [5-9]. Interruption of blood supply caused by ischemia which rapidly damages the intestinal epithelium, increases the intestinal permeability. Restoration of blood flow from the reperfusion stage can initiate a cascade of pathophysiologic responses that lead to additional cell or tissue injury [2]. Loss of intestinal barrier function has been implicated as a potential source of multiple organ failure under such conditions, which caused high mortality in hospital.

Tight junctions (TJs), which are located at the apical membrane, facing the lumen and the basolateral surface and in contact with the intestinal organisms, regulate the passage of ions, water and molecules through the paracellular pathway [10, 11]. The dynamic regulation of TJs under many physiological and pathological processes appears to be important, and the injury of TJs can lead to an increase in intestinal permeability and the dyshomeostasis of the intestinal barrier function (IBF). A loss of epithelial integrity could potentially lead to septic complications.

Hypoxia is a characteristic of many physiological and pathological processes and in a variety

of clinical conditions, such as inflammatory bowel disease [12], celiac disease [13] and intestinal I/R [14], and can induce the disruption of TJs and lead to the alternation of the paracellular permeability. During hypoxia, the accumulation of the extracellular nucleoside adenosine increases, mainly through the regulation of cellular metabolism [15]. Adenosine exerts its function through an interaction with the cell surface G protein-coupled receptors A1, A2A, A2B and A3, and A2BAR is the predominant adenosine receptor expressed in intestinal epithelial cells during the hypoxia [16]. Recently, studies focused on A2BAR [17-20] demonstrated that this receptor has a protective role in maintaining the homeostasis of the epithelial and endothelial barrier function. However, many studies also reported a detrimental role of the A2BAR in which its activation contributes to the inflammation and barrier dysfunction of several organs and tissues [21]. Carman et al using A_2 receptor knockout mice showed that activation of A_2 receptor resulted in opening of blood brain barrier, results in cellular changes in vitro including decreased transendothelial electric resistance, and alterations in tight junction molecules [22]. Studies from Gündüz also showed that exposure of endothelial monolayers (RCEC) to NECA, a non-selective agonist of AR, resulted in complete derangement of actin cytoskeleton which appeared as scrambled knots resulting in loss of cell demarcation and cell shrinkage [23]. Our laboratory has recently demonstrated a critical role of the HIF-1 signaling pathway to mediate an I/R injury in the intestine. Interestingly, there is a HIF-1alpha binding site in the promoter region of the A2b receptor gene [16]. This strongly suggests that A2BAR may well play a critical role in mediation of ischemic injury to the intestine.

Although the functional and biological effects of the A2BAR in the intestine have been studied, the regulation of A2B in the intestinal barrier function has not been well characterized. In this study, we hypothesized that the A2BAR is involved in the regulation of the intestinal barrier function under conditions of intestinal stress which typically results in loss of epithelial barrier disruption. We utilized two distinct but well characterized models to approach this hypothesis, intestinal I/R and hypoxic conditions.

Materials and methods

Reagents

1-Propyl-8-(4-sulfophenyl) xanthine potassium salt hydrate (PSB1115), polyclonal anti-ZO-1, anti-A2BAR and monoclonal anti-occludin antibodies were purchased from Santa Cruz Biotechnology (USA). Polyclonal anti-claudin-1 was obtained from Abcam (USA). Anti-GAPDH antibody was purchased from Goodhere Biotechnology (Hangzhou, China). The secondary antibodies were obtained from Beyotime (Jiangsu, China). Fetal bovine serum (FBS) was purchased from Hyclone (Thermo, USA).

Animals

Studies reported here conformed to the guidelines for the care and use of laboratory animals established by the University Committee on Use and Care of Animals at the Third Military Medical University, and the whole protocol was approved by this committee. Male, 6-8-week-old, specific pathogen-free, C57BL/6 mice were purchased from the Laboratory Animal Center (Third Military Medical University, Chongqing, P.R. China), maintained in temperature, humidity and light-controlled conditions, and then randomized into Sham group, I/R group and I/R+PSB1115 group. PSB1115 (10 mg/kg intraperitoneally) was given in I/R+PSB1115 group 20 min before the operation.

Intestinal ischemia/reperfusion injury

After intraperitoneal anesthesia with 40 mg/kg pentobarbital, the abdomen was opened at the midline, and the superior mesenteric artery (SMA) was occluded for 20 min using non-traumatic vascular clamps, followed by reperfusion for 1 h, 3 h and 6 h. Animals in the sham group underwent identical procedures without SMA occlusion (Sham). In all experiments, at least 6 animals were included in each group, and all experimental assays were repeated at least 3 times from specimens derived from each animal.

Immunohistochemistry

Anti-Adenosine A2BAR antibody (Santa Cruz Biotechnology; USA) was used for immunohistochemistry staining. The tissues were fixed in 4% paraformaldehyde, cut into 5-mm sections,

treated with 0.5% hydrogen peroxide in methanol, blocked for 45 min, and subsequently incubated with anti-A2BAR (1:200; Santa Cruz), or IgG (negative control) overnight at 4°C. These sections were incubated with biotinylated goat anti-rabbit IgG for 60 min, followed by incubation with streptavidin-enzyme conjugate for 15 min at room temperature. The peroxidase activities were developed using diaminobenzidine. After counterstaining with hematoxylin, the localization of A2BAR was examined under light microscope (630 ×).

Cell culture model under hypoxia

Caco-2 (human colon carcinoma) cells were obtained from China Center for Type Culture Collection (Beijing, China) and maintained in Eagle's Minimum Essential Medium (MEM) medium supplemented with 20% fetal bovine serum (FBS, Gemini Bioproducts, Woodland, CA), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO_2 humidified incubator. The culture medium was changed every two days.

For the hypoxia (1% oxygen concentration) experiment, cells were seeded on 6-well plates at a density of 1×10^6 cells/well. When the cells reached 70-80% confluence, they were cultured overnight with serum-free MEM medium. Prior to the experiment, the cells were pretreated with or without PSB1115 (10 μM) for 15 min and then cultured under normoxic or hypoxic conditions for 6 h. The following groups were analyzed:1) untreated cells (normoxia); 2) untreated cells (hypoxia); and 3) cells pretreated with PSB1115 (10 μM) and then cultured under hypoxia.

Reverse transcription-PCR

To identify the influence of the PSB1115 on the expression of TJ proteins, total RNA was extracted from the monolayers of Caco-2 cells using the Trizol extraction method (Takara, Dalian, China). cDNA synthesis was then performed using reverse transcription according to the manufacturer's protocol (Takara, Dalian, China). The PCR reaction contained 0.4 μM sense and 0.4 μM antisense primers. The polymerase chain reaction amplification was performed using following protocol: 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 60°C or 57 °C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min.

The primer sequences listed in **Table 1** were synthesized by Takara (Dalian, China), and the housekeeping gene β-actin was used as an internal control. The levels of claudin-1, occludin and ZO-1 mRNAs were semi-quantified by RT-PCR analysis tools and normalized to the β-actin levels.

Western blot analyses

Total proteins were extracted using an SDS total protein extraction kit (Beyotime, Jiangsu). The protein concentration was determined according to the Bradford method using the BCA assay reagent (Beyotime, China). Samples (25 μg protein) were loaded onto SDS-PAGE gels and separated at 120 V for 70 min for occludin, 120 V for 40 min for claudin-1 and 200 mA for 120 min for ZO-1. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore). After blocking with 5% bovine serum albumin in TBS-T (50 mMTris–HCl [pH 7.5], 140 mM NaCl, and 0.1% Tween) for 2 h, the membranes were incubated overnight at 4°C with polyclonal rabbit antibodies against A2BAR (1:250,Santa Cruz), claudin-1 (1:500, Abcam) and ZO-1 (1:500, Santa Cruz), monoclonal anti-occludin (1:250, Santa Cruz) and anti-GAPDH (1:1000, Goodhere Biotechnology, Hangzhou, China). The membranes were washed three times with TBST for 5 min and then incubated with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies at room temperature for 2 h. After washing 6 times with TBST for 5 min each, the proteins were detected using ECL reagents (Boster, Wuhan, China).

Immunofluorescence

Caco-2 cells were cultured on acid-washed coverslips and exposed to hypoxic conditions (2% O_2, 5% CO_2, 94% N_2, 37°C and 90% humidity). The samples were then washed twice in PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the samples were incubated with 5% bovine serum albumin and then immunostained with mouse anti-occludin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-ZO-1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-A2BAR (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-clau-

Table 1. Primers of the genes and the size of the products

Genes	Primer	Size of Products
Mouse A2BAR	Sense: 5'-GGGGTGGAACAGTAAAGACAG-3'	166 bp
	Antisense: 5'-TATGAGCAGTGGAGGAAGGACAC-3'	
Mouse Occludin	Sense: 5'-AGATTCCTCTGACCTTGAGTGTGG-3'	109 bp
	Antisense: 5'-TTTCCTGCTTTCCCCTTCGTG-3'	
Mouse Claudin-1	Sense: 5'-CAGCATGGTATGGAAACAGAATTG-3'	151 bp
	Antisense: 5'-GGACAGGAGCAGGAAAGTAGGA-3'	
Mouse ZO-1	Sense: 5'-AACACAGCCTCCAGAGTTTGACAG-3'	178 bp
	Antisense: 5'-AATGCCACGAGCTGTAGCCACT-3'	
Mouse β-actin	Sense: 5'-TGACGTGGACATCCGCAAAG-3'	205 bp
	Antisense: 5'-CTGGAAGGTGGACAGCGAGG-3'	
Human A2BAR	Sense: 5'-AATCCGATGCCAAAGGCAAG-3'	166 bp
	Antisense: 5'-CAGATACCTGGCCATCTGTGTC -3'	
Human occludin	Sense: 5'-AGCAGCGGTGGTAACTTTGAGA-3'	118 bp
	Antisense: 5'-ACTCGCCGCCAGTTGTGTAG-3'	
Human Claudin-1	Sense: 5'-GGTACGAATTTGGTCAGGCTCTC-3'	118 bp
	Antisense: 5'-CTTGGTGTTGGGTAAGAGGTTGTT-3'	
Human ZO-1	Sense: 5'-ACTCCCACTTCTCCAAAAACTCTT-3'	154 bp
	Antisense: 5'-GACTCACAGGAATAGCTTTAGGCA-3'	
Human β-actin	Sense: 5'-CCACGAAACTACCTTCAACTCC-3'	147 bp
	Antisense: 5'-GTGATCTCCTTCTGCATCCTGT-3'	

apical and bottom compartments was changed every day.

Before the monolayers were exposed to hypoxia, PSB-1115 was added to the medium in the apical and basolateral aspect of intestinal epithelial cells. The integrity of the confluent polarized monolayers was assessed by measuring the TER under normoxia and after hypoxia. TER (ohms per square centimeter) = Total resistance-Blank resistance) (ohms)/Area (square centimeter).

TER, which represents the intestinal epithelial barrier function and tissue viability [24], was determined based on Ohm's law. The permeability of the small intestine was evaluated by TER.

din-1 (1:500, Abcam, USA) antibodies or incubated with PBS as a negative control overnight at 4°C. After washing with PBS, the samples were incubated with Cy3-labeled anti-rabbit antibody (1:400, Beyotime, Jiangsu, China) or Cy3-labeled anti-mouse antibody (1:400, Beyotime, Jiangsu, China) or FITC-labeled anti-rabbit antibody (1:400, Beyotime, Jiangsu, China) for 30 min at 37°C. After washing in PBS, the samples were incubated with DAPI (4',6-diamidino-2-phenylindole) nuclear stain solution (Beyotime, Jiangsu, China) for 3 min. All of the samples were analyzed using a confocal laser-scanning microscope (Leica TCS SP5, Leica, Wetzlar, Germany).

Transepithelial electrical resistance (TER) measurements

TER was measured using a Millipore electric resistance system (ESR-2, Millipore). Caco-2 monolayers were grown on Millicell filters (0.33 cm^2 area, 0.4 μm pore diameter, and 6.5 mm diameter) at a density of 5×10^4 cells/cm^2 for up to 5-6 days until the TER achieved a stable value of 80-120 Ω/cm^2 to obtain an integrated cell monolayer. Additionally, the media in the

The mucosal membrane was mounted in modified Using chambers (Physiologic Instruments, San Diego, CA) as flat sheets on a segment holder, with an exposed tissue area of 0.3 cm^2. Each half cell (mucosal and serosal) was filled with 5 mL of preheated 37°C Kreb's Ringer solution buffer. The buffer contained 110.0 mM NaCl, 3.0 mM $CaCl_2$, 5.5 mM KCl, 1.4 mM KH_2PO_4, 29.0 mM $NaHCO_3$, and, 1.2 mM $MgCl_2$ and was adjusted to a pH of 7.4, continuously oxygenated with O_2/CO_2 (95%/5%), and stirred by gas flow in the chambers. One pair of Ag/AgCl-electrodes (Physiologic Instruments) with 3 M KCl in 3% agar bridges was used to measure transepithelial potential difference, and another pair of Pt electrodes was used for current passage. The spontaneous potential difference across the intestinal membrane was maintained at 5 mV by an automated voltage clamp, and the injected short circuit current (Isc) was monitored continuously as an indication of net active ion transport. The transmembrane resistance in X cm^2 was determined by using Ohm's law. Baseline Isc was recorded after a 20-min equilibration period.
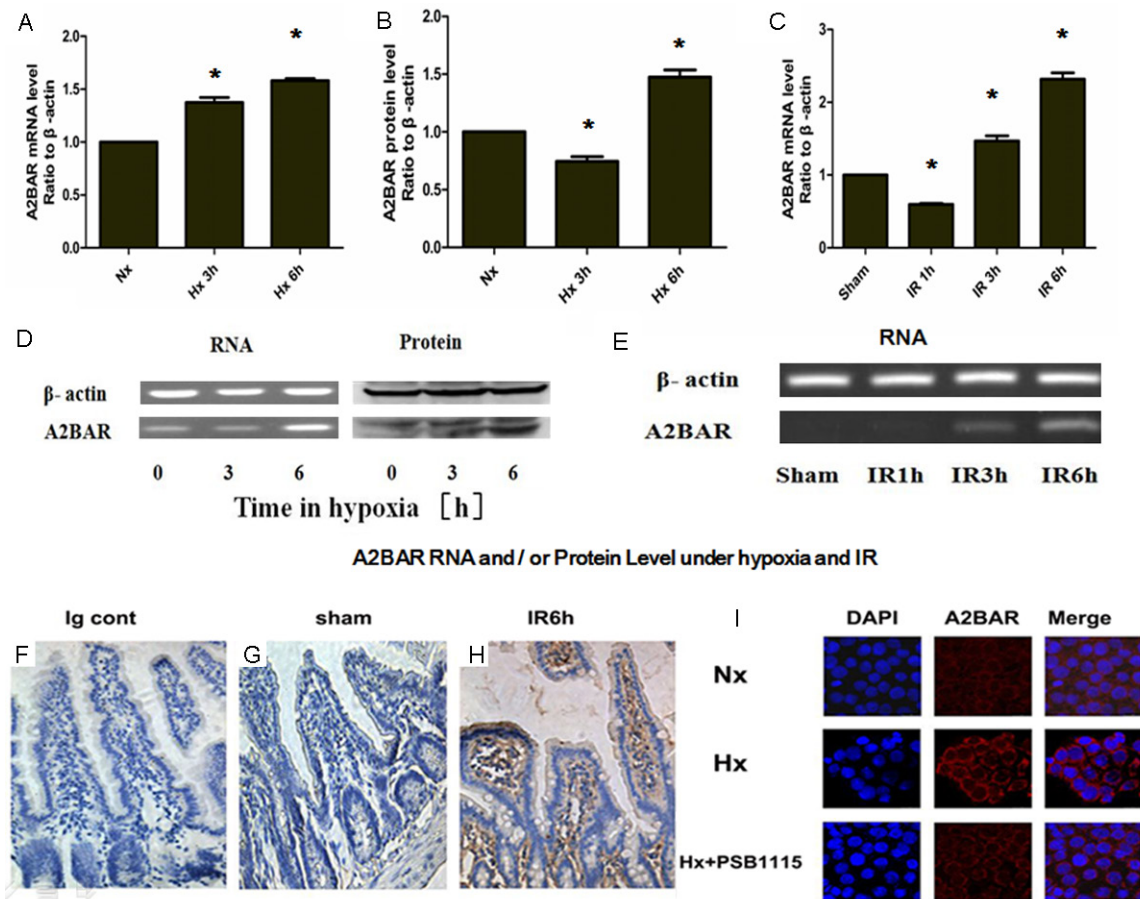
Figure 1. Changes in A2BAR expression under hypoxia and intestinal I/R models. A. Caco-2 cells were exposed to normoxia or hypoxia for indicated time. Total RNA was isolated, and A2BAR mRNA levels were determined by RT-PCR. Data were calculated relative to β-actin and expressed as fold change relative to normoxia. B. Induction of intestinal epithelia A2BAR protein by hypoxia. Shown here is a representative Western blot of A2BAR following incubation for indicated periods of hypoxia, with β-actin as a control. C. Induction of intestinal epithelial A2BAR mRNA levels by IR. Results are derived from three experiments (*: Different from normoxia and Sham group, P < 0.01). F-H. Immunohistochemistry for A2BAR. A2BAR expression was detected in I/R 6 h, compared with the Sham group. F. Negative control; G. Sham group exhibit weak staining; H) The structures of intestinal villus were significantly damaged of I/R injury when compared with the sham group (n = 6 per group). I. Induction of A2BAR in Caco-2 cell lines in normoxic or hypoxic conditions. Immunocytofluorescence labeling of A2BAR (red) in Caco-2 cells cultured for 6 h under normoxia, hypoxia and hypoxia plus PSB1115 (10 μM) is shown. Cellular nuclei are labeled with DAPI (blue). The positive fluorescence of A2BAR (red) is higher under the hypoxic condition than under the normoxic condition.

Tissues were bathed on the serosal and mucosal sides with Ringer solution. Bathing solutions were oxygenated (95% O_2, 5% CO_2) and circulated in water-jacketed reservoirs at 37°C. After equilibration in Ussing chambers for 20 min, experiments were run for up to 1.5-2 h. TER was calculated on the basis of the spontaneous potential difference and short-circuits current.

Statistical analysis

The results are expressed as the mean ± SD from 3-4 experiments performed in triplicate or quadruplicate. All of the data presented were analyzed by using ANOVA. For each test, *P* values < 0.05 were considered to be statistically significant.

Results

A2BAR is induced by hypoxia both in vitro and I/R in vivo models

Based on previous studies showing A2BAR expression is selectively inducted by hypoxia and ischemia [25-27], we using RT-PCR and Western Blot to detected A2BAR expression
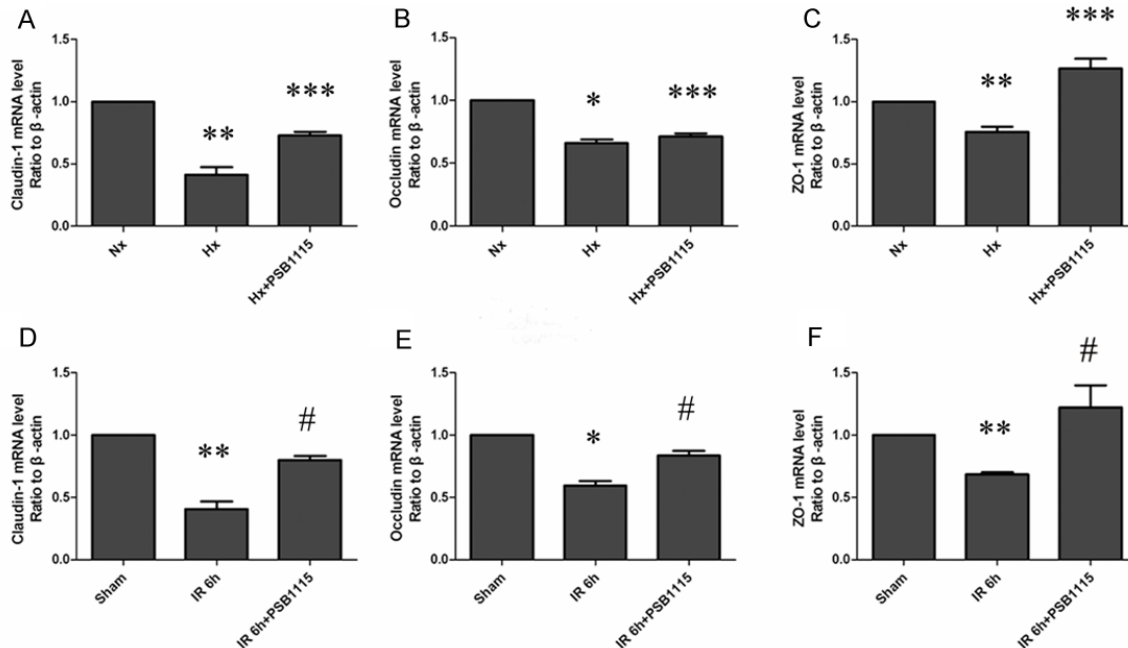
Figure 2. Role of an A2BAR antagonist on TJ mRNA levels under hypoxia and I/R. Caco-2 cells were pretreated with or without PSB1115, and the monolayers were then subjected to hypoxia for 6 h. The mRNA levels of the TJs were determined using RT-PCR. The data are the mean ± SD (n = 6). A-C. Hypoxia down-regulated claudin-1, occludin and ZO-1 mRNA expression, and PSB1115 attenuated the down-regulation of these TJs as induced by hypoxia. D-F. I/R down-regulated claudin-1, occludin and ZO-1 mRNA expression, and PSB1115 attenuated the down-regulation of TJ mRNA expression induced by I/R (*P < 0.05, **P < 0.01 compared to the Nx group and Sham group; ***P < 0.05, compared to the Hx. #P < 0.01, compared to the I/R group).

under hypoxia and intestinal I/R conditions. As shown in **Figure 1A**, analysis of intestinal epithelial A2BAR mRNA by RT-PCR revealed a time-dependent induction of A2BAR by hypoxia (P < 0.01) and I/R (P < 0.01), with maximal changes of 1.58 ± 0.02 fold increase at 6 h after I/R compared with the Sham group (P < 0.01). Extension of these findings at the protein level by Western blot revealed that total levels of A2BAR were also increased in a time-dependent manner induced by hypoxia compared to normoxia (P < 0.01) (**Figure 1B**, **1D**); and induced by I/R compared to Sham animals (P < 0.01) (**Figure 1C**, **1E**).

To further confirm the changes of A2BAR expression, immunohistochemistry was used to detect A2BAR expression in Sham group and I/R group (**Figure 1F-H**), immunofluorescence was used to detect A2BAR expression in cell cultures under normoxic and hypoxic conditions (**Figure 1I**). Sham and normoxia groups exhibit weak staining of A2BAR, while the structures of intestinal villi were significantly damaged by I/R when compared with the Sham group. High magnification reveals that A2BAR

expression localizes to the cytomembrane of intestinal epithelia (**Figure 2C**). The hypoxia group exhibit strong staining of A2BAR when compared with the normoxia group.

All these results confirm that hypoxia and I/R prominently increase A2BAR expression.

A2BAR antagonist attenuates changes in TJ protein expression caused by hypoxia and intestinal I/R

In vitro study: To investigate the expression of TJs under hypoxia, we used RT-PCR to detect the expression of claudin-1, occludin and ZO-1 in Caco-2 monolayers. As shown in **Figure 2A-C**, hypoxia led to a significant reduction in of claudin-1, occludin and ZO-1 expression (0.59 ± 0.048, P < 0.01, 0.34 ± 0.018, P < 0.05, and 0.24 ± 0.039, P < 0.01, respectively) when compared with normoxic conditions. Pretreatment with the A2BAR antagonist, PSB1115, before being subjected to hypoxia exhibited much higher levels of claudin-1, occludin and ZO-1 when compared to the group without
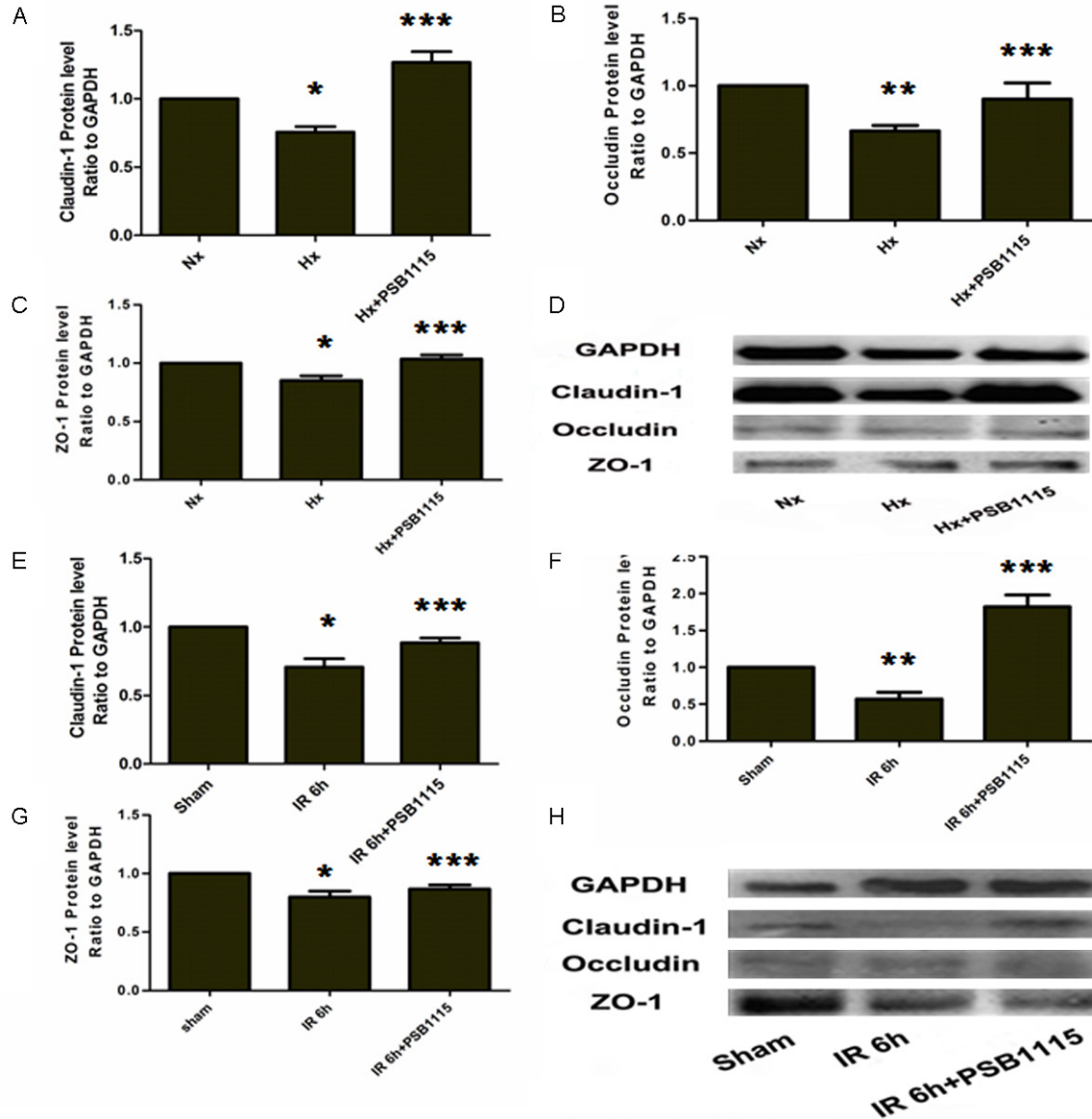
Figure 3. Effect of an A2BAR antagonist on the abundance of TJ proteins in Caco-2 cells exposed to hypoxia and I/R intestinal tissue. A-D. Caco-2 cells were cultured in MEM for 7 days under normoxia, incubated for 24 h in serum-free medium, and then exposed to hypoxia in the absence or presence of PSB1115 (10 μM) for 6 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies against claudin-1, occludin and ZO-1. The amount of each TJ protein was normalized to the GAPDH and then expressed relative to the corresponding normalized value for the normoxic condition. E-H. Claudin-1, occludin and ZO-1 was detected in Sham, I/R and I/R+PSB1115 groups. The amount of each TJ protein was normalized to the GAPDH and then expressed relative to the corresponding normalized value for the Sham group. The data are the means ± SD from three independent experiments. (*P < 0.05, **P < 0.01, compared to the Nx group and Sham group; ***P < 0.05, compared to the Hx group and IR group).

PSB1115 pretreatment under hypoxia (0.73 ± 0.027 vs 0.41 ± 0.061, P < 0.05, 0.71 ± 0.027 vs 0.66 ± 0.027, P < 0.01, and 1.27 ± 0.079 vs 0.76 ± 0.039, P < 0.05). However, no significant changes in occludin mRNA expression was found in the Hx+PSB1115 group (0.71 ± 0.027) when compared with the Hx group (0.66 ± 0.027) (P < 0.01) (**Figure 2B**).

To clarify further these changes in TJ protein expression, claudin-1, occludin and ZO-1 protein expressions were also examined. **Figure 3A-D** shows that the protein levels of claudin-1, occludin and ZO-1 were reduced in Caco-2 cells under hypoxia exposure for 6 h and restored by PSB1115 pretreatment. Compared to the Nx group, the claudin-1, occludin and ZO-1 protein
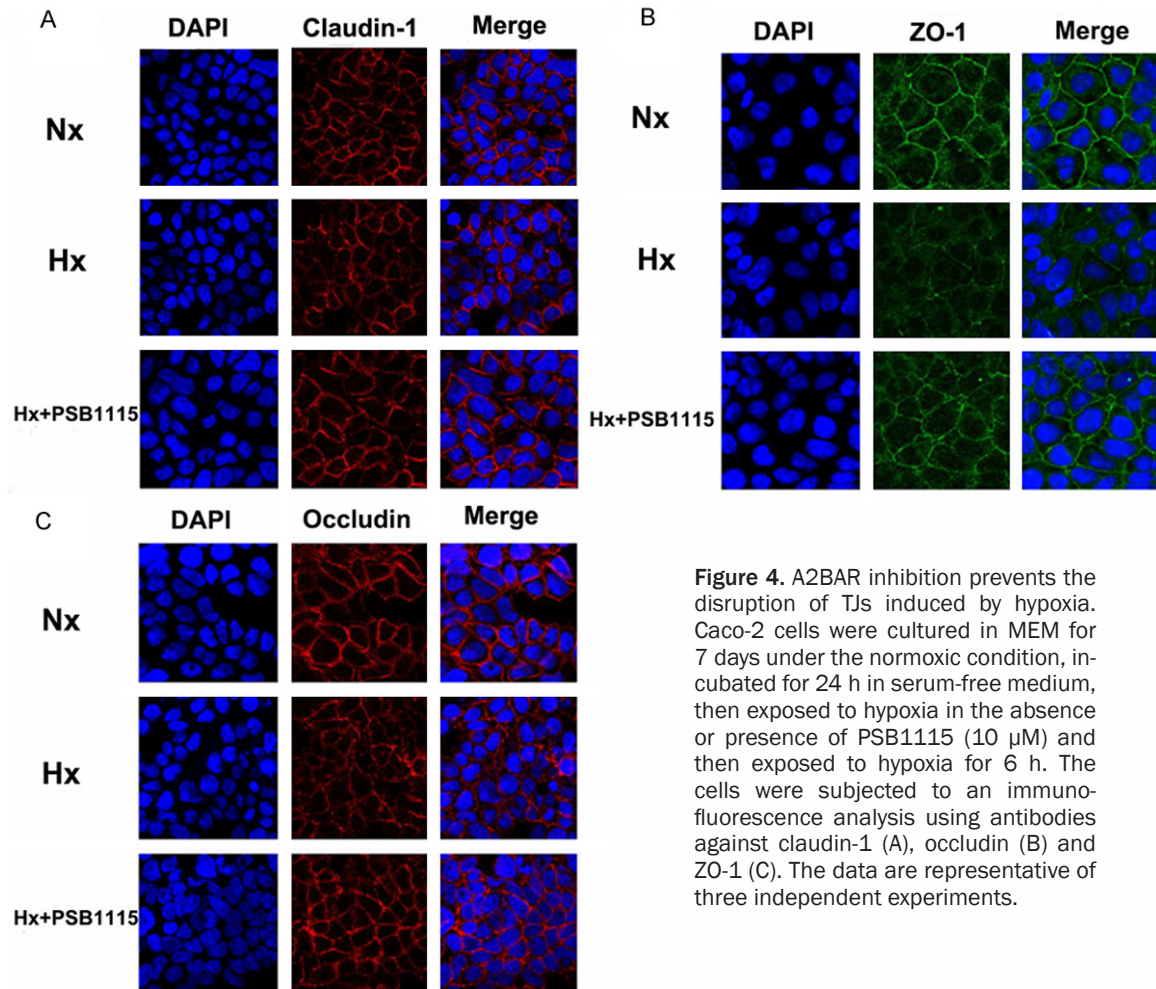
Figure 4. A2BAR inhibition prevents the disruption of TJs induced by hypoxia. Caco-2 cells were cultured in MEM for 7 days under the normoxic condition, incubated for 24 h in serum-free medium, then exposed to hypoxia in the absence or presence of PSB1115 (10 μM) and then exposed to hypoxia for 6 h. The cells were subjected to an immuno-fluorescence analysis using antibodies against claudin-1 (A), occludin (B) and ZO-1 (C). The data are representative of three independent experiments.

expression under hypoxia (**Figure 3A-C**) showed a reduction of approximately 25% (P < 0.05), 34% (P < 0.01) and 25% (P < 0.05), respectively. In the Hx+PSB1115 group, claudin-1, occludin and ZO-1 expression significantly increased by approximately 42% (P < 0.05), 24% (P < 0.05) and 50% (P < 0.05), respectively were found (**Figure 3A-C**). The above data indicated that the TJ protein expression, which was down-regulated under the hypoxic condition, can be prevented by the A2BAR antagonist.

In vivo study: As shown in **Figure 2D-F**, intestinal I/R induced significant decreases in claudin-1, occludin and ZO-1 mRNA expression when compared with the Sham group, respectively. The group pretreated with PSB1115 before being operation exhibited high levels of claudin-1, occludin and ZO-1 when compared to the group without PSB1115 pretreatment under I/R conditions (0.80 ± 0.04 vs 0.40 ± 0.06, P < 0.01, 0.84 ± 0.04 vs 0.60 ± 0.03, P <

0.05, and 1.22 ± 0.18 vs 0.68 ± 0.02, P < 0.01) (**Figure 2D-F**).

Figure 3E-H shows that the protein levels of claudin-1, occludin and ZO-1 were reduced under I/R in the 6 h group, and restored by PSB1115 pretreatment. Compared to the Sham group, the claudin-1, occludin and ZO-1 protein expression under I/R condition (**Figure 3E-H**) showed a reduction of approximately 23% (P < 0.05), 33% (P < 0.01), and 20% (P < 0.05) respectively. In the I/R+PSB1115 group, occludin expression significantly increased by approximately 125% (P < 0.05). However, no significant changes in claudin-1 and ZO-1 expression in the I/R+PSB1115 group (elevated by approximately 18%, P < 0.05 and 7%, P < 0.05) (**Figure 3E** and **3G**) were found. The above data indicated that the TJ protein expression, which was down-regulated under I/R conditions and can be prevented by the A2BAR antagonist.
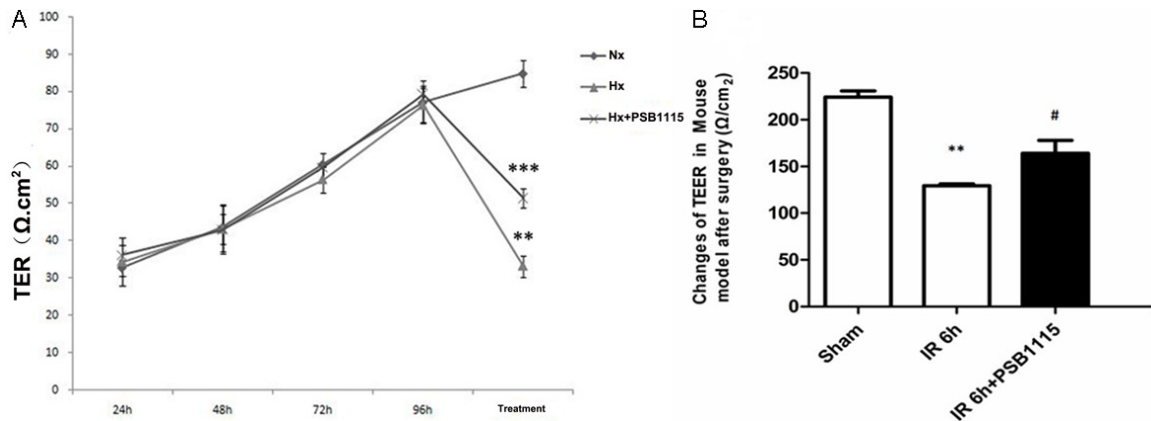
Figure 5. Effect of an A2BAR antagonist on the hypoxia-induced the changes in the TER in Caco-2 monolayers and I/R-induced changes in TER in intestinal epithelia. Hypoxia-induced reduction in the TER in Caco-2 monolayers and IR-induced reduction the TER in intestinal epithelia can be prevented by A2BAR antagonist pretreatment. The results are expressed as the mean ± SD (n = 6). A. **P < 0.01, compared to the Nx group; ***P < 0.05, compared to the Hx group. B. **P < 0.01, compared to the Sham group; #P < 0.01, compared to I/R group.

Taken together, these data suggested that both hypoxia and I/R could down-regulate TJ protein expression, and inhibition of A2BAR could attenuate changes in TJ protein expression induced both by hypoxia and intestinal I/R.

Alternations in TJs induced by the A2BAR antagonist under hypoxia

To examine further the role of A2BAR on the cellular distribution of TJ proteins under the hypoxia condition, immunofluorescence was used. As shown in **Figure 4A-C**, claudin-1, occludin and ZO-1 under the normoxic condition were distributed primarily near the cell membrane in the epithelial cells and localized to the borders of the adjacent cells. In contrast, after the monolayers were exposed to hypoxia for 6 h, claudin-1, occludin and ZO-1 showed a typical rearrangement, with diffuse and discontinuous staining and significant disruptions (**Figure 4A-C**). However, this effect of hypoxia on the distribution of claudin-1, occludin and ZO-1 at the adjacent cell was prevented by the pretreatment with the A2BAR antagonist, PSB-1115.

Taken together, our results indicate that in vitro hypoxia is associated with rearrangements and the disruptions of the TJ proteins, and that an A2BAR antagonist can attenuate the hypoxia-induced the changes of TJ protein.

A2BAR antagonist prevents the reduction of TER under hypoxia and intestinal I/R

In vitro study: To verify the role of the A2BAR in TJ function, the epithelial permeability was detected by measuring TER to assess the functional integrity. After culturing for 4 days under normoxic conditions, the monolayers were exposure to hypoxia for 6 h, resulting in a decrease in TER. As shown in **Figure 5A**, compared with the monolayers under normoxia (84.8 ± 3.64 Ω/cm^2), hypoxia resulted in a significant decrease in the TER (33.23 ± 2.84 Ω/cm^2) (P < 0.05). PSB1115 treatment significantly attenuated this TER decrease (51.4 ± 2.60 Ω/cm^2) (P < 0.05) when compared to the monolayers in the absence of PSB1115 under hypoxia (33.23 ± 2.84 Ω/cm^2) (P < 0.05). In contrast, PSB1115 had no effect on the TER in Caco-2 monolayers for maintaining the IBF under normoxia (**Figure 5A**) (P < 0.05). These data demonstrate that hypoxia induces an increase in intestinal permeability and that the inhibition of A2BAR could significantly attenuate this loss of intestinal barrier function.

In vivo study: To further confirm the role of A2BAR in the regulation of intestinal barrier function, we next investigated the changes of intestinal epithelial barrier function by measuring TER with Ussing chamber instrumentation [24]. Baseline TER (0 min after tissue mounting) was 224.17 ± 6.76 Ω/cm^2 in the control group and 129.41 ± 2.02 Ω/cm^2 in the I/R group. Intestinal I/R resulted in a significant decrease in the TER (94.46 ± 4.75) (**Figure 5B**). However, A2BAR antagonist pretreatment significantly attenuated the reduction in the TER as compared to the I/R group (163.98 ± 14.07 vs. 129.41 ± 2.02 Ω/cm^2, P < 0.001) (**Figure 5B**). These results suggest that A2BAR

antagonist pretreatment significantly improved the hypoxia and intestinal I/R-induced epithelial barrier dysfunction.

Discussion

In the present study, we addressed the role of A2BAR in the regulation of intestinal barrier function under hypoxic and intestinal I/R conditions. We demonstrate that, in the presence of A2BAR antagonist, PSB1115, the expression levels of claudin-1, occludin and ZO-1 were increased under hypoxia and I/R. When the intestinal epithelial cells or mice were pretreated with PSB1115, the TER also increased significantly compared to the epithelial cells and mouse in the absence of PSB1115 under hypoxic and I/R condition, to levels similar to that in control conditions. Taken together, our studies provide evidence that the inhibition of the A2BAR can prevent the disruption of intestinal tight junctions and maintain the intestinal barrier function under hypoxic and intestinal I/R conditions.

The intestinal epithelial barrier provides the primary determinant of intestinal permeability. Along with the plasma membrane, the intercellular tight junctions (TJs) are the primary cellular determinants of epithelial barrier function [28]. Due to the functional and structural integrity of TJs, intestinal permeability is typically low under normal conditions. However, any physiological or pathological changes that induce alternations in TJ expression and/or arrangement may result in increased permeability of the intestine. The disruption of tight junction structure can be both a cause and an effect of many diseases, and intestinal epithelial barrier dysfunction can be found in many disease states, such as inflammatory bowel disease [12], celiac disease [13], intestinal I/R [14, 29] and other hypoxia conditions [29-31].

Intestinal I/R injury is a serious and frequent clinical problem in trauma, burn and the perioperative period in surgical patients. Many major surgical procedures, including aorto vascular surgery, intestinal or liver transplantation, as well as hepatic and pancreatic resections, require an obligate period of intestinal ischemia that is an important determinant of short- and long-term outcomes [32-34]. Several changes may occur in the intestinal mucosa after acute intestinal I/R, including an increase

in the mucosal epithelial permeability and a decline in intestinal barrier function. Damage to the mucosa includes epithelial shedding, bacterial translocation, disordered mucosal permeability, and alteration of the absorptive function [35]. During intestinal I/R, alterations of intestinal blood supply and inflammatory activation can result in severe intestinal hypoxia.

Hypoxia is a common state in many physiological and pathophysiological process. Previous studies from Eltzsching [36] demonstrate that hypoxia can markedly induce the accumulation of extracellular adenosine and mediate cell function through the activation of different adenosine receptors [37]. The A2BAR, a G protein-coupled receptor, is the predominant adenosine receptor expressed in intestinal epithelial [38] and endothelial cells [18, 26, 39, 40] during hypoxia. Depending on the tissue and cell type, the biological effect of the A2BAR presents a protective or detrimental effect under different conditions. This dichotomous effect of A2BAR activation has also been observed in studies focusing on the heart and brain in addition to the gut. For example, a protective role of the A2BAR has been demonstrated to against endotoxin-induced sepsis by regulating inflammation and vascular adhesion [41] and against cardiac fibrosis by mediating the proliferation of cardiac fibroblastic [42]. Its protective role also can be found in dampens hypoxia-induced vascular leakage [19, 20]. Treatment with a selective A2BAR agonist attenuated intestinal I/R injury in a mouse model, indicating the-A2BAR as a potential therapeutic target during intestinal I/R injury [16]. Previous studies demonstrated that hypoxia could disrupt TJ gene expression and induce the loss of barrier function [29]. All these studies suggest that the activation of the A2BAR is involved in the regulation of intestinal TJ expression and the maintenance of the epithelial barrier function.

Our experimental paradigm used 6 h of hypoxia or 6 h of I/R as treatment models, and we showed a decrease in claudin-1, occludin and ZO-1 expression after hypoxia and I/R. We also demonstrated that hypoxia and I/R is associated with the disruption and rearrangement of TJ proteins. Pretreatment with A2BAR antagonist before hypoxia or intestinal I/R could prevent this down-regulation of claudin-1, occludin and

ZO-1 expressions and the loss of barrier function.

In addition, we investigated the role of A2BAR in the distribution of TJs using immunofluorescence staining. Claudin-1, occludin and ZO-1 are localized to the borders of adjacent cells under the normoxic condition, however, after the exposure to hypoxia, claudin-1, occludin and ZO-1 showed a typical rearrangement, with diffuse and discontinuous staining and significant disruption. Interestingly, this effect of hypoxia on the distribution of claudin-1, occludin and ZO-1 in the adjacent cells was prevented by the A2BAR antagonist, PSB1115 pretreatment. This study suggests that the activation of A2BAR is integrally involved in this hypoxia-induced TJ protein loss and rearrangement.

To better determine the role of the A2BAR in TJ function, the epithelial permeability was also detected by measuring TER to assess the functional integrity of our cell culture model and animal model. We found that the pretreatment with A2BAR antagonist before cells were exposed to hypoxia and before the mouse being operated attenuated the hypoxia and IR-Induced changes in the TER. These results indicate that inhibit the A2BAR can maintain the intestinal epithelial barrier function under hypoxia and intestinal I/R.

In conclusion, the present study has demonstrated novel insights into the role of A2BAR in modulation of intestinal barrier function under hypoxic or intestinal I/R condition. Activation of A2BAR under hypoxic or intestinal I/R condition results in low expression of TJ proteins and high epithelial permeability. Thus, inhibition of A2BAR with specific antagonist could significantly attenuate these changes. Future investigation is needed to elucidate the molecular mechanism responsible for the role of the A2BAR in modulates intestinal epithelial permeability and TJ expression under hypoxic and I/R condition.

Acknowledgements

Address correspondence to: Dr. Hua Yang, Department of General Surgery, Xinqiao Hospital, Third Military Medical University, Chongqing 400037, China. Tel: (86) 23-687-55705; E-mail: huayang@tmmu.edu.cn

References

[1] Daniel C, Sartory NA, Zahn N, Radeke HH and Stein JM. Immune modulatory treatment of trinitrobenzene sulfonic acid colitis with calcitriol is associated with a change of a T helper (Th) 1/Th17 to a Th2 and regulatory T cell profile. J Pharmacol Exp Ther 2008; 324: 23-33.

[2] Higuchi S, Wu R, Zhou M, Marini CP, Ravikumar TS and Wang P. Gut hyperpermiability after ischemia and reperfusion: attenuation with adrenomedullin and its binding protein treatment. Int J Clin Exp Pathol 2008; 1: 409-418.

[3] Berland T and Oldenburg WA. Acute mesenteric ischemia. Curr Gastroenterol Rep 2008; 10: 341-346.

[4] Ogra PL and Welliver RC Sr. Effects of early environment on mucosal immunologic homeostasis, subsequent immune responses and disease outcome. Nestle Nutr Workshop Ser Pediatr Program 2008; 61: 145-181.

[5] Kenway-Lynch CS, Das A, Pan D, Lackner AA and Pahar B. Dynamics of cytokine/chemokine responses in intestinal CD4+ and CD8+ T Cells during Acute Simian Immunodeficiency Virus Infection. J Virol 2013; 87: 11916-11923.

[6] Visco V, Belleudi F, Marchese C, Leone L, Aimati L, Cardinali G, Kovacs D, Frati L and Torrisi MR. Differential response to keratinocyte growth factor receptor and epidermal growth factor receptor ligands of proliferating and differentiating intestinal epithelial cells. J Cell Physiol 2004; 200: 31-44.

[7] Fukumoto S. [Disorders of phosphate metabolism]. Rinsho Byori 2010; 58: 225-231.

[8] Tatekawa Y, Kanehiro H and Nakajima Y. Duodenal atresia associated with "apple peel" small bowel without deletion of fibroblast growth factor-10 or fibroblast growth factor receptor 2IIIb: report of a case. Surg Today 2007; 37: 430-433.

[9] Mallick IH, Yang WX, Winslet MC and Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci 2004; 49: 1359-77.

[10] Gonzalez-Mariscal L, Betanzos A, Nava P and Jaramillo BE. Tight junction proteins. Prog Biophys Mol Biol 2003; 81: 1-44.

[11] Gonzalez-Mariscal L, Tapia R and Chamorro D. Crosstalk of tight junction components with

signaling pathways. Biochim Biophys Acta 2008; 1778: 729-756.

[12] Edelblum KL and Turner JR. The tight junction in inflammatory disease: communication breakdown. Curr Opin Pharmacol 2009; 9: 715-720.

[13] Schulzke JD, Bentzel CJ, Schulzke I, Riecken EO and Fromm M. Epithelial tight junction structure in the jejunum of children with acute and treated celiac sprue. Pediatr Res 1998; 43: 435-441.

[14] Li QR, Zhang Q, Wang CY, Liu XX, Qu LL, Gu LL, Li N and Li JS. Altered distribution of tight junction proteins after intestinal ischaemia/reperfusion injury in rats. J Cell Mol Med 2009; 13: 4061-4076.

[15] Trowbridge JM, Rudisill JA, Ron D and Gallo RL. Dermatan sulfate binds and potentiates activity of keratinocyte growth factor (FGF-7). J Biol Chem 2002; 277: 42815-42820.

[16] Kong T, Westerman KA, Faigle M, Eltzschig HK and Colgan SP. HIF-dependent induction of adenosine A2B receptor in hypoxia. FASEB J 2006; 20: 2242-2250.

[17] Hart ML, Henn M, Kohler D, Kloor D, Mittelbronn M, Gorzolla IC, Stahl GL and Eltzschig HK. Role of extracellular nucleotide phosphohydrolysis in intestinal ischemia-reperfusion injury. FASEB J 2008; 22: 2784-2797.

[18] Yang D, Zhang Y, Nguyen HG, Koupenova M, Chauhan AK, Makitalo M, Jones MR, St Hilaire C, Seldin DC, Toselli P, Lamperti E, Schreiber BM, Gavras H, Wagner DD and Ravid K. The A2B adenosine receptor protects against inflammation and excessive vascular adhesion. J Clin Invest 2006; 116: 1913-1923.

[19] Eckle T, Faigle M, Grenz A, Laucher S, Thompson LF and Eltzschig HK. A2B adenosine receptor dampens hypoxia-induced vascular leak. Blood 2008; 111: 2024-2035.

[20] Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC and Colgan SP. Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. J Exp Med 2004; 200: 1395-1405.

[21] Kolachala V, Ruble B, Vijay-Kumar M, Wang L, Mwangi S, Figler H, Figler R, Srinivasan S, Gewirtz A, Linden J, Merlin D and Sitaraman S. Blockade of adenosine A2B receptors ameliorates murine colitis. Br J Pharmacol 2008; 155: 127-137.

[22] Carman AJ, Mills JH, Krenz A, Kim DG and Bynoe MS. Adenosine receptor signaling modulates permeability of the blood-brain barrier. J Neurosci 2011; 31: 13272-13280.

[23] Taraban VY, Slebioda TJ, Willoughby JE, Buchan SL, James S, Sheth B, Smyth NR, Thomas GJ, Wang EC and Al-Shamkhani A. Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia. Mucosal Immunol 2011; 4: 186-196.

[24] Smith PL. Methods for evaluating intestinal permeability and metabolism in vitro. Pharm Biotechnol 1996; 8: 13-34.

[25] Weissmuller T, Eltzschig HK and Colgan SP. Dynamic purine signaling and metabolism during neutrophil-endothelial interactions. Purinergic Signal 2005; 1: 229-239.

[26] Eltzschig HK, Ibla JC, Furuta GT, Leonard MO, Jacobson KA, Enjyoji K, Robson SC and Colgan SP. Coordinated adenine nucleotide phosphohydrolysis and nucleoside signaling in posthypoxic endothelium: role of ectonucleotidases and adenosine A2B receptors. J Exp Med 2003; 198: 783-796.

[27] Eltzschig HK, Grenz A, Osswald H, Eckle T, Yang D, Zhang H, Tran ZV, Klingel K and Ravid K. The reno-vascular A2B adenosine receptor protects the kidney from ischemia. Plos Medicine 2008; 5: 968-986.

[28] Marchiando AM, Graham WV and Turner JR. Epithelial Barriers in Homeostasis and Disease. Annu Rev Pathol 2010; 5: 119-144.

[29] Brown RC, Mark KS, Egleton RD, Huber JD, Burroughs AR and Davis TP. Protection against hypoxia-induced increase in blood-brain barrier permeability: role of tight junction proteins and NFkappaB. J Cell Sci 2003; 116: 693-700.

[30] Brown RC and Davis TP. Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells. Biochem Biophys Res Commun 2005; 327: 1114-1123.

[31] Yamagata K, Tagami M, Takenaga F, Yamori Y and Itoh S. Hypoxia-induced changes in tight junction permeability of brain capillary endothelial cells are associated with IL-1beta and nitric oxide. Neurobiol Dis 2004; 17: 491-499.

[32] Brandt LJ and Boley SJ. AGA technical review on intestinal ischemia. American Gastrointestinal Association. Gastroenterology 2000; 118: 954-968.

[33] Debus ES, Muller-Hulsbeck S, Kolbel T and Larena-Avellaneda A. Intestinal ischemia. Int J Colorectal Dis 2011; 26: 1087-1097.

[34] Halm MA. Acute gastrointestinal complications after cardiac surgery. Am J Crit Care 1996; 5: 109-118; 119-120.

[35] Faioni EM, Ferrero S, Fontana G, Gianelli U, Ciulla MM, Vecchi M, Saibeni S, Biguzzi E, Cordani N, Franchi F, Bosari S and Cattaneo M. Expression of endothelial protein C receptor and thrombomodulin in the intestinal tissue of patients with inflammatory bowel disease. Crit Care Med 2004; 32: 266-270.

[36] Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, Robson SC and Colgan SP. Endogenous adenosine produced during hypoxia

attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. Blood 2004; 104: 3986-3992.

[37] Jacobson KA and Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 2006; 5: 247-264.

[38] Strohmeier GR, Reppert SM, Lencer WI and Madara JL. The A2b adenosine receptor mediates cAMP responses to adenosine receptor agonists in human intestinal epithelia. J Biol Chem 1995; 270: 2387-2394.

[39] Fischer S, Wiesnet M, Marti HH, Renz D and Schaper W. Simultaneous activation of several second messengers in hypoxia-induced hyperpermeability of brain derived endothelial cells. J Cell Physiol 2004; 198: 359-369.

[40] Stenfeldt AL and Wenneras C. Danger signals derived from stressed and necrotic epithelial cells activate human eosinophils. Immunology 2004; 112: 605-614.

[41] Feoktistov I, Ryzhov S, Zhong H, Goldstein AE, Matafonov A, Zeng D and Biaggioni I. Hypoxia modulates adenosine receptors in human endothelial and smooth muscle cells toward an A2B angiogenic phenotype. Hypertension 2004; 44: 649-654.

[42] Chen Y, Epperson S, Makhsudova L, Ito B, Suarez J, Dillmann W and Villarreal F. Functional effects of enhancing or silencing adenosine A2b receptors in cardiac fibroblasts. Am J Physiol Heart Circ Physiol 2004; 287: 2478-2486.