A Novel Role of A2AR in the Maintenance of Intestinal Barrier Function of Enteric Glia from Hypoxia-Induced Injury by Combining with mGluR5

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During acute intestinal ischemia reperfusion (IR) injury, the intestinal epithelial barrier (IEB) function is often disrupted. Enteric glial cells (EGCs) play an important role in maintaining the integrity of IEB functions. However, how EGCs regulate IEB function under IR stimulation is unknown. The present study reveals that the adenosine A2A receptor (A2AR) is important for mediating the barrier-modulating roles of EGCs. A2AR knockout (KO) experiments revealed more serious intestinal injury in A2AR KO mice than in WT mice after IR stimulation. Moreover, A2AR expression was significantly increased in WT mice when challenged by IR. To further investigate the role of A2AR in IEB, we established an in vitro EGC-Caco-2 co-culture system. Hypoxia stimulation was used to mimic the process of in vivo IR. Treating EGCs with the CGS21680 A2AR agonist attenuated hypoxia-induced intestinal epithelium damage through up-regulating ZO-1 and occludin expression in cocultured Caco-2 monolayers. Furthermore, we showed that A2AR and metabotropic glutamate receptor 5 (mGluR5) combine to activate the PKCα-dependent pathway in conditions of hypoxia. This study shows, for the first time, that hypoxia induces A2AR-mGluR5 interaction in EGCs to protect IEB function via the PKCα pathway.

Keywords: A2AR, intestinal epithelial barrier, mGluR5, enteric glial cells, hypoxia

INTRODUCTION

Ischemia-reperfusion (IR) injury of the intestine is a fatal syndrome in abdominal surgeries involving aortic aneurism, small bowel or liver transplantation, cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis (Mallick et al., 2004). Acute intestinal IR injury is one of the most important causes of disruption to the intestinal epithelial barrier (IEB), initiates the systemic inflammatory response syndrome, and leads to multiple organ disorders (Vollmar and Menger, 2011; Lu et al., 2012). For these reasons, increasing attention has been focused on the underlying mechanisms of intestinal IR and promising protective strategies.

Traditionally, enteric glial cells (EGCs), the most abundant cell type in the intestinal nervous system, have been proposed to provide trophic and supportive effects for enteric neurons (Aube et al.,...
Duco Schriemer et al. revealed that A2ARi regulates (Neunlist et al., 2008). In transgenic mice, the conditional intestinal deletion of EGCs results in the development of fulminant inhibition of increased iNOS activity (Xiao et al., 2011). We also functions in response to lipopolysaccharide (LPS) stimulation by found that EGC-released glial-derived neurotrophic factor (GDNF) is closely involved in the IEB protective mechanisms of EGCs in acute IR stimulation (Xiao et al., 2014). However, the precise mechanisms by which EGCs regulate IEB in IR injury have not been elucidated.

The Adenosine A2A receptor (A2A-R), one of four G protein-coupled adenosine receptors (including A1-R, A2A-R, A2B-R, and A3-R), binds adenosine and induces activation of adenylate cyclase, promoting cAMP synthesis and producing corresponding biological effects (Welihinda et al., 2016). A2A-R is involved in the regulation of several physiological functions, including in the gastrointestinal system (Fornai et al., 2009). A2A-R has diverse and important roles in the intestine, including gut motor functions, acetylcholine release, cholinergic contraction modulation, and enteric nervous system regulation (Duarte-Araújo et al., 2004; Antonioli et al., 2006; Fornai et al., 2009; Schriemer et al., 2016). Dusco Schriemer et al. revealed that A2A-R is a key regulator of terminal neuronal differentiation in GDNF-treated enteric neural crest cells (ENCCs) (Schriemer et al., 2016). However, there is relatively little information about the role of A2A-R in EGCs. Therefore, this study was designed to investigate the role of A2A-R in EGC-mediated IEB regulation.

**MATERIALS AND METHODS**

**Cell Culture and Co-Culture**

Rat EGC/PK060399egfr (CRL-2690™) and human intestinal epithelial cells Caco-2 (HTB-37™) were obtained from the American Type Culture Collection. EGC/PK060399egfr and Caco-2 cells were grown in high glucose DMEM and MEM, respectively, supplemented with 10% FCS, 2 mM L-glutamine, and 100 U/100 μg/ml penicillin–streptomycin. Cells were incubated in a 5% CO2 humidified incubator at 37°C. The EGC-Caco-2 co-culture system was established as described previously by our laboratory (Xiao et al., 2014). Caco-2 cells were seeded on Millicell® filters (0.4 μm pore diameter; Millipore; Billerica, MA) at a density of 5 × 104 cells/cm² for up to 4–5 days. EGCs were seeded at an equal density in 6 or 24 well tissue culture plates to avoid any possible direct cell contact with Caco-2 cells. During the co-culture period, half of the culture medium in the apical and basal compartments was changed daily.

**Mice**

Global A2AR homozygous KO mice (A2AR knockout mice) with C57BL/6J background were provided by Dr. Yuanguo Zhou (Research Institute of Surgery, Daping Hospital, Army Medical University, Chongqing, China). Specific pathogen-free wild-type (WT) C57BL/6J mice were purchased from the Laboratory Animal Center of the Army Medical University. All mice were housed and maintained in laminar flow cabinets under specific pathogen-free conditions.

**In Vitro Hypoxia Experiments**

For hypoxia experiments, cells were subjected to hypoxia in a CO2 incubator (Forma® Series II Water Jacketed CO2 Incubators; Thermo Scientific) with 94% nitrogen, 5% CO2, and 1% oxygen and incubated at 37°C for 6 h. Re-oxygenation was initiated by replacing the media and exposing the cell monolayers to 37°C plus 5% CO2 for 1 h. Control cells were maintained at 37°C in an atmosphere with 5% CO2.

**Intestinal Ischemia/Reperfusion Model**

Male mice (8–10 weeks old) were fasted for 12 h and were free to drink water prior to surgery. The animals were intraperitoneally injected with 40 mg/kg of pentobarbital anesthesia and an aseptic laparotomies diaphragm line was placed. The following specific surgical procedures were performed as previously described (Xiao et al., 2014).

**Western Blot Analysis**

Cells and tissues were lyzed in cold RIPA buffer for 30 min and centrifuged at 13,000×g for 30 min at 4°C. Protein concentration was determined using a BCA assay reagent (Beyotime). The primary antibodies used were rabbit anti-ZO-1 (1:800), rabbit anti-Occludin (1:1000), mouse anti-A2AR (1:500), rabbit anti-PKCα (1:1000), rabbit anti-Na+/K+ ATPase (1:1000), and rabbit anti-GAPDH (1:1,000). Protein expression was measured in optical density units and normalized to GAPDH expression.

**Immunofluorescence Staining**

The small intestine tissues were embedded with OCT compound (Tissue-Tek, Sakura Finetek, Torrance, CA, United States). Consecutive frozen sections (5 μm in thickness) were obtained and fixed in 4% paraformaldehyde for 20 min at room temperature. After 30 min pre-incubation with a blocking solution containing 5% bovine serum albumin, sections were incubated overnight at 4°C with primary antibody against GFAP (Abcam), A2AR (Abcam), or ZO-1 (Abcam). After washing in PBS, sections were incubated with fluorescence-conjugated secondary antibodies at 37°C for 1 h. After washing in PBS, sections were incubated with DAPI nuclear stain solution for 5 min. All images were obtained using a TCS-SP5 confocal microscope (Leica, Germany).

**Coimmunoprecipitation**

Cells were harvested and lyzed in standard immunoprecipitation (IP) buffer containing either 1% 3-(3-cholamidopropyl) dimethylammonio] propane sulfonic acid (Chaps) or 1% Triton X-100 (for ERAD substrates), or 2% digitonin (for ERAD machinery) for 1 h on ice. Cells were centrifuged at 16,000 ×g for 10 min, and the supernatant was used for immunoprecipitation.
experiments. Co-IP was performed using protein A-agarose beads (EMD Millipore) with anti-A2AR (Santa Cruz Biotechnology, Inc.), anti-mGluR5 antibodies (Cell signaling), or anti-D2R antibodies (Santa Cruz Biotechnology, Inc.) following the usual method.

**Transepithelial Electrical Resistance (TER) and Permeability Measurements**

The TER of cells was determined via a Millipore electric resistance system (ERS-2; Millipore). Caco-2 cell monolayers were grown in Millicell inserts (0.33 cm² area, 0.4 μm pore diameter, and 6.5 mm diameter) and the culture medium was replaced before TER measurement. To calculate the actual resistance of the cell monolayer, the mean resistance of filters without cells was subtracted from the monolayer measurement, and the difference between the filter and monolayer areas was corrected.

The intestinal mucosa TER was measured by Ussing chambers (Physiologic Instruments, San Diego, CA). The excised intestinal tissues were bathed in 5 ml Krebs buffer (110.0 mM NaCl, 3.0 mM CaCl₂, 5.5 mM KCl, 1.4 mM KH₂PO₄, 29.0 mM NaHCO₃, and 1.2 mM MgCl₂; pH 7.4) on both the mucosal and serosal sides. The TER was measured as previously described (Liu et al., 2018).

**Statistical Analysis**

All experimental data are shown as the Mean ± SD. Statistical significance was determined by unpaired two-tailed Student t test analysis using GraphPad Prism version 7.0 software (San Diego, CA). If not otherwise stated, all experiments included three independent replications in triplicate. *p* < 0.05 was considered statistically significant.

**RESULTS**

**Acute IR Treatment Significantly Activated A2AR Expression in Intestinal Mucosa EGC**

To study the effect of A2AR on EGC, we first examined the expression of A2AR in different pathological conditions. Lipopolysaccharide (LPS) and hypoxia treatments were used to
stimulate EGCs in vitro. As shown in Figure 1A, A2A R expression dramatically increased in EGCs following LPS and hypoxia stimulation, with the effect of hypoxia being obvious than that of LPS. Therefore, hypoxia treatment was used to study the effect of A2A R. As demonstrated previously, GFAP is a specific marker of activated glial cells (Xiao et al., 2014). A2A R and GFAP immunofluorescent staining colocalization were used to observe changes in A2A R expression in EGCs. Acute IR-treated mice showed a moderate decrease in GFAP-positive intestinal EGCs compared to sham-treated mice (Figure 1B). However, the A2A R levels increased significantly in GFAP-positive EGCs after IR treatment. Together, these results indicate that hypoxia stimulation can activate the A2A R-mediated signaling pathway in mucosal EGCs in the intestine.

Activation of A2A R in EGCs Efficiently Prevents Barrier Dysfunction of Caco-2 Monolayers Under Hypoxia Stimulation

To further explore the role of A2A R in IEB modulation under acute hypoxia stimulation, we used an A2A R agonist and inhibitor separately in an in vitro EGC-Caco-2 co-culture system. The tight junctions (TJs) are primary determinants of IEB function (Lee, 2015). As shown in Figure 2A, western blot analysis revealed an almost 50% drop in ZO-1 and occludin expression in the hypoxia group and a similar reduction on ZO-1 and occludin expression in the A2A R inhibitor ZM241385 group. However, the A2A R agonist CGS21680 significantly prevented hypoxia-induced TJs destruction. Further study with immunocytochemistry (ICC) confirmed the western blot results (Figure 2B). Additionally, TER measurement analysis produced similar results. CGS21680 pretreatment effectively blocked TER decrease under hypoxia stimulation when compared to the ZM241385 pretreatment group (528.6 ± 18.11 Ω cm² vs. 289.6 ± 12.63 Ω cm² for the CGS21680 and ZM241385 pretreatment groups, respectively) (Figure 2C). Together, these results showed that A2A R plays a protective role in the EGC barrier-protecting effect on the IEC monolayer under hypoxia stimulation.

A2A R Deficiency Aggravates IR-Induced IEB Injury

To further confirm the role of A2A R in the barrier protective of EGCs during hypoxia stimulation, A2A R KO mice were treated with IR. Western blot analysis of ZO-1 and occludin expression was assessed in the small intestine (Figure 3A). Acute IR treatment led to a substantial decrease in ZO-1 and occludin expression in A2A R KO mice compared to WT mice. Immunofluorescence analysis also revealed a similar reduction in ZO-1 and occludin expression in the intestinal mucosa after IR stimulation (Figure 3B). The functional impact of A2A R knockdown on tight junctions in the small intestine was further evaluated by determining the TER value using Ussing chambers. As shown in Figure 3E, intestinal I/R caused a marked TER decrease in A2A R KO mice (78.63 ± 3.407 Ω cm²) compared with WT mice (94.5 ± 3.151 Ω cm²). Histological examination of intestinal tissues revealed that IR-treated A2A R KO mice showed more increased intestinal villus fracturing and epithelial removal
than did WT mice (Figures 3C,D). Together, these results strongly suggest that A2AR has a significant protective effect in IR-induced IEB injury.

Hypoxia Induces the Interaction between A2AR and mGluR5

There are synergistic interactions between A2A,R, mGluR5, and the dopamine D2 receptor (D2R) in central nervous system (CNS) related diseases (Ferraro et al., 2012; Fernández-Dueñas et al., 2013). However, whether this relationship exists in the enteric nervous system has yet to be determined. It has been suggested that in micro glial cells, A2AR combines with D2R in low glutamate concentration and combines with mGluR5 in high glutamate concentration (Dai et al., 2010; Beggio et al., 2016). Therefore, we used high glutamate concentrations as a positive control. We studied the relationship among them under hypoxia in EGCs. When exploring the effect of oxygen concentration, the band corresponding to D2R was coimmunoprecipitated by anti-A2AR antibodies, and an A2AR band was coimmunoprecipitated by anti-D2R antibodies. Together, these results indicate that A2AR and D2R interact in normoxic but not in hypoxic conditions (Figure 4A). However, an opposite relationship was observed between A2AR and mGluR5. As shown in Figure 4B, the band corresponding to mGluR5 was coimmunoprecipitated by anti-A2AR antibodies, and an A2AR band was also coimmunoprecipitated by anti-mGluR5 antibodies. These results indicate that A2AR and D2R interact in hypoxia but not in normoxia, demonstrating that A2AR interacts with D2R under normoxic conditions and interacts with mGluR5 under hypoxic conditions.

mGluR5 Inhibition Attenuates the Protective Effect of A2AR on the IEB from Hypoxia Induced Damage

To confirm that A2AR regulates the IEB via a mGluR5-dependent pathway, we tested the responsiveness of the IEB to 100 μM of the MPEP selective mGluR5 antagonist under hypoxia. Western blot analysis of ZO-1 and occludin expression revealed that treatment with MPEP significantly inhibited CGS21680-mediated activation of ZO-1 and occludin expression after hypoxia induced damage (Figure 4C). Similar results were observed in TER measurements: CGS21680 pretreatment effectively prevented the decrease of TER from hypoxia stimulation, while, MPEP blocked CGS21680-induced potentiation (454.3 ± 15.7 Ω cm² vs. 391 ± 8.441 Ω cm²) (Figure 4D). Together,
these results indicate that the protective effect of A2AR on the IEB after hypoxia stimulation is dependent on mGluR5.

**The PKCα Signaling Pathway Is Required for the Protective Function of A2AR on IEB**

The PKCα signaling pathway is associated with an A2AR–mGluR5 interaction-associated proinflammatory effect (Dai et al., 2013; Li et al., 2017). Therefore, we next tested whether PKCα is required for A2AR-mediated IEB protection. PKC family isoforms can translocate to multiple subcellular localizations in response to hypoxia in different cell lines (Yu et al., 2015). Consistent with this, western blots showed increased PKCα expression in the membrane after hypoxia stimulation in EGCs (Figure 5A). These results were confirmed by immunocytochemistry analysis showing that PKCα translocates from the cytoplasm to the cell membrane in EGCs after hypoxia stimulation (Figure 5B). We then used PKCα inhibitor, chelerythrine chloride, to observe whether the protective effect of CGS21680 on IEB was affected. As shown in Figure 5C, chelerythrine chloride significantly reduced ZO-1 and occludin protein levels in CGS21680-pretreated EGCs. As expected, chelerythrine chloride pretreatment also blocked CGS21680-promoted TER (533 ± 24.2 Ω cm² vs. 390.9 ± 24.14 Ω cm²) (Figure 5D). Together, these results suggest that A2AR exerts its protective effects on IEB via the PKCα signaling pathway.

**DISCUSSION**

Our research has previously demonstrated that EGCs enhance IEB functions under acute intestinal injury (Xiao et al., 2014). In this context, our data provide the first evidence that EGCs protect IEB by activating A2AR. The A2AR agonist significantly improved the barrier functions of Caco-2 monolayers following exposure to HR stimulation. Moreover, in A2AR KO mice, intestinal tissue damage was accelerated, including the structural and mucosal barrier defects, following intestinal I/R. We found that A2AR combines with mGluR5 under hypoxic conditions to exert a protective effect on IEB. This data also shows that A2AR and mGluR5 combine to activate the PKCα-dependent signaling pathway. Together, these results show that A2AR plays a critical role in the barrier protective mechanism of EGCs under acute intestinal hypoxia stimulation.

EGCs are involved in the regulation of IEB function. However, the precise mechanisms by which EGCs function in the regulation...
of IEB remain unclear. Increasing evidence indicates that EGCs and astrocytes share morphological features and electrophysiological properties and express similar proteins, including GFAP and S100β, leading to the idea that EGCs might share many features of the central nervous system astrocytes (Le Berre-Scoul et al., 2017). The similarities between EGCs and astrocytes indicate that these two glial cell types may regulate barrier functions through common molecular mechanisms (Jiang et al., 2005; Xiao et al., 2014). It is reported that primary cell cultures of either astrocytes or enteric glia can induce barrier properties across endothelia and epithelia (Savidge et al., 2007). Jiang et al. previously reported that implantation of enteric glia accelerates normal spinal cord vasculature repair processes at the site of injury and promotes functional blood-brain barrier (BBB) induction (Jiang et al., 2005). However, there are no mice with selective inactivation of EGCs-derived A2AR, we cannot comprehensively show that EGCs protect IEB via the A2AR pathway. We exposed an in vitro EGC-Caco-2 co-culture system to hypoxia treatment to detect the role of A2AR in enteric glia. Our results show that activation of A2AR in EGCs prevents damage to the IEB during hypoxia.

A2AR activation is closely related to a variety of neurological diseases and is an important component of the adenosine signaling pathway (Stone et al., 2009). Recently, many studies have suggested that A2AR also plays an important protective role in enteritis (Warren et al., 2012; Antonioli et al., 2018). However, there is relatively little information about the role of A2AR in intestinal IR damage. A2AR inactivation can prevent IR by regulating the inflammatory response and excitotoxic cascades in the brain, kidney, lung, and blood vessels (Vincent and Okusa, 2015; Li et al., 2013; Mohamed et al., 2012; Mohamed et al., 2016; Gui et al., 2009; Cunha, 2005). Due to the similarities between the brain and the intestine, we speculate that A2AR may also have protective effects on intestinal IR damage. Our data shows that IEB damage is accelerated in A2AR KO mice. However, because there are no mice with selective inactivation of EGCs-derived A2AR, we cannot comprehensively show that EGCs protect IEB via the A2AR pathway. We exposed an in vitro EGC-Caco-2 co-culture system to hypoxia treatment to detect the role of A2AR in EGCs. Our results show that activation of A2AR in EGCs prevents damage to the IEB during hypoxia.

To clarify the mechanism by which A2AR influences IEB functions under acute intestinal epithelium hypoxia injury, we explored how A2AR works in the brain. Functional A2AR-mGlu5R heteromeric complexes have been reported in the central nervous system (Beggiato et al., 2016; Temido-Ferreira et al., 2018). Beggiato et al. found that A2AR and mGlu5R interact synergistically to modulate D2R-mediated control of striatopallidal GABA neurons (Beggiato et al., 2016). Additionally, Dai et al. reported that A2AR-mGlu5R interplay is critical for the proinflammatory effect in bone marrow-derived cells (BMDCs) after acute lung injury (Dai et al., 2013). Consistent with our expectation, we observed that A2AR combined with mGluR5 in EGCs suffering from hypoxia.

mGluR5 is a G-protein-coupled receptor that exerts its physiological roles through intracellular chemical-messenger signaling cascades (Power et al., 2016). In general, mGluR5 represents a promising target for studying neuro-protective agents of potential application in neurodegenerative diseases (Li et al., 2017). However, little data exists supporting the function of
mGluR5 in the intestine, especially in relation to its role in IEB regulation. In the intestinal mucosa, mGluR5 is only observed in EGCs (Nasser et al., 2007). EGCs are involved in the occurrence of inflammatory bowel disease through c-Fos and ERK1/2 phosphorylation induced by mGluR5 (Nasser et al., 2007). In the present study, we demonstrated that mGluR5 plays a key role in the protection of IEB by A2AR. The proinflammatory effect of mGluR5 is not mediated by PKC signaling, but instead uses the PKA pathway (Dai et al., 2013). Giaroni et al. reported that the PKCα antagonist significantly inhibits intestinal mucosal injury induced by IR (Giaroni et al., 2011). These studies provide further support for our results that A2AR protects the IEB by a PKCα dependent pathway.

Taken together, our results suggest a model for A2AR in the maintenance of intestinal barrier function. Upon intestinal hypoxia injury, A2AR combines with mGluR5 to protect IEB function via the PKCα pathway in EGCs (Figure 6). Although there are complex interactions between A2AR and mGluR5 that remain to be fully understood, our findings are important for a better understanding of the role of EGCs in regulating IEB. Additionally, these findings offer new insight into the clinical use of A2AR modulators for IR-induced intestinal injury.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Animal Ethics Committee of the Army Medical University.

**AUTHOR CONTRIBUTIONS**

LS and XL contributed equally to this work. LS and XL conceived the study and analyzed the data. HG, SC, XF, and CZ performed the research. HY and WX wrote the manuscript.

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**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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