

Adenosine A₁ receptor agonism protection mechanism in intestinal ischemia/reperfusion injury via activation of PI3K/Akt signaling

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Abstract. Intestinal ischemia/reperfusion (I/R) injury is a common clinical problem with a high mortality rate, resulting from loss of blood flow to an intestinal segment. Adenosine serves a protective role in intestinal I/R injury; however, its potential mechanism is not completely understood. The present study aimed to investigate the protective effects of adenosine A₁ receptor (A₁R) agonists CPA and LUF6941 and whether their mechanisms are associated with the PI3K/Akt signaling pathway. To simulate intestinal I/R injury, a cell oxygen-glucose deprivation/reoxygenation (OGD/R) model was established and the human colon cancer cell line (Caco-2) was incubated with A₁R agonists before OGD/R treatment. The viability of Caco-2 cells was detected by PI and Cell Counting Kit-8 assay, apoptosis was detected using flow cytometry and western blotting was used to analyze protein expression levels of PI3K, Akt and p53 in Caco-2 cells. A₁R agonist pretreatment protected Caco-2 cells against OGD/R-induced cell damage and activated PI3K/Akt signaling. Additionally, apoptosis was inhibited by downregulating phosphorylation of p53 protein, as evidenced by increased cell viability. These findings suggested that A₁R agonists decreased OGD/R damage in Caco-2 cells, which may be due to their anti-apoptotic effects and activation of the PI3K/Akt/p53 signal pathway.

Introduction

Intestinal ischemia/reperfusion (I/R) injury is a serious clinical complication, associated with abdominal and thoracic vascular surgery, small bowel transplantation, traumatic hemorrhagic

shock, cardiopulmonary bypass and strangulated intestinal obstruction, that increases the incidence rate and mortality of intestinal mucosa injury and intestinal necrosis (1,2). Intestinal ischemia is caused by local or systemic factors, such as mechanical vascular obstruction, hypovolemia, hypotension, hypoxia or sepsis, which lead to cytopathy and death due to oxygen and nutrient deprivation (3). Blood reperfusion following intestinal ischemia also leads to cell damage and death, mainly due to the accumulation of oxygen free radicals and other cytotoxic substances resulting in lipid peroxidation of the cell membrane (4).

Several drugs have been proposed to decrease or prevent the cellular dysfunctions caused by intestinal I/R injury, including drugs that interfere with adenosine (ADO) receptors (ARs) (5,6). In mammals, ATP degradation produces ADO that blocks potentially destructive inflammatory cascades and decreases activation of platelets, leukocytes and endothelial cells by mediating four subtypes of AR: A₁, A_{2A}, A_{2B} and A₃ (7,8). A previous study concluded that ADO blocks intestinal I/R injury and an A₁R agonist (CPA) ameliorated intestinal contractile dysfunction induced by I/R. It was shown that intestinal I/R injury was limited by decreasing oxidative stress, lowering neutrophil infiltration and increasing reduced glutathione content (9,10). In addition, activation of A₁R has been shown to have cardioprotective, renal and pulse protective effects on I/R injury (11-14).

Upon activation of A₁R, specific signal transduction pathways are modulated, including the phosphatidylinositol 3-kinase (PI3K)/activated protein kinase B (Akt) signaling pathways. The activation of A₁R in cells and tissue leads to increased expression levels of Akt, resulting in an anti-apoptotic effect (15,16). Studies have shown that pharmacological phosphorylation of select reperfusion survival promoting kinases, such as PI3K and Akt, protects the heart from I/R injury following ischemia (17,18). Therefore, it was hypothesized that A₁R agonism may protect against intestinal I/R injury, not only by ameliorating intestinal contractile dysfunction but also by activating the PI3K/Akt signaling pathway.

In the present study, two selective A₁R agonists were used with different residence times. CPA (N⁶-cyclopentyl-adenosine) and LUF6941 [2-amino-6-((2-(4-chlorophenyl) thiazol-4-yl) methylthio)4-(4-methoxyphenyl)pyridine-3,5-dicarbonitrile]

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were examined to assess their protective effects on enterocytes in an I/R model. Copeland *et al* (19) defined residence time as the period when the drug and receptor form a complex after the drug binds. Previous investigations have shown that A₁R agonists with different residence times could produce different anti-lipolytic effects (20,21). The present study aimed to determine whether A₁R agonists CPA and LUF6941 protect cells by activating the PI3K/Akt signaling pathway.

Materials and methods

Cell culture. The human colon carcinoma cell line Caco-2 cell line (Jiangsu Laisen Institute of Biotechnology Co, Ltd.) was used for this study. The cell line was grown in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% HEPES in a humidified atmosphere at 5% CO₂ and 37°C. Cells were used at 60-70% confluence and plated at the density of 1x10⁶ cells per ml in a 96-well flat-bottomed plate 1 day before assay.

Oxygen-glucose deprivation/reoxygenation (OGD/R) model construction and experimental groups. To simulate intestinal I/R injury, a cell OGD/R model was established as previously described (22,23). Briefly, Caco-2 cells cultured in glucose-free Earle balanced salt solution (Leagene Biotechnology; Beijing Regen Biotechnology Co., Ltd.) in a microaerophilic system at 37°C, 5% CO₂, 1% O₂ and 94% N₂ for 8 h to achieve OGD. Media was then replaced with culture medium EBSS containing high sugar (glucose 4.5 g/l)(Leagene Biotechnology; Beijing Regen Biotechnology Co., Ltd.) and cells were returned to normal oxygen culture conditions (5% CO₂, 37°C) for 20 h to achieve reoxygenation. The control group was kept in normal oxygen conditions (5% CO₂, 37°C) for the same period time. A previous study showed that 1x10⁻⁶ M agonists CPA and LUF6941 produces strong biological activity (20). Agonists were diluted in EBSS to 1x10⁻⁶ M concentration from the stock solution and added at the beginning of OGD/R. ADO deaminase at 1 U/ml was used to remove endogenously released adenosine from Caco-2 cells during OGD/R before the addition of the agonists (24). Cells were divided into following groups: i) Control; ii) OGD/R; iii) CPA (OGD/R + 1x10⁻⁶ M CPA) and iv) LUF6941 (OGD/R + 1x10⁻⁶ M LUF6941).

Cell viability assay and imaging of Caco-2 cells. PI assay was used to detect non-viable cells in all groups. At the end of OGD/R, 5 mM PI (Sigma-Aldrich; Merck KGaA) was added to each well (1x10⁶ cells) and incubated for 15 min at room temperature in the dark. Cells were imaged using an inverted fluorescence microscope (10x magnification) connected to a SPOT RT camera (Axio Imager A2; Zeiss GmbH) and DG-4 lamp box (Sutter Instruments, Novato, CA) at 488 nm excitation wavelength and 617 nm emission filter. From each well, two images were taken and PI-positive cells were quantified using Image J 1.44 software (National Institutes of Health). Data were normalized to the OGD/R treatment group. Duplicate wells were used for each experiment and each experiment was repeated 3 times.

Cell counting kit-8 (CCK-8) assay. CCK-8 assay (Beyotime Institute of Biotechnology) was used to determine cell viability according to the manufacturer's protocols. Briefly, Caco-2

cells were seeded in 96-well plates (150 μl/well, 1x10⁴ cells) and pretreated with 1x10⁻⁶ M CPA or LUF6941, as aforementioned. Following OGD/R, 10% CCK-8 reagent was added to each well and cells were incubated for 2 h at 37°C. Absorbance was recorded at 450 nm using a microplate reader (Varioskan LUX; Thermo Fisher Scientific, Inc.). Data were normalized to the control group.

Western blotting. Caco-2 cell protein was obtained using protein lysis buffer (RIPA lysis buffer; Beyotime) containing 1 mmol/l phenylmethylsulfonyl fluoride (PMSF; Beyotime) and phosphatase inhibitors (Beyotime). Protein concentrations of cell lysates were determined using a BCA protein assay kit (Beyotime). Equal amounts of protein (20 μg/lane) were electrophoresed on 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were blocked with 5% BSA (Cell Signaling Technology) for 2 h at room temperature, before incubation at 4°C overnight with the following primary antibodies (all from Cell Signaling Technology) at a 1:1,000 dilution: PI3K (cat. no. 3811S), Akt (cat. no. 9272S), phosphorylated (p)-Akt (cat. no. 9275S), p53 (cat. no. 9282S), p-p53 (cat. no. 9284S) and GAPDH (cat. no. 5174S; Cell Signaling Technology, Inc.). Membranes were washed by PBS buffer and incubated with horseradish peroxidase-conjugated second antibody (1:2,000; cat. no. ab97051; Abcam) at room temperature for 1 h. Immunoreactive protein bands were observed with a chemiluminescence kit (Sigma-Aldrich; Merck KGaA) and quantified using Image J software (National Institutes of Health). GAPDH was used as the loading control.

Flow cytometry. Apoptosis was assessed using the PE Annexin V Apoptosis Detection kit I (cat. no. 559763; Becton, Dickinson and Company) according to the manufacturer's protocols. Briefly, Caco-2 cells were suspended in binding buffer at a concentration of 1x10⁶ cells/ml. Aliquots of 100 μl cell suspension (1x10⁵ cells) were transferred to 5 ml culture tubes and 5 μl PE Annexin V and 5 μl 7-AAD were added to each tube. The cells were gently vortex and incubated in darkness at room temperature (25°C) for 15 min. An additional 400 μl binding buffer was added to each tube. Finally, cell early and late apoptosis were analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter, Inc.) with FlowJo 8.7.1 software (FlowJo LLC).

Statistical analysis. All data are presented as the mean ± SEM and all experiments were performed three times. Statistical differences between groups were determined by one-way ANOVA followed by post hoc Dunnett's test. Statistical analysis was performed using SPSS 22.0 (IBM, USA) and GraphPad Prism 7.0 (GraphPad software, San Diego, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

CPA and LUF6941 increase cell viability after OGD/R. To investigate whether A₁R agonists have a protective effect on intestinal I/R injury, an OGD/R model was established and the cell viability was examined using PI assay. Incubation of Caco-2 cells in a microaerophilic system for 8 h significantly

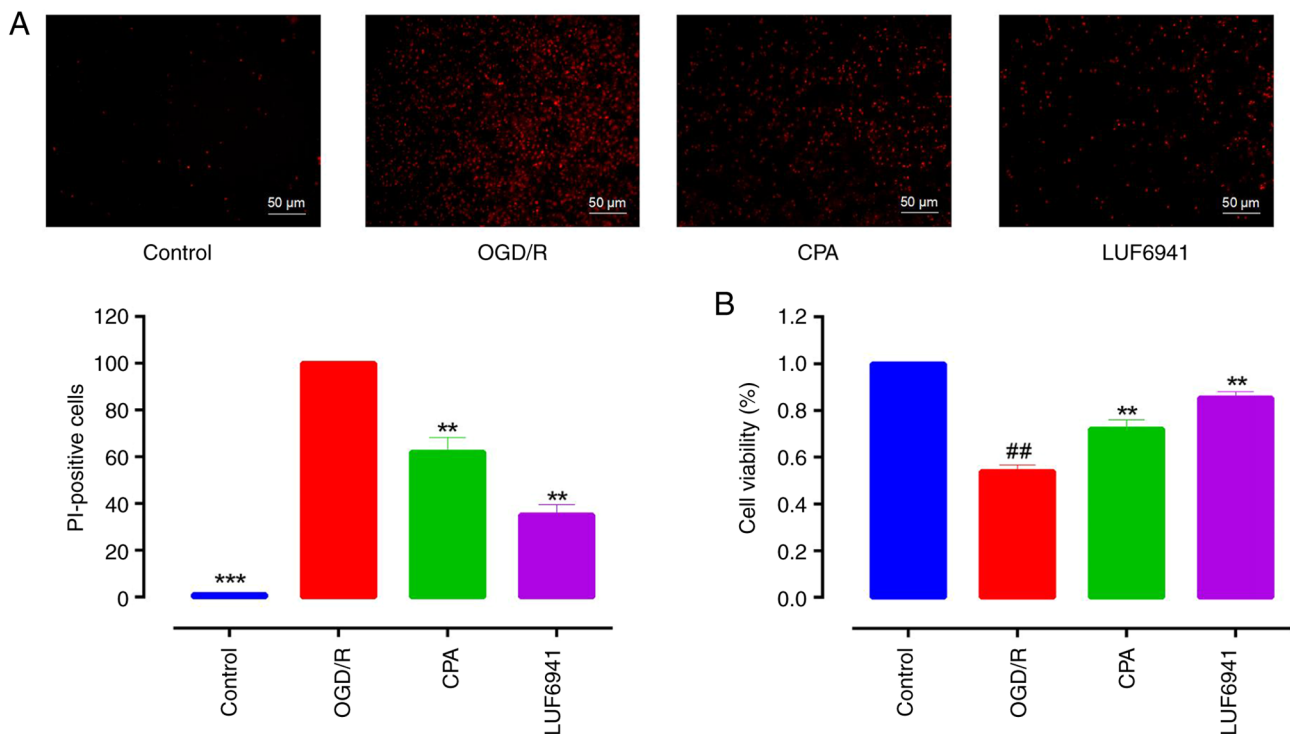


Figure 1. Effect of adenosine A₁ receptor activation on cell survival during OGD/R. Caco-2 cells were incubated with CPA or LUF6941 in OGD/R. (A) Caco-2 cells were stained with PI to measure non-viable cells. (B) Cell viability was determined using CCK-8 assay. Data are shown as the mean \pm SEM and experiments were performed three times. ** $P < 0.01$, *** $P < 0.001$ vs. OGD/R. ## $P < 0.01$ vs. control. OGD/R, oxygen-glucose deprivation/reoxygenation.

increased the proportion of non-viable cells. The OGD/R group was assigned as the control to which other treatment groups were normalized and compared. CPA and LUF6941 significantly decreased the proportion of non-viable cells (Fig. 1A; 62.04 ± 6.20 and $35.14 \pm 4.43\%$, respectively; both $P < 0.01$). To evaluate the level of cell injury, CCK-8 assay was used to determine cell viability. Consistent with PI assay results, CPA and LUF6941 significantly increased the viability of OGD/R-treated Caco-2 cells (Fig. 1B; 72.02 ± 2.38 and $85.40 \pm 1.51\%$, respectively; both $P < 0.01$).

Effect of CPA and LUF6941 pretreatment on PI3K/Akt signaling. A previous study demonstrated that the PI3K/Akt signaling pathway is activated following ischemia to promote proliferation (17). Here, A₁R agonists activated PI3K/Akt signaling pathway members in intestinal I/R injury (Fig. 2). Western blotting showed that PI3K and p-Akt/Akt levels increased significantly when cells were treated with CPA (PI3K, 1.58 ± 0.13 ; p-Akt/Akt, 1.65 ± 0.22 ; both $P < 0.05$) and LUF6941 (PI3K, 2.05 ± 0.19 ; p-Akt/Akt, 1.92 ± 0.88 ; both $P < 0.01$). In addition, A₁R agonists significantly decreased levels of p-p53/p53 compared with the OGD/R group (CPA, 0.75 ± 0.03 ; LUF6941, 0.60 ± 0.03 ; both $P < 0.01$). These results indicated that the PI3K/Akt signaling pathway, activated by A₁R agonists, exhibited protective effects during intestinal I/R injury and A₁R agonists significantly inhibited the degree of phosphorylation of p53 protein.

CPA and LUF6941 decrease Caco-2 apoptosis after OGD/R. To investigate the effect of A₁R agonists on the physiological function of Caco-2 cells after OGD/R, an Annexin

V-PE/7-AAD double staining assay was performed to assess the apoptotic activity of Caco-2 cells after CPA and LUF6941 treatment (Fig. 3). Flow cytometry showed that, compared with the OGD/R group, Caco-2 cell apoptosis significantly decreased in the CPA and LUF6941 groups (30.48 ± 1.20 and $27.37 \pm 1.59\%$, respectively; both $P < 0.01$). Early apoptotic cells were also significantly decreased in the CPA ($21.66 \pm 1.45\%$; $P = 0.0312$) and LUF6941 ($20.36 \pm 2.03\%$; $P = 0.0306$) groups compared with the OGD/R group. Late apoptotic cells were observed in all groups but there was no significant difference between the CPA group and OGD/R group ($P = 0.1198$). However, there was a significant difference ($P < 0.05$) between the LUF6941 and OGD/R group.

Discussion

The present study showed that A₁R agonists CPA and LUF6941 protected against OGD/R in intestinal epithelial Caco-2 cells, which was associated with activation of the PI3K/Akt signaling pathway. To simulate clinical intestinal I/R injury, an OGD/R model was established to evaluate the survival rate of Caco-2 cells after intestinal I/R injury. The proportion of apoptotic Caco-2 cells pretreated with A₁R agonists significantly compared with the OGD/R group. CPA and LUF6941 were able to significantly decrease apoptosis in Caco-2 cells after OGD/R.

Although the two agonists showed significant anti-apoptotic effects in early apoptosis, there were differences in late apoptosis. LUF6941 inhibited late apoptosis, while CPA had no significant effect. It was hypothesized that this result may be associated with residence time of the drug (25).

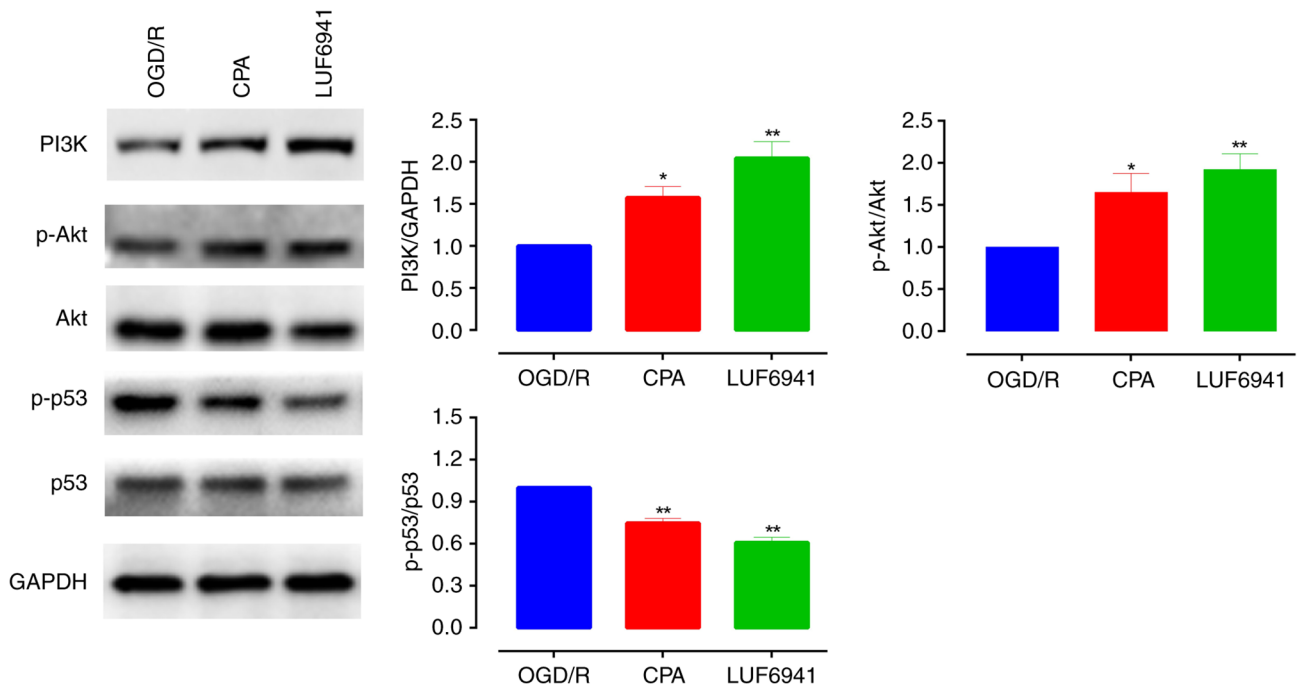


Figure 2. A₁R agonists activate the PI3K/Akt signaling pathway during intestinal ischemia/reperfusion injury. The protein expression of PI3K, p-Akt/Akt and p-p53/p53 was measured using western blotting. Data are shown as the mean \pm SEM and experiments were performed three times. * $P < 0.05$, ** $P < 0.01$ vs. OGD/R. OGD/R, oxygen-glucose deprivation/reoxygenation; PI3K, phosphatidylinositol 3-kinase; Akt, activated protein kinase B; p-, phosphorylated.

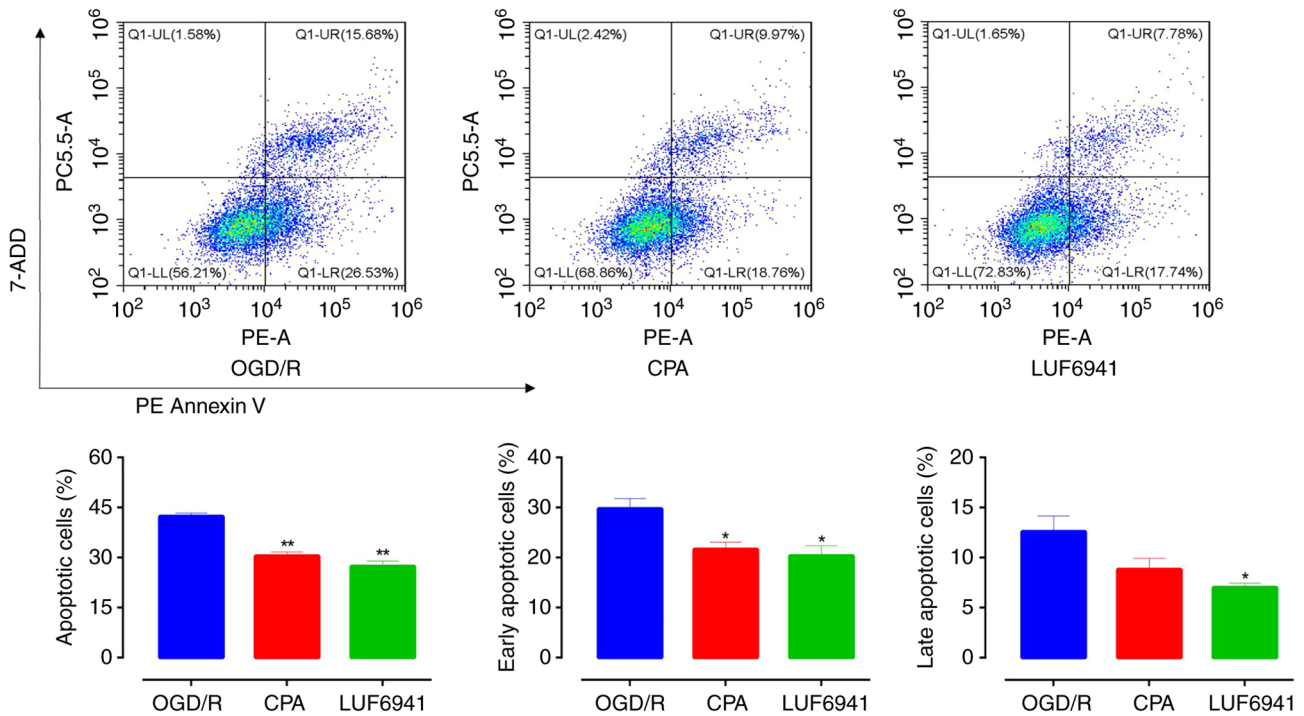


Figure 3. Apoptosis detected by flow cytometry. Apoptosis levels were analyzed by the Annexin V-PE/7-AAD staining assay in Caco-2 cells with or without A₁R agonists. Data are shown as the mean \pm SEM and experiments were performed three times. * $P < 0.05$, ** $P < 0.01$ vs. OGD/R. OGD/R, oxygen-glucose deprivation/reoxygenation.

Previous research suggests that enduring drug-target interaction produces sustained functional response, while short drug-target engagement leads to a more short-lived duration of drug action (26,27). Previous investigation has shown that A₁R agonists with longer residence times sustain an anti-lipolytic

effect (20). LUF6941 has a longer residence time than CPA and this longer residence time produces a sustained wash-resistant anti-lipolytic effect in rat adipocytes (20). Thus, it was hypothesized that differences in the anti-apoptotic effect of the A₁R agonists may be associated with residence time of the drug

during OGD/R. With a long residence time, LUF6941 had an inhibitory effect throughout apoptosis, while CPA, with a shorter residence time, appeared to only play a role in the early stages of apoptosis. Therefore, CPA and LUF6941 improve survival of Caco-2 cells; however, the degree of improvement was different.

To determine the underlying mechanisms involved in inhibition of Caco-2 cell apoptosis induced by A₁R agonists, protein expression levels of PI3K, Akt and p53 were analyzed using western blotting. Levels of activated Akt and the expression of upstream PI3K were significantly increased following OGD/R in the A₁R agonist-treated cells. A₁R agonists were also shown to inhibit intestinal cells apoptosis by down-regulating phosphorylation level of p53 protein. Previous studies have shown that activated Akt promotes survival of intestinal epithelial cells and increases tolerance of the heart, brain and other organs to I/R damage by inhibiting apoptotic pathways (17,28-30). Apoptosis is the main mechanism of intestinal cell death during I/R (31). p53 protein serves a key role in mediating apoptosis and blocking p53-mediated Akt activation in response to DNA damage results in decreased cell viability (32). In the present study, A₁R agonist pretreatment significantly enhanced the activation of Akt and decreased cell apoptosis in the intestinal I/R model. Therefore, activation of Akt may be involved in the improvement of recovery from I/R injury induced by A₁R agonism.

There are, however, some limitations to the design of the current study. Only the mechanisms of A₁R agonist protection at the cellular level following I/R *in vitro* were studied, however, Taha *et al* (33) showed that ADO was able to attenuate motor and neural dysfunctions of small bowel caused by ischemia, but not by reperfusion, in rabbits. Meanwhile, Lee *et al* (12) showed that A₁R activation or blockade is associated with decreased and enhanced inflammatory responses, respectively, following renal I/R injury in mice. After intestinal I/R, A₁R may have anti-inflammatory effects in addition to inhibiting cell apoptosis and the association between these mechanisms requires further study. Mandl and Depping (34) reported that low oxygen also activates the hypoxia-inducible factor (HIF) pathway. However, to the best of our knowledge, no studies have reported that CPA and LUF6941 affect the HIF pathway. In future studies, the effect of CPA and LUF6491 treatment on this pathway will be investigated.

In summary, pretreatment with A₁R agonists had a protective effect against intestinal I/R injury by activating PI3K/Akt signaling. A₁R agonists inhibited Caco-2 cell apoptosis by downregulating phosphorylation of p53 protein *in vitro*. These results indicated that the intestinal I/R protection induced by A₁R agonists may, at least in part, be attributed to an anti-apoptotic effect mediated by activating the PI3K/Akt/p53 signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY conceived the study and designed the experiments. YY and QX performed the experiments. YY, QX and ZM were responsible for the acquisition, analysis and interpretation of the data. QX and ZM wrote and revised the article. YY and QX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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