Protective effects of Notoginsenoside R1 on intestinal ischemia-reperfusion injury in rats

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1Department of Integration of Chinese and Western Medicine, School of Basic Medical Sciences, Peking University, Beijing, China; 2Tasly Microcirculation Research Center, Peking University Health Science Center, Beijing, China; 3Key Laboratory of Microcirculation, State Administration of Traditional Chinese Medicine, Beijing, China; and 4Key Laboratory of Stasis and Phlegnm of State Administration of Traditional Chinese Medicine, Beijing, China

Submitted 18 April 2013; accepted in final form 6 November 2013

Li C, Li Q, Liu YY, Wang MX, Pan CS, Yan L, Chen YY, Fan JY, Han JY. Protective effects of Notoginsenoside R1 on intestinal ischemia-reperfusion injury in rats. Am J Physiol Gastrointest Liver Physiol 306: G111–G122, 2014. First published November 14, 2013; doi:10.1152/ajpgi.00123.2013.—Intestinal ischemia and reperfusion (I/R) is a clinical problem occurred for diverse causes with high mortality. Prophylaxis and treatment of intestinal I/R remains a challenge for clinicians. The purpose of the present study was to explore the role of Notoginsenoside R1 (R1), a major component form Panax notoginseng, in management of intestinal I/R injury. Intestinal I/R was induced in male Sprague-Dawley rats by clamping the superior mesenteric artery for 90 min followed by reperfusion for 60 min or 3 days. R1 (10 mg·kg⁻¹·h⁻¹) was administered either 20 min before ischemia or 20 min after reperfusion. Intestinal microcirculation was evaluated by intravitral microscopy over 60 min reperfusion. Sixty minutes or 3 days after reperfusion, rats were killed for histological examination of the jejunum tissue and immunohistochemical localization of myeloperoxidase and CD68. ATP, ADP, and AMP content in jejunum tissue was assessed by ELISA. Activation of nuclear factor-κB (NF-κB) and expression of ATP5D and tight junction proteins were determined by Western blotting. The results demonstrated that R1 is capable of attenuating intestinal I/R-induced microvascular hyperpermeability, inflammatory cytokine production, NF-κB activation, and loss of tight junction proteins, as well as improving energy metabolism during I/R. The results of the present study suggest R1 as an option in protecting against intestinal I/R injury.

Microcirculation; inflammation; adenosine 5’-triphosphate; nuclear factor-κB; tight junction

Intestinal ischemia-reperfusion (I/R) injury may occur in diverse conditions, such as mesenteric artery embolism, small bowel transplantation, cardiopulmonary bypass and abdominal aortic aneurysm surgery, and traumatic or hemorrhagic shock (4, 16). Intestinal I/R may lead to sepsis, systemic inflammatory response syndrome, and multiple organ failure, carrying high morbidity and mortality (1, 25).

Generation of oxygen radicals and the starting of a proinflammatory cascade have been considered as the two major mechanisms for the pathogenesis of intestinal I/R injury. Intensive effort has been made to develop treatments targeting oxidative stress and inflammatory reaction (13, 28). However, the result so far remains unsatisfied. It appears that oxidative stress and the resultant inflammatory cascade are complex and multifactorial, most of which take place during reperfusion. Once this cascade initiates, it would be difficult to interrupt. Thus, a tempting strategy to cope with intestinal I/R injury would be to interfere in the pathogenetic process by acting at multiple links, particularly in the early phase.

Intestinal I/R injury starts with ischemia, which results in hypoxia and malnutrition leading to energy metabolism disorder characterized by ATP deficiency. Lack of ATP impacts a range of ATP-consuming processes, including cation pumps and F-actin polymerization. The cation pump disorder drives cells toward necrosis, whereas disarrangement of F-actin causes barrier breakdown of epithelium and endothelium. The energy metabolism disorder has been noted in intestinal I/R injury and used as a parameter for assessment of intestinal damage and viability after gut I/R and bowel transplantation (18, 27). However, few, if any, reports are available to protect or treat intestinal I/R injury by modulating energy metabolism.

Notoginsenoside R1 (R1, Fig. IA) is a major component of Panax notoginseng, a Chinese medicine commonly used for treatment of cardiovascular diseases in China. R1 is known to have anti-inflammatory, anti-oxidative, and anti-apoptotic potential (12, 21, 29, 30). Our previous in vivo study has shown that R1 attenuates hepatic microcirculatory disturbances induced by gut I/R (5). However, there is no report with respect to the effect of R1 on intestinal I/R injury. The present study was designed to investigate the role of R1 in intestinal I/R injury, with focus particularly on its potential for modulation of energy metabolism and regulation of microvascular permeability.

Materials and Methods

Materials. R1 was obtained from Feng-Shan-Jian Medical (Kunming, China). ATP, ADP, AMP, and malondialdehyde (MDA) ELISA kits were all from Huanya Biomedicine Technology (Beijing, China). The antibodies against inhibitory-κB-α (IκB-α) and nuclear factor-κB (NF-κB) p65 were from Cell Signaling Technology (Beverly, MA). The antibody against CD68, claudin-5, occludin, and zonula occludens (ZO)-1 were from Abcam (Cambridge, MA). The antibody against ATP5D was purchased from Santa Cruz (Santa Cruz, CA). The antibody against myeloperoxidase (MPO), BCA protein assay kit, and NE-PER nuclear and cytoplasmic extraction reagents were from Thermo Scientific (Fremont, CA). All other chemicals were of the highest commercial grade available.

Animals. Male Sprague-Dawley rats weighing 200–220 g were obtained from the Animal Center of Peking University Health Science Center. Animals were raised at a temperature of 20 ± 2°C with
12:12-h light-dark cycles and fed with standard rat chow and water. Animal care was in compliance with institutional guidelines of the Peking University Animal Research Committee, and experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the Health Science Center of Peking University (LA2010-001).

Surgical protocols. Animals were fasted for 16 h with free access to water before the experiments. After anesthetization with intraperitoneal injection of pentobarbital sodium (50 mg/kg), the femoral vein was cannulated for drug infusion. To induce intestinal I/R injury, the rats underwent a midline laparotomy, and the superior mesenteric artery (SMA) was isolated and clamped for 90 min with an atraumatic arterial clamp to occlude splanchnic circulation. During this procedure, the animals were placed on a heating pad to maintain body temperature at 37°C. Following occlusion, the clamps were removed for reperfusion. The reperfusion of the intestine was confirmed by recurrence of the pulses and pink color. Sixty minutes or 3 days after reperfusion, animals were killed, and jejunum 0–60 cm proximal to the ileocecal junction was removed, washed in saline, and used for histological examination and biochemical studies. In another set of studies, rats were observed for 3 days following reperfusion to determine survival rate.

Experimental group. Rats were randomly allocated into 10 groups, 10 animals each, as follows: groups 1 and 2) sham groups (60 min or 3 days). Rats underwent an identical surgery but without SMA occlusion, and received continuous intravenous infusion of normal saline; groups 3 and 4) sham + R1 groups (60 min or 3 day): rats underwent an identical surgery as rats in sham groups, and received a continuous infusion of R1 (10 mg·kg⁻¹·h⁻¹ iv) for 170 min starting from 20 min before ischemia; group 5) I/R 60 min group: rats were subjected to 90 min intestine ischemia and 60 min reperfusion and received continuous intravenous infusion of normal saline; group 6) R1 + I/R 60 min group: rats were subjected to intestine I/R like the animals in the I/R 60 min group and received continuous intravenous infusion of R1 (10 mg·kg⁻¹·h⁻¹ for 170 min) starting from 20 min before I/R; group 7) I/R 60 min + R1 group: rats were subjected to intestine I/R like the animals in the I/R 60 min group and received continuous intravenous infusion of R1 (10 mg·kg⁻¹·h⁻¹) for 40 min starting from 20 min after initiation of reperfusion; group 8) I/R 3 day group: rats underwent an identical
procedure to the animals in the I/R 60 min group, except for reperfusion for 3 days instead of 60 min; group 9) R1 + I/R 3 day group: rats underwent an identical procedure to the animals in the R1 + I/R 60 min group, except for reperfusion for 3 days instead of 60 min; and group 10) I/R 3 day + R1 group: rats underwent an identical procedure to the animals in I/R 60 min + R1 group, except for reperfusion for 3 days instead of 60 min.

The R1 was dissolved in normal saline and used at a dose based on a previous in vivo study (5). All intravenous infusions were performed at a constant rate of 2 ml/h.

**Intravital microscopy.** Rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and placed on an observation board in the lateral position. After laparotomy, the small intestine was exteriorized gently and mounted on an adjustable Plexiglas microscope stage within a thermocontrolled (37°C) box. The jejunal region 10–20 cm proximal to ileocaecal junction was selected for observation under an inverted intravital microscope (TE2000-E; Nikon, Tokyo, Japan) assisted by a 3 CCD color camera (JK-TU53H; Toshiba, Tokyo, Japan). The dynamics of small intestine microcirculation was recorded using a DVD recorder (DVR-R25; Malata, Xiamen, China).

The intestinal surface was moisturized with 37°C physiological saline throughout the procedure. Fluorescein isothiocyanate-conjugated albumin (FITC-albumin, 50 mg/kg; Sigma, St. Louis, MO) was administrated via the femoral vein after the observation of microcirculation dynamics. Fluorescent images were acquired under an excitation light (455 nm wavelength) at various time points after injection of FITC-albumin. The image analysis software Image-Pro Plus (version 6.0; Media Cybernetics, Rockville, MD) was used off-line to measure fluorescence intensities of FITC-albumin inside the lumen of the selected postcapillary venules (Iv) and in the surrounding interstitial area (II). The ratio of Ii/Iv was calculated as an indication for albumin leakage (2). To determine diameters of venules, Image-Pro Plus was calibrated with a micrometer specifically designed for the camera and monitor. In addition, the velocity of red blood cells (RBCs) in the venule was recorded at a rate of 1,000 frames/s using a high-speed video camera system (Fastcam-ultima APX; Photron, Tokyo, Japan).

The recordings were replayed at a rate of 25 frames/s from the video camera system (Fastcam-ultima APX; Photron, Tokyo, Japan), and the recordings were replated at a rate of 25 frames/s from the high-speed stored images. RBC velocity in the venule was measured with Image-Pro Plus software at baseline (before ischemia) and 0, 20, 40, and 60 min after reperfusion (31).

**Intestinal blood flow.** Intestinal blood flow (IBF) was measured by using a Laser-Doppler Perfusion Imager (PeriScan PIM3 System; PERIMED, Stockholm, Sweden) equipped with a computer at baseline, immediately before reperfusion, 60 min or 3 days after reperfusion. For this purpose, the small intestine was exposed, and a computer-controlled optical scanner directed a low-powered He-Ne laser beam over the exposed intestine. The scanner head was positioned in parallel to the surface of the intestine at a distance of 18 cm. At each measuring site, the beam illuminated the tissue to a depth of 0.5 mm. A color-coded image to denote specific relative perfusion level was displayed on a video monitor, and all images were evaluated with the software LDPIwin 3.1. The magnitude of IBF was represented by different colors, with blue to red denoting low to high. A rectangle region (2 cm x 1 cm, 10–20 cm proximal to the ileocaecal junction) of interest that included the main branch of the microcirculatory network was outlined on each image and used to calculate the area-averaged flux. The data were presented as a mean flux from the measured region of interest in perfusion units. IBF was measured region of interest in perfusion units. The magnitude of IBF was represented by different colors, with blue to red denoting low to high. A rectangle region (2 cm x 1 cm, 10–20 cm proximal to the ileocaecal junction) of interest that included the main branch of the microcirculatory network was outlined on each image and used to calculate the area-averaged flux. The data were presented as a mean flux from the measured region of interest in perfusion units.

**Macro- and microscopic assessment of jejunum.** At 60 min after reperfusion, the jejunum of each animal was examined and grossly graded for edema and discoloration according to a previously published scale (32). For the gross jejunal pathology of the animals in I/R 3 day groups, we established a six-point scoring system: 0, normal mucosal; 1, normal size villi with dilated lymphatic; 2, normal size villi with dilated lymphatic and hyperemia; 3, hypertrophic villi, edematous, hematose, crypt glands proliferating and extending along villi; 4, short regenerated villi and proliferative crypt gland; 5, ulcerated area covered with a layer of simple epithelium under which crypt gland proliferating; 6, ulcerated area only covered with a layer of simple epithelium; 7, ulcer involving submucosa; 8, ulcer involving muscularis.

**Immunohistochemistry of MPO and CD68.** After 60 min or 3 days of reperfusion, jejunum tissues were fixed in 4% paraformaldehyde. Serial paraffin sections (5 μm) were prepared. After deparaffinization, endogenous peroxidase was quenched with 0.3% H2O2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. Non-specific binding was minimized by incubating the section in 5% normal goat serum in PBS for 30 min. The sections were then incubated overnight with MPO antibody (Thermo Scientific) or CD68 antibody (Abcam). Next, specific antibody was detected by incubation with horseradish peroxidase-conjugated secondary antibody and revealed with the 3,3′-diaminobenzidine substrate kit. PBS was used, instead of primary antibody, as negative control. Photographs were taken at ×100 magnification. Five visual fields were selected from each section for analysis of the protein expression using Image-Pro Plus software.

**MPO activity and MDA level in the jejunum tissue.** MPO activity, an indicator of neutrophil infiltration, was measured using an EnzChek Myeloperoxidase Activity Assay Kit, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Briefly, jejunum tissues were collected after 60 min or 3 days of reperfusion, homogenized, and suspended in 50 μl of PBS. Lysates were incubated for 30 min at room temperature in the working solution, according to the manufacturer’s instructions. Fluorescence was measured at 530 nm after excitation at 485 nm using the microplate reader Elx800 (BIO-TEK, Winooski, VT) at room temperature. The background fluorescence measured for each zero-MPO control reaction was subtracted from each fluorescence measurement before plotting.

MDA levels in the jejunum tissue were determined as an indicator of lipid peroxidation, as described previously (3). Jejunum tissue was collected after 60 min or 3 days of reperfusion and homogenized. The MDA level was determined with a rat MDA ELISA Kit (DZE30266; Huayna Biomedicine Technology), according to the manufacturer’s instructions.

**In situ detection of intestinal mucosal epithelial apoptosis.** Sixty minutes or 3 days after reperfusion, the jejunal tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The apoptosis of intestinal mucosal epithelial cells was detected by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling method (TUNEL), as described previously (11). Cell death was assessed using an assay kit (Roche, Basel, Switzerland), and the nuclei were labeled with Hoechst 33342 (Invitrogen, Camarillo, CA). The numbers of the TUNEL-positive cells in the five fields were counted, and the average was calculated and expressed as cell number per field.

**Determination of cytokines.** Sixty minutes or 3 days after reperfusion, blood was collected via the abdominal aorta and anticoagulated with 3.8% sodium citrate. The serum was isolated by centrifugation.
(Allegra 64R Centrifuge; Beckman-Coulter, Fullerton, CA) at 1,300 g for 10 min at 4°C and stored at -80°C. The concentration of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 was determined with ELISA kits (Diaclone Research, Cell Sciences, Canton, MA) according to the manufacturer’s instructions. All determinations were performed in duplicate.

Assessment of energy metabolism. Sixty minutes or 3 days after reperfusion, rats were perfused with saline under anesthesia, and the small intestines were removed. The jejunum tissue was homogenized and centrifuged at 20,000 g, 4°C, for 10 min. The content of ATP, ADP, and AMP in the supernatant was assessed with ELISA kits, according to the manufacturer’s instructions.

Western blot analysis for ATP5D, IkB-α, NF-κb p65, ZO-1, occludin, and claudin-5. An in situ jejunal loop (60 cm length) was isolated in each rat, the luminal contents were thoroughly washed out with a sufficient amount of ice-cold saline, and the jejunum was then divided into several parts of the same length. The tissue was homogenized in 10X volume of ice-cold RIPA buffer (Cell Signaling Technology) and sonicated for 10 s as described previously (7). The lysate was centrifuged, and protein content in the supernatant was determined using the BCA protein assay (Thermo Scientific). For the levels of NF-κB p65 in the nuclear fraction, the nuclear extracts were prepared with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) following the manufacturer’s instructions. Equal amounts

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of protein in each sample were applied to individual wells of polyacrylamide gels, and proteins were separated by electrophoresis. Proteins were transferred electrophoretically to Immobilon-P PVDF membranes (Millipore, Bedford, MA) at 200 mA for 60 min. Membranes were incubated in TBST plus 5% nonfat dry milk for 1 h at room temperature to block nonspecific binding sites. Membranes were incubated with primary antibodies (1:1,000 dilution) in TBST + 5% nonfat dry milk at 4°C overnight, washed three times with TBST for 10 min each, and incubated with peroxidase-conjugated secondary antibodies (1:5,000 dilution) in TBST + 5% nonfat dry milk for 1 h at room temperature. The bands were detected by enhanced chemiluminescence, and the band intensities were quantified by densitometry and expressed as mean area density using the Quantity One image analyzer software (Bio-Rad, Richmond, CA). Mean area density was expressed for all protein blots relative to β-actin or Histone-3 expression. Presented blots are representative of at least three independent experiments performed on at least two independent sample lysate preparations.

Data analysis. Statistics were undertaken with SPSS 15.0 software (SPSS, Chicago, IL). Survival time was compared by the Kaplan-Meier log-rank test. The other data were expressed as means ± SE, and one-way ANOVA (Tukey post hoc test) or two-way ANOVA (Bonferroni post hoc test) was used for multiple comparisons. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days on the tissue section collected from all of the animals in each group. Therefore, all data are means of numbers that themselves are means of triplicate measurements for these parameters. A P value <0.05 was considered statistically significant.

RESULTS

R1 reduces tissue injury, leukocyte infiltration, and apoptosis in the jejunum after intestinal I/R. All sham-operated rats survived the entire 3-day reperfusion period. In contrast, occlusion of the SMA blood flow for 90 min impacted the survival of the animals. At 12 h after reperfusion, 10 of 20 saline-treated I/R-injured rats had died, and, by 3 days, 17 of 20 rats had succumbed. Both pre- and posttreatment with R1 statistically significantly reduced the mortality at 72 h after reperfusion (Fig. 1B). At 60 min and 3 days after reperfusion,
we killed rats in the sham and I/R injury groups and assessed their jejunal pathology. On gross examination, the jejunal of the sham-ligation rats, regardless of treatment with saline or R1, exhibited a healthy pink color and were firm and largely free of contents (Fig. 1C1). On the other hand, the jejunal of rats in I/R 60 min and I/R 3 day groups was both hemorrhagic and edematous, and fragile, and contained a substantial volume of bloody fluid (Fig. 1, C2 and C5). R1 pre- or posttreatment obviously alleviated the gross appearance of rat jejunal 60 min and 3 days after I/R injury (Fig. 1, C3, C4, C6, and C7; see Gross Score, Fig. 1D).

Histologically, the jejunal mucosal epithelium of rats in the saline- or R1-treated sham groups was intact with clearly visible goblet cells (Fig. 2A1), whereas the epithelium of I/R injury rats was badly destroyed at 60 min or 3 days after reperfusion with few villi retaining their normal architecture (Fig. 2, A2 and A5; see Histological Score, Fig. 4A). There was a significant protective effect of the R1 pre- or posttreatment on jejunal mucosal epithelium injury after 60 min and 3 days of I/R (Fig. 2, A3, A4, A6, and A7; see Histological Score, Fig. 4A).

As a marker enzyme of neutrophils, MPO expression in jejunal tissue was assessed by immunohistochemistry and MPO activity assay. Only a few cells exhibited MPO-positive staining in the sham group (Fig. 2B1). Ninety minutes of ischemia followed by 1 h of reperfusion evoked a significant increase in infiltration of neutrophils (Fig. 2B2). On day 3 of reperfusion, the number of MPO-positive cells declined when compared with that at 60 min of reperfusion, but it remained larger than that in the sham group (Fig. 2B5). Nonetheless, the I/R-induced increase in the number of MPO-positive cells in jejunum was diminished significantly by pre- or posttreatment with R1 after 3 days of I/R (Fig. 2, B6 and B7, and Fig. 4B). Moreover, the neutrophil infiltration evaluated by jejunal MPO activity assay confirmed that pre- or posttreatment with R1 significantly attenuated neutrophil infiltration in the intestinal tissue after 3 days of I/R (Fig. 4E).

Infiltration of monocytes is a critical step in the development of I/R-induced injury. Immunohistochemistry staining for CD68 is shown in Fig. 2C to show monocyte infiltration. Only a few cells were CD68 positive in the sham group (Fig. 2C1).

Fig. 5. Effects of R1 on albumin leakage, venular diameter, red blood cell (RBC) velocity, and intestinal blood flow. A: representative images of albumin leakage. B: quantitative evaluation of albumin leakage presented as percent change. C and D: quantitative evaluation of venular diameter and RBC velocity, respectively, in each group. E: representative color images of intestine blood flow acquired by Laser-Doppler Perfusion Imager. F: quantitative evaluation of intestinal blood flow. Data are means ± SE (n = 10). *P < 0.05 vs. sham group; #P < 0.05 vs. I/R group.
In contrast, the number of CD68-positive cells increased prominently in I/R injury groups (Fig. 2, C2 and C5). Treatment with R1 blunted monocyte infiltration significantly on 60 min or 3 days after reperfusion (Fig. 2, C3, C4, C6, and C7, and Fig. 4C).

In addition, MDA levels were measured in the jejunal tissues as an indicator of lipid peroxidation. As shown in Fig. 4F, the content of MDA increased significantly after I/R injury compared with the sham group. Treatment with R1 apparently restrained the increase in MDA level evoked by I/R injury after 3 days of reperfusion.

To investigate whether intestinal injury is associated with apoptotic cell death, we conducted TUNEL staining for jejunum tissues. After 60 min or 3 days of reperfusion, jejunum tissues demonstrated a high number of apoptotic cells (Fig. 3). In contrast, apparently less TUNEL-positive cells were observed in the tissues from rats pre- or posttreated with R1 at 60 min or 3 days after reperfusion (Figs. 3 and 4D). Similarly, no apoptotic cells were observed in the jejunum from sham rats. R1 protects rats against I/R-induced microvascular hyperpermeability and improves microvascular blood flow. I/R caused a significant increase in FITC-albumin leakage from venules compared with shams, indicative of a microvascular hyperpermeability. Pre- or posttreatment with R1 significantly reduced I/R-induced microvascular hyperpermeability at 40 and 60 min after reperfusion (Fig. 5A). R1 alone had no effect on microvascular permeability compared with control. The quantified results of FITC-albumin leakage from the jejunal venules is presented in Fig. 5B, which confirmed the results from the survey. The venular diameter of the I/R group significantly decreased during 60 min of reperfusion. There was no significant difference of the venular diameter in the R1 pre-treatment or posttreatment group at any time point compared with the I/R group (Fig. 5C). RBC velocity in the venule was determined with the high-speed video camera and Bio-image analysis system. As shown in Fig. 5D, the RBC velocity of the I/R group significantly decreased at I/R 0 min and returned to a normal level at I/R 20 min. Pre- or posttreatment with R1 had no significant influence on the change of RBC velocity induced by I/R.

IBF was acquired by a Laser-Doppler Perfusion Imager in each group. As shown in Fig. 5E, the IBF decreased in both I/R 60 min and I/R 3 day groups compared with the sham group. Treatment with R1 obviously attenuated the I/R-induced decrease in IBF on day 3 of reperfusion. The above results were verified by quantitative evaluation (Fig. 5F).

![Fig. 6. Effects of R1 on inhibitory-κB-α (IκB-α) degradation and nuclear factor-κB (NF-κB) p65 expression in the jejunum after I/R. Shown are the representative Western blots for the effects of R1 on IκB-α and p65 nuclear translocation after I/R 60 min (A) and I/R 3 day (D). The blot image in A and D is representative of 4 animals in a group, whereas the densitometry in B, C, E, and F is an averaged result for the 4 animals. Data are means ± SE (n = 4). *P < 0.05 vs. sham group; #P < 0.05 vs. I/R group.](http://ajpgi.physiology.org/)

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00123.2013 • www.ajpgi.org
**R1 reduces the inflammatory response induced by I/R.** IkB-α was distinctly detectable in the jejunum from rats in the sham groups, whereas, in rats from both I/R 60 min and I/R 3 day groups, the levels of IkB-α reduced significantly (Fig. 6, A–F). Moreover, NF-κB p65 subunit nuclear translocation in the jejunum tissue of both I/R 60 min and I/R 3 day groups increased significantly compared with the sham groups. Of notice, R1 administration significantly attenuated the degradation of IkB-α and nuclear translocation of p65 after I/R (Fig. 6, A–F).

A number of inflammatory mediators were found implicated in the I/R-induced jejunum injury. Compared with sham rats, I/R injury resulted in a marked increase in the levels of TNF-α, IL-1β, and IL-6 in serum after both 60 min and 3 days of reperfusion (Fig. 7, A–C), which were significantly attenuated by pre- or posttreatment with R1 (Fig. 7, A–C). Furthermore, the level of the anti-inflammatory cytokine IL-10 increased in the serum of rats from I/R 60 min or I/R 3 day groups. R1 pre- or posttreatment further enhanced the release of IL-10 evoked by I/R on day 3 of reperfusion with statistical significance (Fig. 7D). The levels of jejunal TNF-α, IL-1β, and IL-6 also markedly increased after both 60 min and 3 days of reperfusion. This increase in jejunal TNF-α, IL-1β, and IL-6 levels was significantly attenuated in the R1 pre- and posttreatment group (Fig. 8, A–C). We also detected a significant stimulatory effect of R1 on IL-10 levels in the jejunal tissue on day 3 of reperfusion (Fig. 8D).

**Energy metabolism.** To address the energy metabolism in different conditions, we first explored the ratios of ADP/ATP and AMP/ATP in jejunal tissue in different groups. As shown in Fig. 9, A and B, compared with the sham group, I/R 60 min or I/R 3 day group challenge dramatically increased the ratios of ADP/ATP and AMP/ATP in jejunal tissue, indicating a perturbation in the balance of energy metabolism inclining toward ATP catabolism. Interestingly, pre- or posttreatment with R1 significantly prevented ADP/ATP from elevation by I/R 60 min and I/R 3 day. In addition, R1 pretreatment, but not posttreatment, significantly decreased the ratio of AMP/ATP on day 3 of reperfusion.

We next determined the expression of ATP5D, one of the subunits of ATP synthase, in jejunal tissue in response to I/R injury, and the effects of R1 on regulation of this protein expression. As shown in Fig. 9, C and D, the expression of ATP5D reduced significantly in I/R 60 min or I/R 3 day groups compared with the sham group. Pre- or posttreatment with R1 significantly restrained the decline of ATP5D expression evoked by I/R 3 day. In the I/R 60 min group, only R1 pretreatment increased ATP5D expression after I/R injury with significance.

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**Fig. 7.** Effects of R1 on cytokine release. The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-10 in the serum were elevated significantly after 60 min or 3 days of reperfusion in I/R groups compared with sham rats (A–D, respectively). R1 significantly reduced TNF-α, IL-1β, and IL-6 levels (A–C, respectively) but increased the IL-10 level after 3 days of reperfusion (D). Data are means ± SE (n = 10). *P < 0.05 vs. sham group; #P < 0.05 vs. I/R group.
R1 ameliorates I/R-induced damage in intestinal tight junction. To assess tight junction (TJ) disorder in vivo caused by I/R injury, the expression of TJ proteins ZO-1, occludin, and claudin-5 in jejunum tissues was determined by Western blotting. As shown in Fig. 10, $A$–$D$, after 60 min of I/R challenge, there was a marked decrease in the expression of ZO-1, occludin, and claudin-5, which was significantly inhibited only by R1 pre- but not posttreatment. The change of proteins ZO-1, occludin, and claudin-5 after 3 days of reperfusion is depicted in Fig. 10, $E$–$H$, showing a significant decrease in the expression of the three proteins after I/R 3 day, and both pre- and posttreatment with R1 prevented the I/R-induced decrease in ZO-1, occludin, and claudin-5.

**DISCUSSION**

The major findings in the present study are as follows: 1) R1 significantly attenuated I/R-induced intestinal injury in rats, as revealed by both macro- and microscopic examination, as well as by the improvement of survival rate; 2) R1 ameliorated the microvascular hyperpermeability, reduced breakdown of the TJ, and prevented NF-$\kappa$B activation and the subsequent intestinal inflammation and apoptosis after I/R; and 3) most importantly, the present study showed that I/R reduced the expression of ATP5D, one of the ATP synthase subunits, suggesting that energy depletion may participate in I/R-induced intestinal injury via depressing ATP5D expression, which presumably is one of the targets for R1 action, as showed by the role of R1 in the preservation of ATP5D.

In search of an efficacious treatment for I/R, our study has focused on the microcirculation, based on recent evidence that microcirculatory dysfunction is a major feature of intestinal I/R (25). Previous studies have shown that intestinal barrier failure is related to a reduction in intestinal microcirculation and oxygen extraction, resulting in an increase in intestinal permeability and translocation of enteric bacteria to the systemic circulation and extraintestinal organs/tissues (15). In support of this notion, leakage of macromolecules from the systemic circulation into the intestinal lumen increases after I/R injury (22). In the present study, endothelial barrier integrity was determined by measuring the leakage of FITC-labeled BSA from the systemic circulation to the intestinal interstitial space. We found that R1 pre- or posttreatment protected the microcirculation against the barrier breakdown induced by I/R. Consistently, microcirculatory perfusion was found to be dramatically reduced in I/R rats while improved by R1 treatment. Notably, R1 did not significantly alter the vessel diameter during I/R challenge, nor in sham operation, suggesting that the effects of R1 on microvascular permeability and blood flow are not the result of changes in microvascular resistance but rather the result of preservation of endothelial barrier integrity.

Changes in the expression of TJ transmembrane proteins represent an additional way to modulate paracellular permeability. In particular, occludin, members of the claudin family and ZO-1 are of central importance for barrier regulation, and alterations in the mucosal expression of these proteins have been frequently observed in inflammatory bowel diseases (6,
23). We therefore examined the impact of R1 on the expression of TJ proteins ZO-1, occludin, and claudin-5 in jejunal tissue after I/R by using immunoblotting. Our results showed that these proteins decreased in jejunal tissue after I/R. R1 treatment partially reversed the reduction in TJ proteins evoked by I/R 60 min or I/R 3 day, suggestive of a possible mechanism for the protective role of R1. In the present study, posttreatment with R1 had no significant ameliorative effect on the expression of TJ proteins at I/R 60 min; however, microvascular hyperpermeability was preserved by R1 posttreatment equal to pretreatment (Fig. 5, A and B). Whether R1 could inhibit albumin leakage through affecting the structure and function of VE-cadherin, caveolae, or basement membrane of endothelial cells deserves further investigation.

An interesting finding in the present study is the change in energy metabolism. Previous studies have shown that ATP depletion is implicated in tissue damage and viability after gut I/R (18, 27). Studies showed a relationship existing between tissue ATP and mucosal permeability. The current study revealed an ATP reduction in jejunal tissue during ischemia, consistent with results from others (14, 24, 26). Importantly, this reduction was significantly attenuated with R1 pretreatment. Thus, attenuation of ATP reduction may account for the inhibition effect of R1 on I/R-induced intestinal mucosal hyperpermeability.

There are diverse ways to regulate ATP synthesis, such as altering the oxygen supply, regulating the activity of ATP synthase, or modulating anaerobic metabolism. In a previous study, our laboratory observed a decrease in the expression of one subunit of ATP synthase, ATP5D, in heart after I/R, implying that I/R-caused ATP lack is at least in part attributable to downregulation of ATP synthase activity (8). In the present study, we obtained similar results in intestinal I/R. More importantly, R1 treatment was revealed to protect against the decrease in ATP5D level in intestinal tissue after I/R. The detail of the mechanism for the protective effect of R1 on the expression of ATP5D remains to be clarified. Nonetheless, the result of the present study points to a potentially novel target for developing therapy to cope with the intestinal I/R injury.

Previous studies have implicated infiltrating leukocytes as pivotal mediators of intestinal damage resulting from mesenteric I/R (19). Therefore, we also tested the role of leukocyte infiltration in R1 protecting intestinal endothelium barrier func-
tion. Our immunohistochemical data of MPO and CD68 in the intestinal tissue suggested that intestinal I/R induced substantial leukocyte recruitment and that this reduction was significantly attenuated by R1 treatment. Our results also demonstrated that treatment with R1 significantly abolished the activity of MPO on day 3 of reperfusion. Thus, the protective effects of R1 in the present circumstance are probably also attributable to interference with the recruitment of leukocytes in the intestinal mucosa. Furthermore, I/R induced the expression of P-selectin and intercellular adhesion molecule 1 on endothelial cells, which was abolished by treatment with R1 (data not shown), suggesting involvement of attenuation of leukocyte infiltration in the effect of R1 on I/R-evoked endothelium barrier dysfunction.

The release of free radicals occurs during reperfusion, which has been suggested to contribute significantly to the tissue damage and mucosal dysfunction through activation of NF-κB-dependent expression of cytokines known to be involved in the pathophysiology of intestinal I/R injury (20). In agreement with this concept, our data revealed an activation of intestinal NF-κB, as evidenced by the IκB-α degradation and nuclear translocation of the NF-κB p65 subunit in jejunum tissue after I/R, concurrent of enhanced release of TNF-α, IL-1β, and IL-6. Importantly, all the aforementioned alterations induced by I/R challenge were alleviated by R1.

In summary, the present study demonstrated that pretreatment with R1 is capable of reducing intestinal injury invoked by I/R via several pathways, including preservation of intestine energy metabolism, amelioration of intestinal microcirculation, prevention of NF-κB activation, and inhibition of epithelial cell apoptosis. R1 posttreatment starting from 20 min reperfusion protected intestinal tissue injury from I/R as well, and, compared with the pretreatment, there are subtle differences. Effects of R1 posttreatment on ATP5D expression and TJ proteins in the jejunum only manifested in the later phase (I/R 3 day). Our results provide novel evidence for R1 as a promising strategy in reducing I/R-induced intestinal injury.

Fig. 10. Effects of R1 on the expression of tight junction proteins in the jejunum after I/R. Representative Western blots showing the effects of R1 on zonula occludens (ZO)-1, occludin, and claudin-5 expression after I/R 60 min (A) and I/R 3 day (E), with densitometry analysis of each protein shown below in B, C, and D and F, G, and H, respectively. Data are means ± SE (n = 4). *P < 0.05 vs. sham group; #P < 0.05 vs. I/R group.

AJP-Gastrointest Liver Physiol • doi:10.1152/ajpgi.00123.2013 • www.ajpgi.org
REFERENCES


ACKNOWLEDGMENTS

We thank Chuan-She Wang for technical assistance.

DISCLOSURES

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS


REFERENCES