

Transcription factors Nrf2 and NF- κ B contribute to inflammation and apoptosis induced by intestinal ischemia-reperfusion in mice

QING-TAO MENG¹, RONG CHEN¹, CHENG CHEN², KE SU², WEI LI¹, LING-HUA TANG¹, HUI-MIN LIU¹, RUI XUE³, QIAN SUN¹, YAN LENG¹, JIA-BAO HOU¹, YANG WU¹ and ZHONG-YUAN XIA¹

Departments of ¹Anesthesiology and ²Nephrology, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060; ³Department of Anesthesiology, Renmin Hospital of Shiyan City, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

Received March 29, 2017; Accepted September 28, 2017

DOI: 10.3892/ijmm.2017.3170

Abstract. Intestinal ischemia/reperfusion (IIR) is a common pathological event associated with intestinal injury and apoptosis with high mortality. Nuclear factor (NF)-E2-related factor-2 (Nrf2) is a key transcription factor that interacts with NF- κ B and has a vital anti-inflammatory effect. However, whether Nrf2 has a role in IIR-induced apoptosis and the possible underlining mechanisms, such as modulation of the inflammation regulation pathway, have remained to be fully elucidated. In the present study, IIR was identified to cause significant intestinal injury and apoptosis, with high expression levels of inflammatory cytokines, as well as the apoptotic proteins B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and caspase-3, while simultaneously decreasing the protein levels of Bcl-2. The effect was more pronounced after pretreatment of the animals with all-*trans* retinoic acid or brusatol, potent inhibitors of Nrf2. t-Butylhydroquinone, an Nrf2 activator, significantly attenuated IIR-induced intestinal injury and apoptosis, with inhibition of the overexpression of the inflammatory cytokines, Bax and caspase-3 protein and partial restoration of Bcl-2 protein expression. Taken together, these results indicated that increased Nrf2 expression reduced IIR-induced intestinal apoptosis and that the

protective function of Nrf2 may be based on its anti-inflammatory effects through the inhibition of the NF- κ B pathway.

Introduction

Intestinal ischemia reperfusion (IIR) is a life-threatening pathological event associated with various clinical conditions, including vessel occlusion, hernias, necrotizing enterocolitis and septic shock, and is also an adverse effect of small bowel transplantation (1,2). The intestinal mucosa is particularly sensitive to IIR injury due to the anatomical and physiological characteristics of the villus microcirculation. A temporary interruption of blood flow (ischemia) results in endothelial cell barrier dysfunction and proinflammatory cytokine activation. Paradoxically, the restoration of blood flow (reperfusion) and reoxygenation exacerbates the local (epithelial/endothelial) damage and bacterial translocation, leading to systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) (3).

Accumulating evidence has demonstrated that IIR is associated with inflammatory responses and cell death via necrosis and apoptosis (4). Inflammatory responses activate immunocompetent cells and release cytokines, including interleukin-1 β (IL-1 β), IL-6, IL-10 and tumor necrosis factor- α (TNF- α) (5), which in turn aggravate the inflammatory responses to IIR by inducing microcirculation dysfunction and aggravating cell apoptosis and by further recruitment and accumulation of inflammatory cells. Anti-inflammatory therapies significantly attenuate IIR injury.

Nuclear factor (NF) erythroid 2-related factor 2 (Nrf2), a member the of cap 'n' collar/basic region leucine zipper transcription factor family, participates in the modulation of the pathogenesis of numerous diseases by regulating the expression of several antioxidant genes (6,7). After exposure to oxidative stress, Nrf2 dissociates from Keap1, translocates into the nucleus and binds to antioxidant responsive elements (ARE). Various studies have demonstrated that Nrf2 has a strong anti-inflammatory effect in numerous tissues (8,9). NF- κ B has a pivotal role in immune responses by regulating the expression of multiple inflammatory genes (10). As a classical pro-inflammatory factor, NF- κ B has been implicated in the regulation of Nrf2. A recent review summarized that Nrf2 cross-talks with NF- κ B (11). However, in IIR, little is known regarding the anti-inflammatory role of Nrf2 and the possible counter-balancing effects of Nrf2

Correspondence to: Dr Zhong-Yuan Xia, Department of Anesthesiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan, Hubei 430060, P.R. China
E-mail: xiazhongyuanmz@hotmail.com

Abbreviations: IIR, intestinal ischemia reperfusion; SIRS, systemic inflammatory response syndrome; MODS, multiple organ dysfunction syndrome; IL, interleukin; TNF, tumor necrosis factor; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant responsive elements; NF- κ B, nuclear factor- κ B; TUNEL, terminal deoxynucleotidyl transferase deoxyuridinetriphosphate nick end labeling; ATRA, all-*trans* retinoic acid; SMA, superior mesenteric artery; D-LA, D-lactic acid; I-FABP, intestinal-type fatty acid-binding protein

Key words: nuclear factor erythroid 2-related factor 2, nuclear factor- κ B, intestinal ischemia/reperfusion, all-*trans* retinoic acid, brusatol, t-butylhydroquinone, inflammation, apoptosis

and NF- κ B in the coordination of the final fate of innate immune cells. Therefore, the present study investigated the role of Nrf2 in the modulation of inflammation and apoptosis caused by IIR.

Materials and methods

Reagents. The TNF- α (cat. no. H052), IL-1 β (cat. no. H002), IL-6 (cat. no. H007), IL-10 (cat. no. H009), D-lactic acid (D-LA; cat. no. A019-2) and intestinal-type fatty acid-binding protein (I-FABP; cat. no. H266) enzyme-linked immunosorbent assay (ELISA) kits specific for mouse cytokines were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies to Nrf2 (cat. no. sc-722), NF- κ B (cat. no. sc-71675) and phosphorylated inhibitor of NF- κ B (p-I κ B α ; cat. no. sc-101713) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies directed against β -actin (cat. no. 4970) and lamin B1 (cat. no. 13435) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). IRDye 800CW secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA). Brusatol (cat. no. SML1868) and all-*trans* retinoic acid (ATRA; cat. no. R2625), specific antagonists of Nrf2 (12,13), were purchased from Sigma-Aldrich, Merck KGaA (Darmstadt, Germany). t-Butylhydroquinone (t-BHQ; cat. no. 112976), a specific activator of Nrf2 (12), was also purchased from Sigma-Aldrich, Merck KGaA. All of the chemicals used were of the highest grade commercially available.

Animals. This study was approved by the Animal Care Committee of Wuhan University (Wuhan, China) and protocols were in accordance with the National Institutes of Health (NIH) guidelines for the care and use of experimental animals (NIH publication no. 80-23). This study was performed at the animal center of Renmin Hospital of Wuhan University (Wuhan, China). A total of 64 adult male C57BL/6J mice (Hunan Slac JD Laboratory Animal Co., Ltd., Hunan, China; age, 8-10 weeks; weight, 25 \pm 3 g) were housed in individual cages (4 mice/cage) in a climate-controlled room (23 \pm 1 $^{\circ}$ C; relative humidity 60 \pm 5%) with a 12-h light/dark cycle and free access to food and water. The mice were allowed to acclimatize to the environment for 2 weeks prior to the experiments. All of the animals were fasted for 12 h prior to the experiments but had free access to water.

Intestinal ischemia-reperfusion model. All mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). Surgery was performed after the loss of blink and withdrawal reflexes. The mice were then placed in the supine position and allowed to breathe spontaneously. The IIR model was established by superior mesenteric artery (SMA) occlusion (12). In brief, after laparotomy, the SMA was isolated and temporarily occluded with a microvascular clip. The mice were subjected to ischemia (45 min) followed by 120 min of reperfusion by gently removing the clip. After 120 min of reperfusion, the mice were euthanized and the intestinal tissues and blood were collected and processed for further analysis.

Experimental protocol. After surgical preparation, the animals were randomly allocated into 8 groups as follows (n=8 in each group): i) S group: Sham surgical preparation with isolation of the SMA but without occlusion; ii) IIR group: SMA occlusion for 45 min followed by 120 min of reperfusion; iii) A+S group:

Sham surgery plus ATRA treatment; iv) A+IIR group: IIR procedure plus ATRA treatment; v) B+S group: Sham surgery plus brusatol treatment; vi) B+IIR group: IIR procedure plus brusatol treatment; vii) T+S group: Sham surgery plus t-BHQ treatment; viii) T+IIR group: IIR procedure plus t-BHQ treatment. For ATRA treatment, the animals received ATRA [2 mg/ml dissolved in 1% dimethyl sulfoxide (DMSO); 10 ml/kg intraperitoneally per day] for two weeks prior to the experiment (13). Brusatol was diluted with 1% DMSO to 0.5 mg/ml and 4 ml/kg was injected intraperitoneally once every 2 days for 10 days prior to the experiment (14). t-BHQ was diluted with 1% DMSO and 16.7 mg/kg was administered intraperitoneally 3 times/day (every 8 h) for 3 days prior to the experiment, as described previously (12).

Histopathology of the intestinal tissue. After reperfusion, 1 cm of small intestine without adipose tissue was biopsied from the same site from each animal at the distal end of the ileum and fixed in 4% formaldehyde. Sections (4- μ m) were prepared from the paraffin-embedded tissue and assessed by hematoxylin and eosin (H&E) staining (hematoxylin staining for 10-30 sec and eosin staining 1-3 min at 23 \pm 1 $^{\circ}$ C) and light microscopic examination (original magnification, x200; Olympus BX50; Olympus Optical, Tokyo, Japan). Intestinal mucosal damage was evaluated in at least 2 different sections of each specimen using the improved Chiu *et al* scoring method (15), with blinding to the experimental groups, using a 5-point grading scale according to the changes in the villi and the glands of the intestinal mucosa: 0, normal mucosa; 1, development of subepithelial Gruenhagen's space at the tip of a villus; 2, extension of the space with moderate epithelial lifting; 3, massive epithelial lifting with a few denuded villi; 4, denuded villi with exposed capillaries; and 5, disintegration of the lamina propria, ulceration and hemorrhage.

Analysis of intestinal edema. Tissue edema was detected by the wet/dry weight ratio of the biopsied gut segments. At the end of the experiments, 1 cm of small intestine without adipose tissue was taken from the same site in each animal, weighed and then placed in a drying oven at 80 $^{\circ}$ C for 24 h. After this drying procedure the specimens were reweighed, and the ratio of the weight prior to and after drying was calculated.

ELISA. D-LA, I-FABP, IL-1 β , IL-6, IL-10 and TNF- α levels in the intestinal mucosa and in the serum were measured following the standard procedures of the ELISA kits.

Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick end labeling (TUNEL) assay. Apoptosis in the intestinal sections was examined after TUNEL staining with the Click-iT TUNEL Alexa Fluor 488 Imaging assay (cat. no. C10245; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The 4- μ m paraffin-embedded sections were deparaffinized in xylene and double diluted water. The sections were then treated with proteinase K for 20 min at room temperature and subsequently incubated with a mixture of fluorescent labeling solution and TdT enzyme for 1 h in a humidified atmosphere. After washing with phosphate-buffered saline (PBS) and drying, the sections were incubated with DNase I for 10 min in a humidified atmosphere at room temperature. The fluorescein isothiocyanate-labeled TUNEL-positive cells were imaged using fluorescence micros-

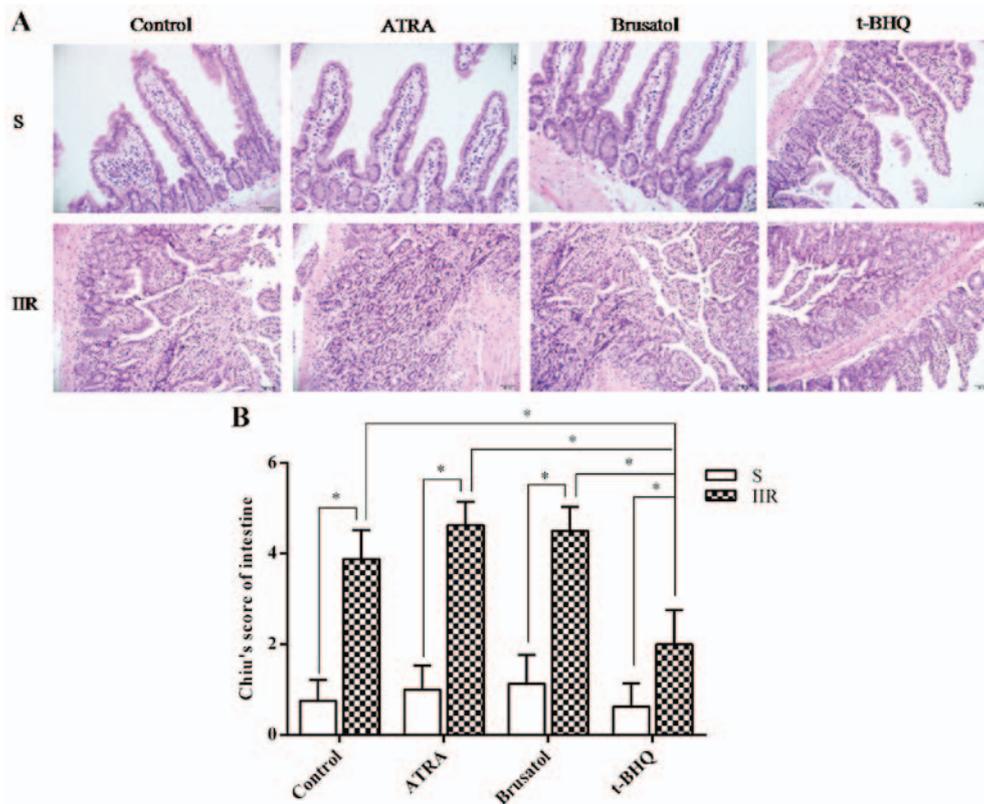


Figure 1. Protective effect of Nrf2 activation on IIR-induced intestinal injury. (A) Histopathological alterations in the intestinal mucosa under a light microscope (magnification, x200; hematoxylin and eosin staining). Superior mesenteric artery occlusion for 45 min followed by 120 min of reperfusion caused epithelial lifting with a small amount of denuded villi with exposed capillaries, disintegration of the lamina propria, ulceration and hemorrhage. These damage-associated features were markedly deteriorated in animals pretreated with ATRA or brusatol. The damage was markedly ameliorated in animals pretreated with the Nrf2 activator t-BHQ. (B) Summary of Chiu's score in different groups. Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. S, sham surgery; IIR, intestinal ischemia/reperfusion; ATRA, all-trans retinoic acid; t-BHQ, t-butylhydroquinone; Nrf2, nuclear factor erythroid 2-related factor 2.

copy. DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) was used to stain the nuclei. The average number of apoptotic cells was calculated from five random fields with Image-Pro Plus software (version 6.0; Media Cybernetics, Rockville, MD, USA).

Immunohistochemical analysis. The 5- μ m paraffin-embedded sections were stained using the streptavidin-biotin complex immunohistochemistry technique for Nrf2, NF- κ B and p-I κ B α detection. A positive signal was visualized by a 3,3'-diaminobenzidine color reaction. The nuclei were stained with hematoxylin. Brown staining in the cytoplasm and the nucleus was considered an indicator of protein expression. Two different sections of each specimen were examined (original magnification, x400; Olympus BX50; Olympus Optical). The results were semi-quantitatively evaluated with Image-Pro[®] Plus version 6.0 according to the optical density values of protein expression. For this purpose, five fields per slide were randomly selected by the viewer for evaluation.

Western blot analysis. Endochylema and cellular nuclear proteins were extracted from frozen intestinal tissues with a nuclear extract kit (cat. no. P0028; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Protein concentration was determined using a BCA assay. Equal amounts (100 μ g per lane) of protein were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 3 h. After electrophoresis, the proteins

were transferred onto polyvinylidene difluoride membranes (cat. no. 88520; Thermo Fisher Scientific, Inc.) at 200 mA for 2 h. The membranes were incubated overnight at 4°C with rabbit anti-mouse polyclonal antibodies to Nrf2 (1:200 dilution), NF- κ B (1:1,000 dilution), p-I κ B- α (1:1,000 dilution), β -actin (1:2,000 dilution) and lamin B1 (1:200 dilution). After washing for three times with Tris-buffered saline containing Tween-20, the membranes were incubated with the corresponding goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 h at room temperature. The intensity of the bands was detected using an Odyssey two-color infrared laser imaging system and densitometry was performed using Odyssey 1.0 software (both LI-COR Biosciences).

Statistical analysis. Values are expressed as the mean \pm standard deviation. GraphPad Prism 5.0 statistical software (GraphPad Software Inc., La Jolla, CA, USA) was used to manage the data and calculate the results. A statistical evaluation of the data was performed by one-way or a two-way analysis of variance, followed by Tukey's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Nrf2 activation reduces IIR-induced intestinal damage. To investigate the underlying mechanisms of the effect of Nrf2 on IIR-induced injury, animals were pretreated with Nrf2 antago-

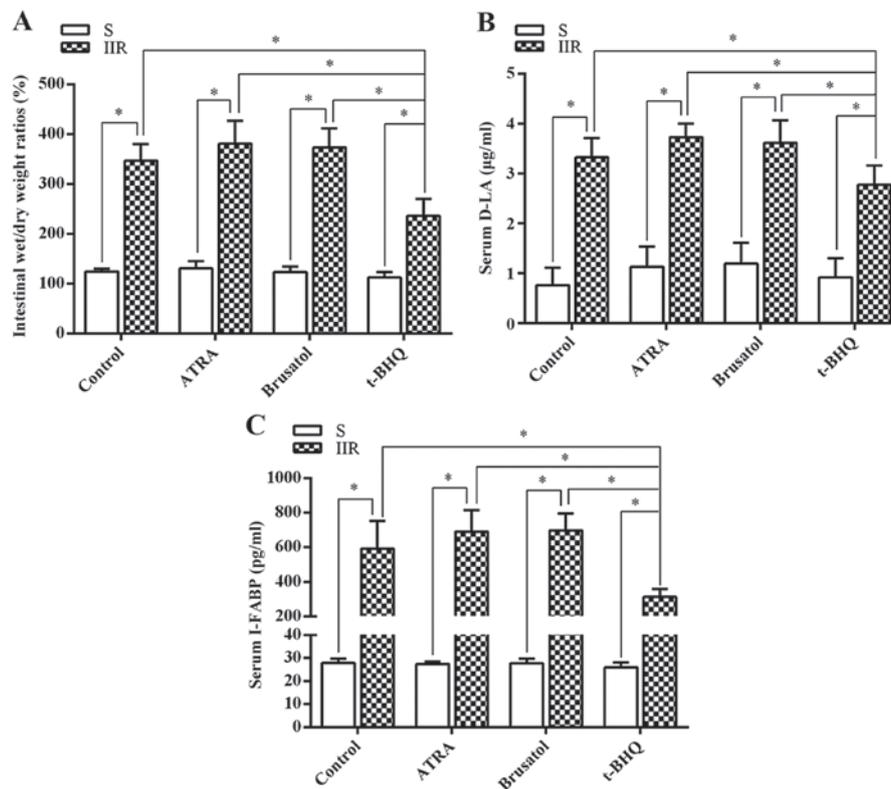


Figure 2. Effect of Nrf2 levels on intestinal permeability and intestinal barrier function. (A) The intestinal water content was determined to reflect gut permeability. The concentrations of (B) D-LA and (C) I-FABP in the serum were detected to determine intestinal epithelial function. Values are expressed as the mean \pm standard deviation (n=8). * P <0.05. D-LA, D-lactic acid; I-FABP, intestinal-type fatty acid-binding protein; S, sham surgery; IIR, intestinal ischemia/reperfusion; ATRA, all-*trans* retinoic acid; t-BHQ, t-butylhydroquinone (Nrf2 activator); Nrf2, nuclear factor erythroid 2-related factor 2.

nists and an Nrf2 activator prior to reperfusion-induced intestinal injury. H&E staining indicated that IIR induced villous edema, inflammatory cell infiltration and capillary congestion, and markedly increased the gap between epithelial cells (Fig. 1A). These effects were dramatically aggravated after administration of an Nrf2 antagonist (ATRA or Brusatol) (Fig. 1). In addition, IIR-induced intestinal injury was significantly attenuated after treatment with the Nrf2 activator t-BHQ (Fig. 1). Chiu's scoring produced similar results to those of H&E staining.

Next, intestinal permeability damage was assessed by determining the intestinal wet/dry weight ratios (Fig. 2A). The intestinal wet/dry weight ratios were significantly higher in the IIR group than in the S group ($P=0.0085$). Compared with the IIR group, the intestinal wet/dry weight ratio was significantly decreased in the group pretreated with the Nrf2 activator t-BHQ ($P=0.021$). Furthermore, the serum levels of D-LA (Fig. 2B) and I-FABP (Fig. 2C) were assessed as biomarkers for the integrity of the intestinal epithelium. Serum levels of D-LA and I-FABP were markedly increased in the IIR group compared with those in the S group ($P=0.0083$ and 0.00009 , respectively) and were significantly decreased in the group pretreated with t-BHQ treatment ($P=0.015$ or 0.0003 , respectively, compared with the IIR group). However, the Nrf2 antagonists had no significant effect on serum D-LA or I-FABP.

Nrf2 regulates inflammatory cytokines in the plasma and intestinal tissues after IIR. Next, the changes in inflammatory cytokine expression in the intestine and serum were investigated. The levels of tissue IL-1 β , IL-6 and TNF- α

in the IIR group were significantly higher than those in the S group ($P=0.021$, 0.0076 and 0.033 , respectively) (Fig. 3A-C). However, the tissue levels of IL-10 were markedly reduced in the IIR group compared with those in the S group ($P=0.044$) (Fig. 3D). In addition, pretreatment with ATRA or brusatol significantly aggravated the IIR-induced increases in IL-1 β , IL-6 and TNF- α levels, while further reducing IL-10 levels. The increases in the levels of IL-1 β , IL-6 and TNF- α , and the decrease of IL-10 induced by IIR were inhibited by pretreatment with t-BHQ ($P=0.032$, 0.017 , 0.026 and 0.023 , respectively) (Fig. 3). The changes in the serum levels of inflammatory cytokines were consistent with those in the intestinal tissue (Fig. 4).

Nrf2 activation attenuates IIR-induced apoptosis. To further investigate the effects of Nrf2 on apoptosis after IIR, the intestine was examined by TUNEL staining (Fig. 5). The amount of TUNEL-positive intestinal cells increased significantly after IIR ($P=0.017$) (Fig. 5A and B). Pretreatment with ATRA and brusatol aggravated IIR-induced apoptosis in epithelial cells. Conversely, the IIR-induced apoptosis of intestinal epithelial cells was inhibited by pretreatment with Nrf2 activator t-BHQ ($P=0.008$).

The expression of apoptosis-associated proteins in the intestine was then examined. It was observed that Bax and cleaved caspase-3 were significantly increased after IIR treatment ($P=0.032$ and 0.046 , respectively) (Fig. 5C, D and F), which was markedly exacerbated by pretreatment with Nrf2 antagonists ATRA ($P=0.037$ and 0.041 , respectively) and brusatol ($P=0.040$

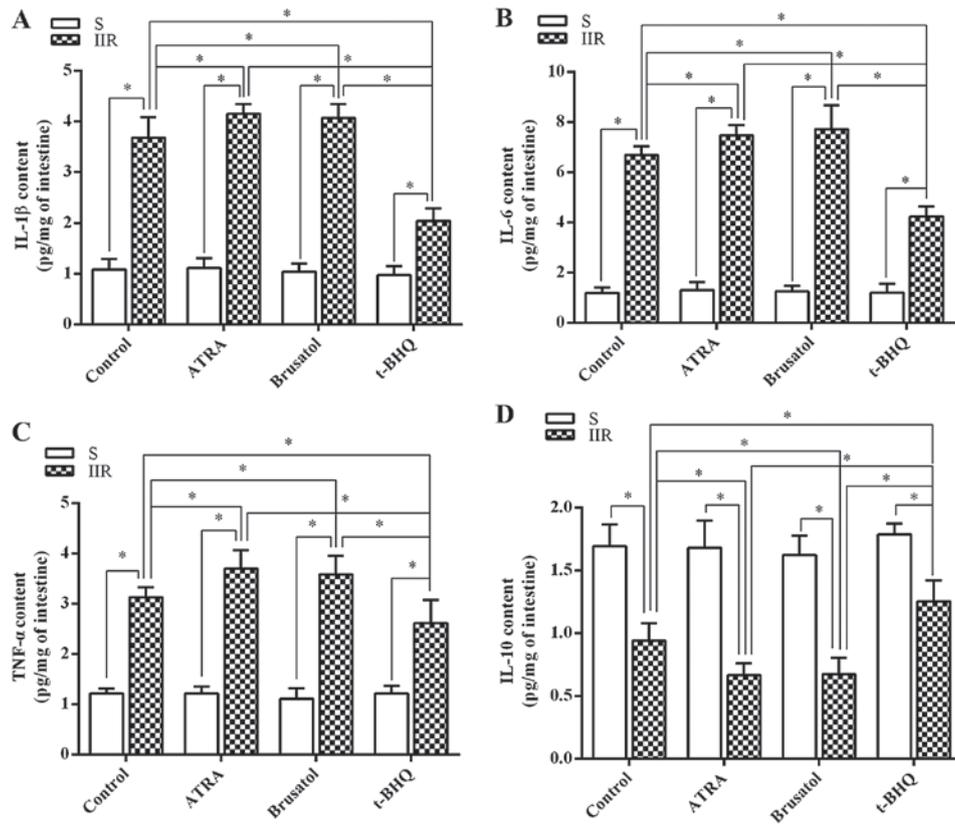


Figure 3. Expression of proinflammatory cytokines (A) IL-1 β , (B) IL-6, (C) TNF- α and (D) IL-10 in intestinal tissue. Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. IL, interleukin; TNF, tumor necrosis factor; S, sham surgery; IIR, intestinal ischemia/reperfusion; ATRA, all-*trans* retinoic acid; t-BHQ, t-butylhydroquinone.

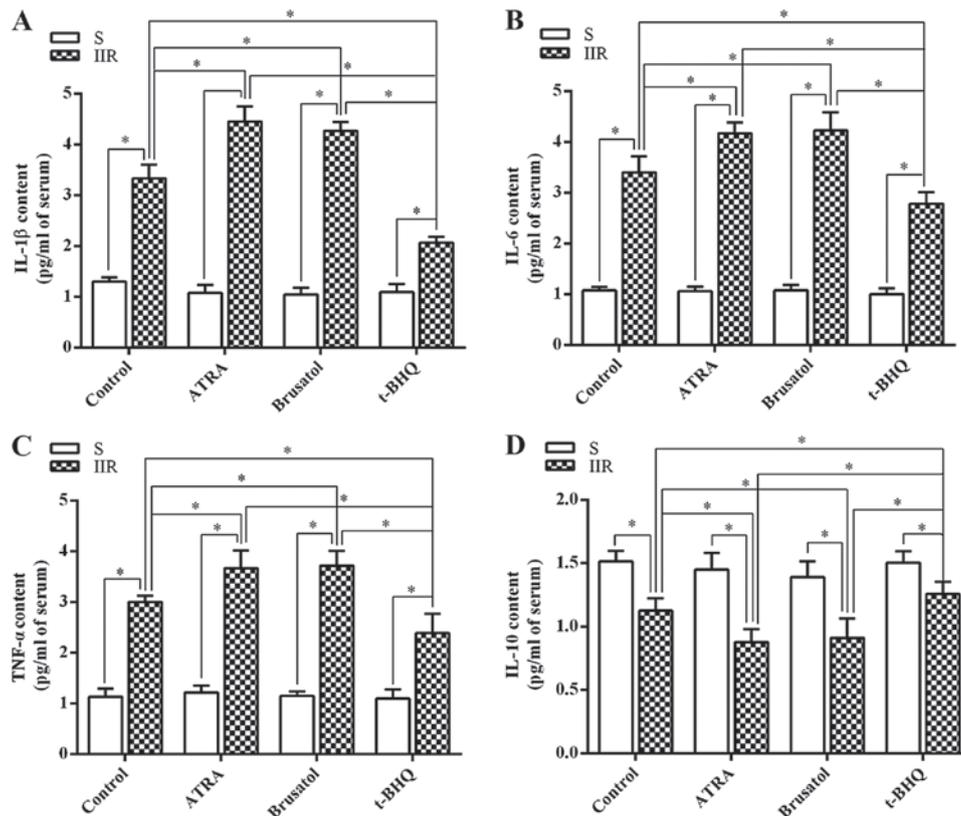


Figure 4. Plasma levels of inflammatory cytokines (A) IL-1 β , (B) IL-6, (C) TNF- α and (D) IL-10 in intestinal tissue. Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. IL, interleukin; TNF, tumor necrosis factor; S, sham surgery; IIR, intestinal ischemia/reperfusion; ATRA, all-*trans* retinoic acid; t-BHQ, t-butylhydroquinone.

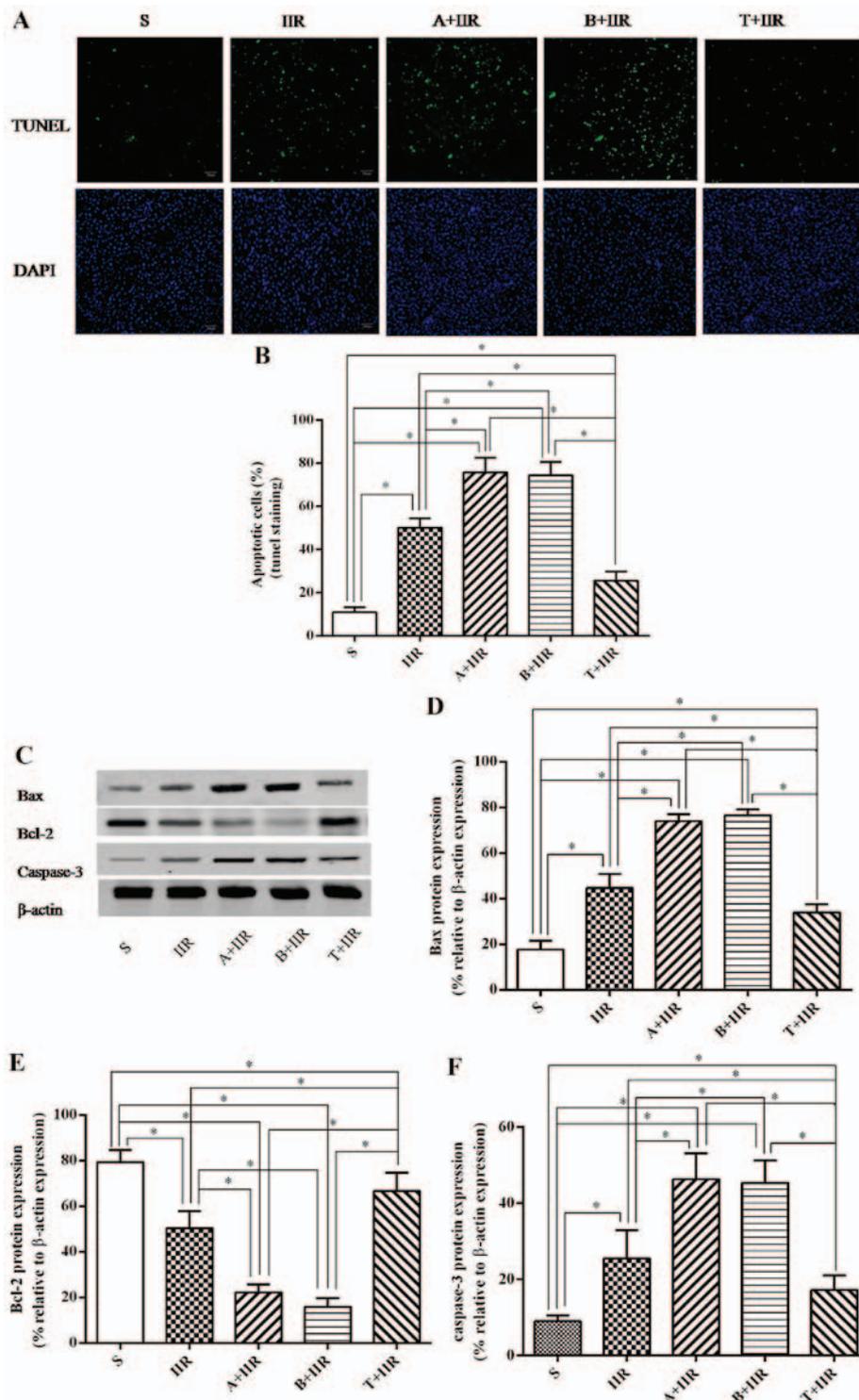


Figure 5. Effect of Nrf2 regulation on IIR-induced apoptosis in intestinal epithelial tissue. (A and B) Apoptosis in the intestine of animals from each group was detected by a TUNEL assay. (A) Representative fluorescence microscopy images (scale bar, 20 μ m) and (B) quantified percentages of TUNEL-stained cells in each group. (C-F) The expression of apoptotic proteins was detected by western blot analysis. (C) Representative western blot image and quantified expression levels of (D) Bax, (E) Bcl-2 and (F) cleaved caspase-3. β -actin was used as a loading control. Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. S, sham surgery; IIR, intestinal ischemia/reperfusion; A, all-*trans* retinoic acid; B, brusatol; T, t-butylhydroquinone (Nrf2 activator); Nrf2, nuclear factor erythroid 2-related factor 2; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; TUNEL, terminal deoxynucleotidyl transferase deoxyuridinetriphosphate nick end labeling.

and 0.035, respectively) (Fig. 5C, D and F). The expression of Bcl-2 in the IIR group was markedly decreased compared with that in the S group (P=0.028), and further significant decreases were observed in the A+IIR and B+IIR groups pretreated with the Nrf2 antagonists (P=0.029 and 0.033, respectively, vs. IIR

group) (Fig. 5C and E). In addition, pretreatment with t-BHQ significantly inhibited the IIR-induced increases in the expression of Bax (P=0.041) (Fig. 5C and D) and the levels of cleaved caspase-3 (P=0.037) (Fig. 5C and F), as well as the decrease in the expression of Bcl-2 (P=0.022) (Fig. 5C and E).

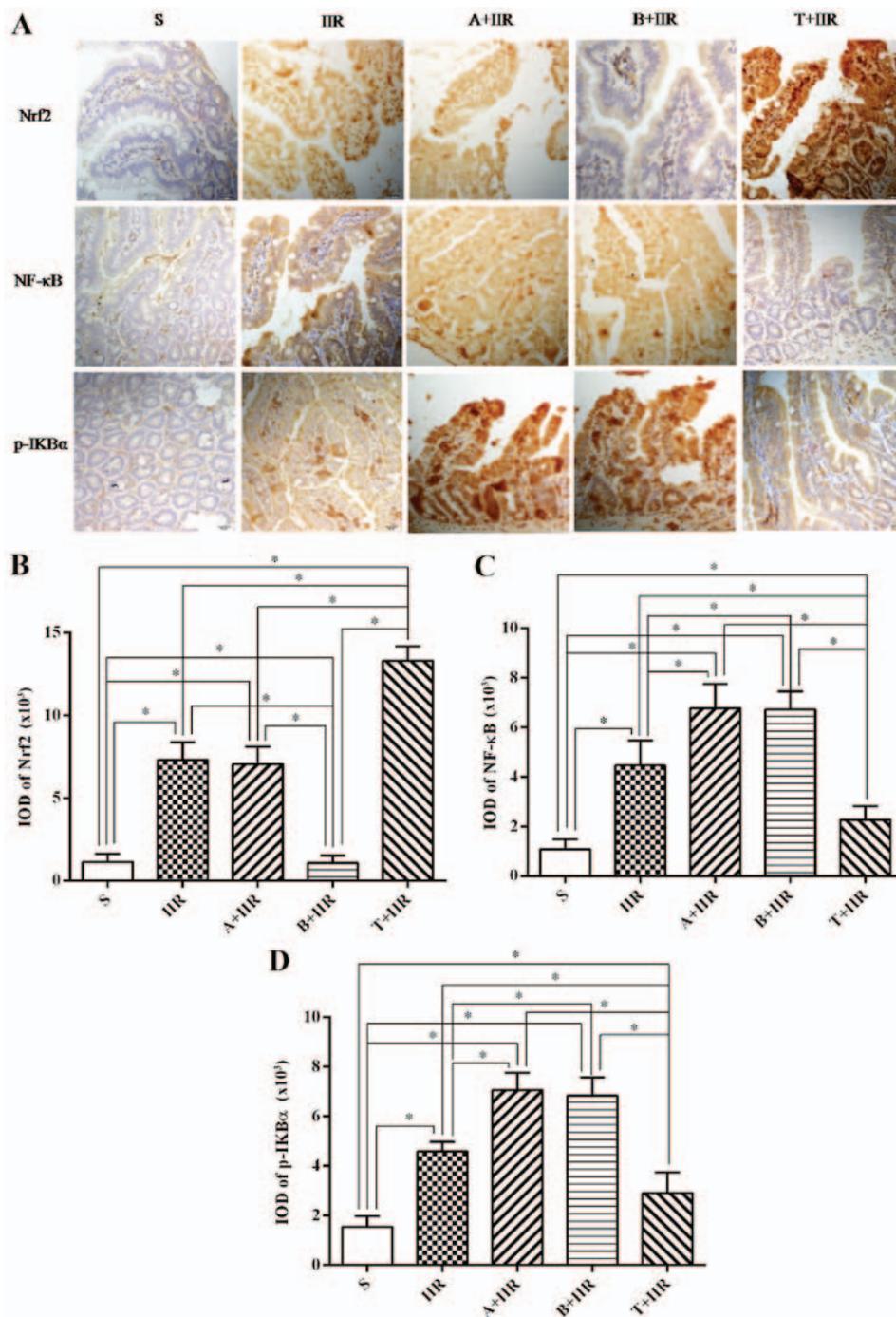


Figure 6. Nrf2 activation is involved in the protection against IIR-induced apoptosis by inhibiting the NF- κ B pathway. (A) Representative immunohistochemical images with staining performed using the streptavidin-biotin complex immunohistochemistry technique. Positive staining was indicated by brownish yellow or dark brown cytoplasm or nuclei. A large proportion of the cytoplasm and nuclei of intestinal tissue cells were stained for Nrf2 in the IIR group as well as in the T+IIR group (scale bar, 20 μ m). Quantified expression of (B) Nrf2, (C) NF- κ B and (D) p-IKB α . Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. S, sham surgery; IIR, intestinal ischemia/reperfusion; A, all-*trans* retinoic acid; B, brusatol; T, t-butylhydroquinone (Nrf2 activator); Nrf2, nuclear factor erythroid 2-related factor 2; NF- κ B, nuclear factor- κ B; p-IKB α , phosphorylated inhibitor of NF- κ B.

Nrf2 activation inhibits the NF- κ B pathway. The Nrf2/ARE pathway is deeply involved in the protection of organs from IIR injury. The present study evaluated the expression of Nrf2 and inflammation-associated proteins in the intestine by immunohistochemical staining and western blot analysis. Positive immunohistochemical staining was indicated by yellow-brown-stained granules. In the IIR group, protein expression was mainly identified in the cytoplasm and the nuclei of intestinal tissue cells. In the group pretreated with t-BHQ, cellular staining for

Nrf2 became lighter after IIR. In the IIR group, a large proportion of the cytoplasm and nuclei of the intestinal tissue cells appeared brownish-yellow or dark brown, and a large proportion of cytoplasm and nuclei of the intestinal tissue cells remained brownish-yellow or dark brown, indicating Nrf2 expression in the group pretreated with t-BHQ prior to IIR (Fig. 6A). After IIR induction, the cytoplasm and the nuclei of the tissues exhibited Nrf2 and NF- κ B expression in the epithelial lamina propria according to immunohistochemical staining (Fig. 6A). Quantification of the

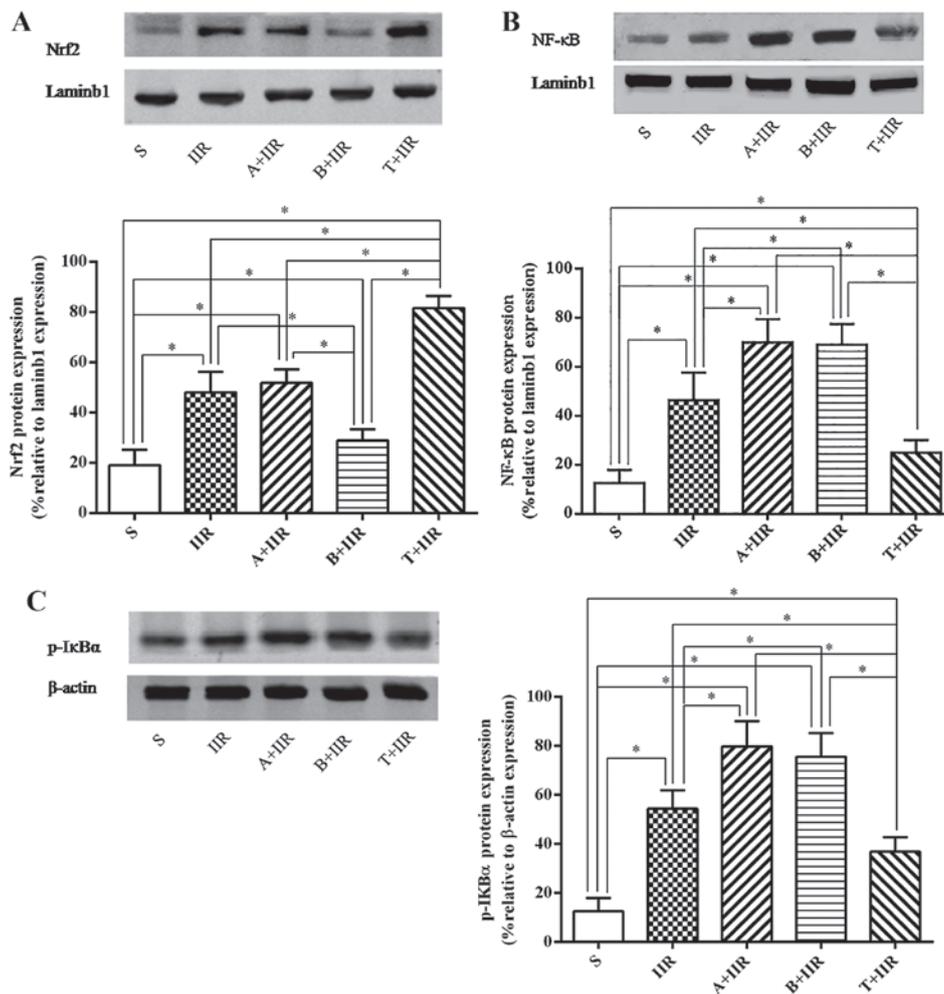


Figure 7. Nrf2 activation is involved in the protection against IIR-induced apoptosis by inhibiting the NF- κ B pathway. Western blot analysis was used to assess the expression of (A) Nrf2 and (B) NF- κ B in the nuclei with lamin B1 as a loading control, as well as of (C) p-I κ B α in the cytoplasm with β -actin used as a loading control. Representative western blot images and quantified expression levels are presented. Values are expressed as the mean \pm standard deviation (n=8). *P<0.05. S, sham surgery; IIR, intestinal ischemia/reperfusion; A, all-*trans* retinoic acid; B, brusatol; T, t-butylhydroquinone (Nrf2 activator); Nrf2, nuclear factor erythroid 2-related factor 2; NF- κ B, nuclear factor- κ B; p-I κ B α , phosphorylated inhibitor of NF- κ B.

staining revealed that the expression of Nrf2 (P=0.034) (Fig. 6B) and NF- κ B (P=0.029) (Fig. 6C) was significantly increased after IIR. Pretreatment with ATRA did not affect the expression of Nrf2 in the cytoplasm and the nuclei of intestinal cells compared with those in the IIR group, while it remained significantly higher than that in the S group (P=0.009) (Fig. 6A and B). However, pretreatment with brusatol abolished the IIR-induced expression with no significant difference compared with that in the S group (P=0.098) (Fig. 6A and B). The levels of NF- κ B and p-I κ B α in the IIR group were much higher than those in the group pretreated with the Nrf2 antagonist ATRA prior to IIR (P=0.025 and 0.022, respectively) (Fig. 6A, C and D). In the group pretreated with t-BHQ, the accumulation of Nrf2 in the nuclei was significantly increased compared with that in the IIR group (P=0.0043) (Fig. 6A and B), while the accumulation of NF- κ B in the nuclei and the levels of p-I κ B α in the cytoplasm decreased significantly compared with those in the IIR group (P=0.014 and 0.028, respectively) (Fig. 6A, C and D).

Furthermore, western blot analysis of the protein expression of nuclear Nrf2 and NF- κ B as well as the cytoplasmic levels of p-I κ B α provided similar results to those obtained by immunohistochemistry (Fig. 7).

Discussion

The present study confirmed that IIR causes severe intestinal tissue damage and cell apoptosis, in accordance with the increased intestinal permeability and reduced integrity of the intestinal epithelia. Furthermore, via Nrf2 antagonist or Nrf2 activator treatment, it was demonstrated that Nrf2 attenuated IIR-induced apoptosis by regulating the systemic inflammatory response. The results of the present study suggested that an activator of Nrf2 had a beneficial effect against IIR-induced intestinal apoptosis through exerting anti-inflammatory effects via inhibiting the NF- κ B pathway.

IIR injury is associated with a wide range of pathological conditions in experimental models and clinical conditions. The original tissue ischemia causes endothelial barrier dysfunction and increased endothelial permeability (16). Subsequent reperfusion has various consequences, including the activation of apoptosis (17) and the stimulation of the innate and adaptive immune responses (18). A self-perpetuating signaling cascade escalates to a vicious cycle of continuously increasing intestinal permeability and bacterial translocation, a stronger inflammatory response, apoptosis and eventual multiple organ

failure. The present study verified that occlusion of the SMA for 45 min followed by reperfusion for 2 h caused significant intestinal injury in mice. This injury resulted in mucosal edema, decreased epithelial cells, villi destruction, inflammatory cell infiltration and a sharp increase in Chiu's score. All of these observations are consistent with those of previous studies (19).

The normal structure and function of the intestinal mucosa is important in the prevention of the translocation of bacteria and other noxious substances (20). Furthermore, the intestinal mucosa is hypersensitive to IIR, which results in intestinal barrier dysfunction. D-LA (21) is a product that is released by numerous microfloras, while I-FABP (22) is only released by damaged intestinal epithelial cells. These factors are known as useful biomarkers for intestinal barrier dysfunction. The molecules that are released upon the disruption of epithelial integrity may be measured and quantified. Upon IIR, significant increases in the serum concentrations of D-LA and I-FABP were detected in the present study, which strongly indicated mucosal integrity impairment and intestinal barrier dysfunction.

Inflammation is an essential part of the innate immune response that prevents tissue damage and helps tissue healing in various ways. However, uncontrollable inflammation gives rise to advanced tissue damage. An IIR challenge leads to the translocation of bacteria and toxins (23), amplifying systemic inflammation and apoptosis and resulting in SIRS and MODS. Ischemia induces the rapid recruitment of inflammatory cells, and the cytokines produced by these inflammatory cells further facilitate an inflammatory status. Intensified inflammation also promotes apoptosis (24). Targeting the inflammatory response is a crucial therapeutic strategy for the treatment of IIR injury. The present results indicated that the proinflammatory cytokines IL-1 β , IL-6 and TNF- α sharply increased in the intestinal tissue and serum after IIR injury, while the anti-inflammatory cytokine IL-10 decreased. Furthermore, IIR markedly increased the number of apoptotic cells, enhanced the expression of Bax and the levels of cleaved caspase-3, and led to downregulation of the expression of Bcl-2 in tissue.

Several signaling pathways are involved in IIR-induced inflammation and apoptosis, reflecting the complexity of the underlying mechanisms. The present study focused on two important transcription factors, Nrf2 and NF- κ B. As a prototypical component of a proinflammatory signaling pathway, NF- κ B induces the transcription of numerous proinflammatory cytokines (TNF- α , IL-1 β and IL-6) and regulates anti-inflammatory cytokines (IL-10) (25). Furthermore, the auto-regulatory loop between NF- κ B and proinflammatory cytokines extensively aggravates the damaging effect of inflammation. Activation of NF- κ B leads to the production of several proinflammatory cytokines (26), including TNF- α and IL-1 β , which in turn further induce the activation of NF- κ B. The present study indicated that IIR induced NF- κ B activation and significantly increased the tissue and serum levels of TNF- α , IL-6 and IL-1 β .

The Nrf2/ARE signaling pathway has a crucial role in antioxidant and anti-inflammatory cellular responses (27). Under physiological conditions, Nrf2 is retained in the cytoplasm by Keap1 and is degraded through ubiquitination. Upon the infliction of oxidative stress, Nrf2 dissociates

from Keap1, translocates to the nucleus and binds to ARE. Therefore, Nrf2 initiates the transcription of genes that code for phase II detoxifying enzymes, such as heme oxygenase-1 and NAD(P)H:quinone oxidoreductase-1 (28). A recent study indicated that the Nrf2/ARE pathway may also serve as an anti-inflammatory modulator (29). Nrf2 activation has also been reported to reduce organ damage and prevent inflammation from hemolysis induction in sickle cell disease (30). Studies have confirmed that CO released by tricarbonyldichlororuthenium (II) dimer mitigates lipopolysaccharide-induced inflammation through the activation of Nrf2 (31). The activation of Nrf2 significantly reduces immune cell infiltration and decreases the expression of the proinflammatory cytokines TNF- α , IL-1 β and IL-6 by inhibiting the NF- κ B signaling pathway. In addition, Nrf2 depletion has been reported to enhance the inflammatory process through the activation of NF- κ B in the brain after traumatic brain injury (32). Distinct patterns of crosstalk between NF- κ B and Nrf2 have been explored in different cell types (11). The present study indicated that the expression of intestinal Nrf2 was markedly increased after IIR treatment. IIR also induced tissue damage, and increased the expression of NF- κ B and the levels of p-I κ B α . These observations suggested that IIR induces overexpression of Nrf2 through the innate immune response to counteract tissue injury, but the protective effects of these factors are not sufficient to fully protect the tissue. In the present study, two different potent inhibitors, ATRA and brusatol, were applied, in order to provide further evidence for the importance of Nrf2 in the protection from IIR-induced injury. Administration of Nrf2 antagonists markedly exacerbated intestinal injury, augmented the inflammatory response and apoptosis, and increased the levels of NF- κ B and p-I κ B α . ATRA inhibits the function of Nrf2 by stimulating the formation of Nrf2:retinoic acid receptor α -containing complexes that do not bind to ARE, while brusatol enhances Nrf2 degradation. Furthermore, treatment with t-BHQ, an activator of Nrf2, was observed to attenuate IIR-induced tissue injury, mitigate intestinal barrier dysfunction, reduce proinflammatory cytokines and apoptotic factors, and inhibit I κ B kinase/I κ B phosphorylation and NF- κ B nuclear translocation. NF- κ B was also reported to inhibit Nrf2 at the transcriptional level (11). The present study indicated that activation of Nrf2 dramatically mitigated pathological changes within the intestine and reduced the inflammatory response and apoptosis by inhibiting the NF- κ B pathway, while Nrf2 antagonists have the opposite effect. All of these results highlighted the importance of Nrf2 in the regulation of the inflammatory response and apoptosis during IIR injury and demonstrated the possibility of complex crosstalk between NF- κ B and Nrf2.

In conclusion, the present study indicated that Nrf2 has a critical role in the regulation of inflammation and apoptosis induced by IIR. The beneficial effects of Nrf2 confer anti-inflammatory properties against IIR-induced apoptosis, potentially through the inhibition of the NF- κ B pathway.

Acknowledgements

This study was supported by the Chinese Natural Science Foundation (grant nos. 81671948, 81671891, 81401574 and 81400698).

References

- Huang CY, Hsiao JK, Lu YZ, Lee TC and Yu LC: Anti-apoptotic PI3K/Akt signaling by sodium/glucose transporter 1 reduces epithelial barrier damage and bacterial translocation in intestinal ischemia. *Lab Invest* 91: 294-309, 2011.
- Gerlach UA, Atanasov G, Wallenta L, Polenz D, Reutzel-Selke A, Kloepfel M, Jurisch A, Marksteiner M, Loddenkemper C, Neuhaus P, *et al*: Short-term TNF-alpha inhibition reduces short-term and long-term inflammatory changes post-ischemia/reperfusion in rat intestinal transplantation. *Transplantation* 97: 732-739, 2014.
- Kim M, Park SW, Kim M, D' Agati VD and Lee HT: Isoflurane post-conditioning protects against intestinal ischemia-reperfusion injury and multiorgan dysfunction via transforming growth factor- β 1 generation. *Ann Surg* 255: 492-503, 2012.
- Crafts TD, Hunsberger EB, Jensen AR, Rescorla FJ, Yoder MC and Markel TA: Direct peritoneal resuscitation improves survival and decreases inflammation after intestinal ischemia and reperfusion injury. *J Surg Res* 199: 428-434, 2015.
- Tian S, Guo R, Wei S, Kong Y, Wei X, Wang W, Shi X and Jiang H: Curcumin protects against the intestinal ischemia-reperfusion injury: Involvement of the tight junction protein ZO-1 and TNF- α related mechanism. *Korean J Physiol Pharmacol* 20: 147-152, 2016.
- Komaravelli N, Tian B, Ivanciuc T, Mautemps N, Brasier AR, Garofalo RP and Casola A: Respiratory syncytial virus infection down-regulates antioxidant enzyme expression by triggering deacetylation-proteasomal degradation of Nrf2. *Free Radic Biol Med* 88: 391-403, 2015.
- Gallorini M, Petzel C, Bolay C, Hiller KA, Cataldi A, Buchalla W, Krifka S and Schweikl H: Activation of the Nrf2-regulated antioxidant cell response inhibits HEMA-induced oxidative stress and supports cell viability. *Biomaterials* 56: 114-128, 2015.
- Boyanapalli SS, Paredes-Gonzalez X, Fuentes F, Zhang C, Guo Y, Pung D, Saw CL and Kong AN: Nrf2 knockout attenuates the anti-inflammatory effects of phenethyl isothiocyanate and curcumin. *Chem Res Toxicol* 27: 2036-2043, 2014.
- Park JH, Choi JW, Ju EJ, Pae AN and Park KD: Antioxidant and anti-inflammatory activities of a natural compound, shizukahenriol, through Nrf2 activation. *Molecules* 20: 15989-16003, 2015.
- Zhao W, Sun Z, Wang S, Li Z and Zheng L: Wnt1 participates in inflammation induced by lipopolysaccharide through upregulating scavenger receptor A and NF- κ B. *Inflammation* 38: 1700-1706, 2015.
- Wardyn JD, Ponsford AH and Sanderson CM: Dissecting molecular cross-talk between Nrf2 and NF- κ B response pathways. *Biochem Soc Trans* 43: 621-626, 2015.
- Meng QT, Cao C, Wu Y, Liu HM, Li W, Sun Q, Chen R, Xiao YG, Tang LH, Jiang Y, *et al*: Ischemic post-conditioning attenuates acute lung injury induced by intestinal ischemia-reperfusion in mice: Role of Nrf2. *Lab Invest* 96: 1087-1104, 2016.
- Wang XJ, Hayes JD, Henderson CJ and Wolf CR: Identification of retinoic acid as an inhibitor of transcription factor Nrf2 through activation of retinoic acid receptor alpha. *Proc Natl Acad Sci USA* 104: 19589-19594, 2007.
- Ren D, Villeneuve NF, Jiang T, Wu T, Lau A, Toppin HA and Zhang DD: Brusatol enhances the efficacy of chemotherapy by inhibiting the Nrf2-mediated defense mechanism. *Proc Natl Acad Sci USA* 108: 1433-1438, 2011.
- Chiu CJ, McArdle AH, Brown R, Scott HJ and Gurd FN: Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg* 101: 478-483, 1970.
- Sun Z, Wang X, Deng X, Lasson A, Wallén R, Hallberg E and Andersson R: The influence of intestinal ischemia and reperfusion on bidirectional intestinal barrier permeability, cellular membrane integrity, proteinase inhibitors, and cell death in rats. *Shock* 10: 203-212, 1998.
- Marques GMN, Rasslan R, Belon AR, Carvalho JG, Felice Neto R, Rasslan S, Utiyama EM and Montero EF: Pentoxifylline associated to hypertonic saline solution attenuates inflammatory process and apoptosis after intestinal ischemia/reperfusion in rats. *Acta Cir Bras* 29: 735-741, 2014.
- Yang X, Bai H, Wang Y, Li J, Zhou Q, Cai W, Han J, Zhu X, Dong M and Hu D: Deletion of regulatory T cells supports the development of intestinal ischemia-reperfusion injuries. *J Surg Res* 184: 832-837, 2013.
- Jiang Y, Zhou Z, Meng QT, Sun Q, Su W, Lei S, Xia Z and Xia ZY: Ginsenoside Rb1 treatment attenuates pulmonary inflammatory cytokine release and tissue injury following intestinal ischemia reperfusion injury in mice. *Oxid Med Cell Longev* 2015: 843721, 2015.
- Schneider KM, Bieghs V, Heymann F, Hu W, Drey Mueller D, Liao L, Frissen M, Ludwig A, Gassler N, Pabst O, *et al*: CX3CR1 is a gatekeeper for intestinal barrier integrity in mice: Limiting steatohepatitis by maintaining intestinal homeostasis. *Hepatology* 62: 1405-1416, 2015.
- Sheedy JR, Wettenhall RE, Scanlon D, Gooley PR, Lewis DP, McGregor N, Stapleton DI, Butt HL and DE Meirleir KL: Increased D-lactic acid intestinal bacteria in patients with chronic fatigue syndrome. *In Vivo* 23: 621-628, 2009.
- Khadaroo RG, Fortis S, Salim SY, Streutker C, Churchill TA and Zhang H: I-FABP as biomarker for the early diagnosis of acute mesenteric ischemia and resultant lung injury. *PLoS One* 9: e115242, 2014.
- Diebel ME, Diebel LN, Manke CW, Liberati DM and Whittaker JR: Early tranexamic acid administration: A protective effect on gut barrier function following ischemia/reperfusion injury. *J Trauma Acute Care Surg* 79: 1015-1022, 2015.
- Santamaría B, Ucero AC, Benito-Martin A, Vicent MJ, Orzáez M, Celdrán A, Selgas R, Ruíz-Ortega M and Ortiz A: Biocompatibility reduces inflammation-induced apoptosis in mesothelial cells exposed to peritoneal dialysis fluid. *Blood Purif* 39: 200-209, 2015.
- Ahmad SF, Attia SM, Bakheet SA, Zoheir KM, Ansari MA, Korashy HM, Abdel-Hamied HE, Ashour AE and Abd-Allah AR: Naringin attenuates the development of carrageenan-induced acute lung inflammation through inhibition of NF- κ B, STAT3 and pro-inflammatory mediators and enhancement of I κ B α and anti-inflammatory cytokines. *Inflammation* 38: 846-857, 2015.
- Fan B, Dun SH, Gu JQ, Guo Y and Ikuyama S: Pycnogenol attenuates the release of proinflammatory cytokines and expression of perilipin 2 in lipopolysaccharide-stimulated microglia in part via inhibition of NF- κ B and AP-1 activation. *PLoS One* 10: e0137837, 2015.
- Wang Y, Wang B, Du F, Su X, Sun G, Zhou G, Bian X and Liu N: Epigallocatechin-3-gallate attenuates oxidative stress and inflammation in obstructive nephropathy via NF- κ B and Nrf2/HO-1 signalling pathway regulation. *Basic Clin Pharmacol Toxicol* 117: 164-172, 2015.
- Li L, Dong H, Song E, Xu X, Liu L and Song Y: Nrf2/ARE pathway activation, HO-1 and NQO1 induction by polychlorinated biphenyl quinone is associated with reactive oxygen species and PI3K/AKT signaling. *Chem Biol Interact* 209: 56-67, 2014.
- Park SY, Kim YH and Park G: Cucurbitacins attenuate microglial activation and protect from neuroinflammatory injury through Nrf2/ARE activation and STAT/NF- κ B inhibition. *Neurosci Lett* 609: 129-136, 2015.
- Keleku-Lukwete N, Suzuki M, Otsuki A, Tsuchida K, Katayama S, Hayashi M, Naganuma E, Moriguchi T, Tanabe O, Engel JD, *et al*: Amelioration of inflammation and tissue damage in sickle cell model mice by Nrf2 activation. *Proc Natl Acad Sci USA* 112: 12169-12174, 2015.
- Qin S, Du R, Yin S, Liu X, Xu G and Cao W: Nrf2 is essential for the anti-inflammatory effect of carbon monoxide in LPS-induced inflammation. *Inflamm Res* 64: 537-548, 2015.
- Pan H, Wang H, Wang X, Zhu L and Mao L: The absence of Nrf2 enhances NF- κ B-dependent inflammation following scratch injury in mouse primary cultured astrocytes. *Mediators Inflamm* 2012: 217580, 2012.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.