Original Paper

Salvianolic Acid A Protects Against Oxidative Stress and Apoptosis Induced by Intestinal Ischemia-Reperfusion Injury Through Activation of Nrf2/HO-1 Pathways

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Abstract
Background/Aims: Ischemia-reperfusion (I/R) adversely affects the intestinal mucosa. The major mechanisms of I/R are the generation of reactive oxygen species (ROS) and apoptosis. Salvianolic acid A (SalA) is suggested to be an effective antioxidative and antiapoptotic agent in numerous pathological injuries. The present study investigated the protective role of SalA in I/R of the intestine. ***Methods:*** Adult male Sprague-Dawley rats were subjected to intestinal I/R injury *in vivo*. *In vitro* experiments were performed in IEC-6 cells subjected to hypoxia/reoxygenation (H/R) stimulation to simulate intestinal I/R. TNF-α, IL-1β, and IL-6 levels were measured using enzyme-linked immunosorbent assay. Malondialdehyde and myeloperoxidase and glutathione peroxidase levels were measured using biochemical analysis. Apoptosis was measured by terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling staining or flow cytometry *in vivo* and *in vitro*. The level of reactive oxygen species (ROS) was measured by dichlorodihydrofluorescin diacetate (DCFH-DA) staining. Western blotting was performed to determine the expression of heme oxygenase-1 (HO-1), Nrf2 and proteins associated with apoptosis. The mRNA expressions of Nrf2 and HO-1 were detected by quantitative real-time polymerase chain reaction *in vivo* and *in vitro*. ***Results:*** Malondialdehyde level and myeloperoxidase and glutathione peroxidase, TNF-α, IL-1β, and IL-6 levels group in intestinal tissue decreased significantly in the SalA pretreatment groups compared to the I/R group. SalA markedly abolished intestinal injury compared to the I/R group. SalA significantly attenuated apoptosis and increased Nrf2/HO-1 expression *in vivo* and *in vitro*. However, Nrf2 siRNA

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treatment partially abrogated the above mentioned effects of SalA in H/R-induced ROS and apoptosis in IEC-6 cells. ***Conclusion:*** The present study demonstrated that SalA ameliorated oxidation, inhibited the release of pro-inflammatory cytokines and alleviated apoptosis in I/R-induced injury and that these protective effects may partially occur via regulation of the Nrf2/HO-1 pathways.

Introduction

The small intestinal mucosa acts as a protective barrier from various environmental stimuli, including physical and chemical insults and microbial invasion, and it is likely the most sensitive and vulnerable area to ischemia reperfusion (I/R) injury [1]. The mechanisms of intestinal I/R injury are extensively researched and reviewed in the literature [2]. The production of oxygen free radicals and inflammatory cytokines is one mechanism that leads to intestinal epithelium damage and apoptosis in intestinal I/R injury [3, 4]. Enzymes involved in defense mechanisms that exhibit antioxidant and antiapoptotic activity to protect against free radical-induced damage also play a role [5]. Therefore, elevated levels of antioxidant and antiapoptosis activity may protect against intestinal I/R-induced damage.

Recent data indicated that a key transcription factor for endogenous antioxidative enzymes, NFE2-related factor-2 (Nrf2), plays a central role in the protective effect against oxidative and apoptotic damage [6]. Nrf2 binds to the antioxidant response element (ARE) in the promoter region of several genes, and it is an upstream regulator of the encoding of antioxidative proteins, such as heme oxygenase 1 (HO-1), which antagonize reactive oxygen species (ROS)-induced oxidative stress [7, 8].

Salvianolic acid A (SalA) is a water-soluble component that is extracted from the dried root and rhizome of Salvia miltiorrhiza Bunge (Danshen). SalA exhibits antioxidant, anti-inflammatory, and antiplatelet properties. Fig. 1 shows the chemical structure of SalA. Previous studies indicated the beneficial effects of SalA in preventing oxidative stress, platelet aggregation and hepatic cirrhosis [9]. SalA exhibited protective effects in myocardial and cerebral I/R injury [10, 11]. Salvianolate reduced endotoxin levels, ameliorated injury to the intestinal mucosa, and inhibited the expression of TNF-α and IL-6 mRNA in the small intestine of cirrhotic rats [12]. However, the effects of SalA on intestinal I/R were not reported. The present study, for the first time, examined the protective effects of SalA on the intestinal mucosa following I/R injury in experimental animals and the role of Nrf2/HO-1 pathway activation.

Materials and Methods

Animals and experimental procedures

Adult male Sprague-Dawley rats weighing 200-250 g and raised in an animal house (SPF animal center of Dalian Medical University) were used. The study protocol and experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Dalian Medical University, and the Institutional Ethics Committee of Dalian Medical University approved the protocol. The facility is air-conditioned, and the temperature was
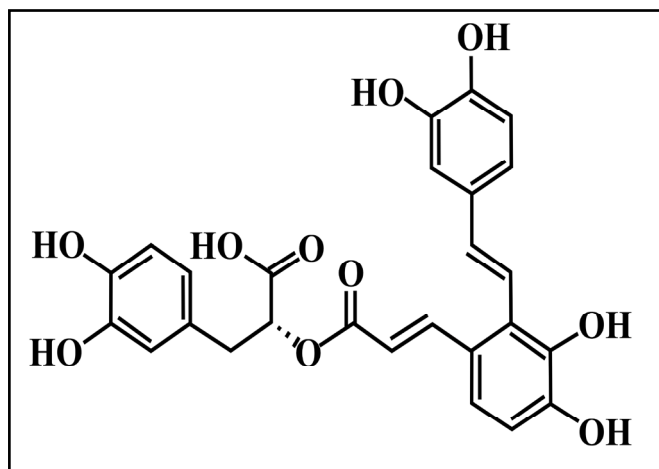

Fig. 1. Chemical structure of SalA.

maintained at 21-24°C, with controlled illumination (12-h light/dark cycle). The rats were fed a standard laboratory diet with water available ad libitum.

The animals were divided into four groups: Control group, I/R Group, SalA(10)+I/R and SalA(20)+I/R (10 animals in each group). Animals in the I/R Group were anesthetized via ether inhalation, and the abdomen was opened using a midline incision. Ischemia to the intestine was induced via clamping of the superior mesenteric artery (SMA) using a microclamp. The abdomen was closed, and the clamp was removed after 60 min to reperfuse the gut. The abdomen was closed. The abdomen was reopened 6 h after reperfusion, and the ileum was removed. Ileum tissues were fixed in 10% formalin. The abdominal cavity was opened in control group, but the bowels and SMA were not clamped. The abdomen was closed, and 2 inches of ileum specimens were collected at the end of the experiments. SalA 10 mg/kg and 20 mg/kg were administered daily for 3 days via gavage in the SalA(10)+I/R and SalA(20)+I/R groups, respectively, followed by I/R as described above.

Histopathological analysis

Fixed ilea from each experimental group were embedded in paraffin blocks. The blocks were cut into 4-μm thick sections and stained with hematoxylin and eosin. Sections were examined using a 10× objective. Injury to the intestinal mucosa was quantified in five sections from each rat and scored using the Chiu score [13].

Measurement of intestinal interleukin-6, tumor necrosis factor-α and interleukin-1β using enzyme-linked immunosorbent assay

Rats were sacrificed, and a segment (300 mg) of the terminal ileum 10 cm proximal to the ileocecal valve was immediately frozen in liquid nitrogen. The intestinal tissues were homogenized using a tissue homogenizer and centrifuged at 12, 000 rpm at 4 °C for 15 min. The supernatant was collected for ELISA. TNF-α, IL-1β, and IL-6 in intestines were measured using commercial ELISA kits (Bio Swamp Life Science Inc., Wuhan, China) in accordance with the manufacturer's protocols.

Biochemical analysis of intestinal tissues

The *in vivo* intestinal tissues of rats were homogenized on ice in normal saline, and the supernatant was collected. Malondialdehyde (MDA) level and myeloperoxidase (MPO) and glutathione peroxidase activities in the supernatants were measured spectrophotometrically according to the manufacturer's protocols (Nanjing Jiancheng Corp., Nanjing, China). The results were calculated as nmol/g protein, U/g protein, and U/g protein, respectively.

Western blot analyses

Intestinal tissues or cells were lysed in RIPA buffer (Beyotime, Jiangsu, China). The protein concentration in each sample was determined using a BCA protein assay kit (Bio-Rad, CA, USA). Protein samples (20 μg) were separated on 12% SDS-PAGE gels and transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with specific antibodies against Nrf2 (1:200), HO-1 (1:200), caspase-3 (1:200), Bax (1:400), Bcl-2 (1:400), and β-actin (1:1000) (Santa Cruz, CA, USA) overnight at 4 °C. Membranes were incubated with anti-rabbit IgG or anti-mouse IgG secondary antibodies (1:2000; ZSGB-Bio, Beijing, China) for 2 h at 37 °C. Proteins were visualized using ECL reagents (Beyotime, Jiangsu, China). The expression of β-actin was used as an internal control.

Total RNA extraction and qRT-PCR

Intestinal tissues (*in vivo*) of rats and IEC-6 cells (*in vivo*) were collected, and total RNA was extracted using TRIzol (Takara, Dalian, China) according to the manufacturer's instructions. The quantity and purity of the obtained total RNA samples were determined using UV spectroscopy (NanoDrop 2000 Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using the TransScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit (TransGen Biotech, Beijing, China). Polymerase chain reaction (PCR) was performed. The following PCR primers were used: Rat Nrf2

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(sense): 5'-CACAGTGCTCCTATGCGTGA-3', antisense: 5'-TTCTGGGCGGCGACTTTAT T-3'; HO-1 (sense): 5'-GGTGATGGCCTCCTTGTACC-3', antisense: 5'-GTGG GGCATAGACTGGGTTC-3'; and β-actin (sense): 5'-GGAAATCGTGCGTGACAT TAAAG-3', antisense: 5'-CGGCAGTGGCCATCTCTT-3'. Real-time PCR analyses were performed to detect mRNA expression using a TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. Samples were run using a 7500 Fast Real-time PCR System (Applied Biosystems). The following reverse transcription conditions were used: 42 °C for 15 min, followed by 5 s at 85 °C for RT/RI inactivation. qPCR was performed using the following conditions: 30 s at 94 °C for denaturation; 5 s at 94 °C for annealing, and 30 s at 60 °C for extension. β-actin was used as a housekeeping gene.

Detection of epithelial apoptosis

Tissues were fixed in 4% paraformaldehyde, and 10-μm frozen sections were prepared. Intestinal epithelial apoptosis was investigated using terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) staining and an *in situ* cell death detection POD kit (Roche, Penzberg, Germany) in accordance with the manufacturer's instructions. The slides were rinsed with phosphate-buffered saline (PBS) for 10 min at room temperature. Slides were incubated with 0.1% Triton X-100 for 8 min, rinsed with PBS for 15 min and 50 μL of the TUNEL reaction mixture was added and incubated for 60 min at 37 °C. Slides were rinsed three times with PBS and examined under a fluorescence microscope. The ratio of TUNEL-positive cells was calculated as follows: (number of apoptotic cells/total number counted) ×100%. Each assay was performed in a blinded manner, and the experiment was repeated three times.

Cell culture and H/R model

We used rat intestinal jejunal crypt cells (IEC-6) exposed to H/R as a model, which is a common model of intestinal I/R injury. IEC-6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) in a humidified atmosphere of air with 5% CO_2 at 37°C Cellular H/R conditions were created via incubation in a microaerophilic system (Thermo Scientific, Waltham, MA) at 5% CO_2 and 1% O_2 balanced with 94% N_2 for 6 h to simulate intestinal I/R *in vivo*. The cells were cultured in normoxic conditions (95% air and 5% CO_2 at 37°C) for 6 h of reoxygenation as previously described [14]. IEC-6 cells were pre-treated or not with SalA, at concentrations ranging from 5 to 20 μg/ml, for variable periods of time (3-24h) and then subjected to H/R.

CCK-8 assay for cytotoxicity

Cell viability and survival were determined using the tetrazolium salt Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc. Tokyo, Japan) according to the manufacturer's recommendations. Briefly, $2×10^3$ cells were plated in 96-well microtiter plates and treated with different SalA concentrations for 6 h. Cells were washed with 100 μL DMEM at various time points after treatment, and 10 μL of CCK-8 was added. The wells were incubated for 2 h in normoxic conditions. Absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay microplate reader (Thermo, Vantaa, Finland). Cell viability was defined relative to an untreated control.

Small interfering RNA transfection

IEC-6 cells were plated on 6-well plates at a density of $1×10^5$ cells per dish. Cells for Nrf2 small interfering RNA (siRNA) transfection were transfected with siRNA complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to manufacturer's instructions, at 50-60% confluency. Cells were changed to fresh culture medium after 6 h, and protein expression was examined using Western blot analysis. The siRNA of Nrf2 was purchased from Genepharma (Shanghai, China).

Measurement of cell apoptosis

Cell apoptosis was assessed using flow cytometry and an Annexin V-FITC/PI Apoptosis Detection Kit from Invitrogen, according to the manufacturer's instruction. The percentage of cell apoptosis was determined using Cell Quest Pro software.

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Measurement of intracellular ROS assay

In vitro IEC-6 cell ROS production was detected using the fluorescent probe dichlorodihydrofluorescin diacetate (DCFH-DA) staining from Molecular Probes (Invitrogen) according to the protocols described in our previous study [15].

Statistical analysis

All data are expressed as the means ± standard deviation (SD) of at least 3 independent experiments. Statistical significance was determined using a nonparametric, 2-tailed Mann-Whitney U test for data with a non-normal distribution. Parametric unpaired two-tailed Student's t tests (to compare two groups) or one-way ANOVA (for more than two groups) was used for data with a normal distribution, and the multiple comparisons between variables were carried out the using "Bonferonni post hoc" test. If the variables were not in conformance with the normal distribution, Kruskal-Wallis test was carried out and the Mann Whitney U test was utilized for the comparison of variables and "Bonferonni" correction test for the significance of statistical data. All analyses were performed using SPSS software (version 19.0; SPSS Inc, Chicago, IL). Graphs were plotted using Prism 5.0 (GraphPad Software, San Diego, CA). A value of $P<0.05$ was chosen to indicate statistical significance.

Results

Histopathological studies

Injury to the intestinal mucosa was quantified using the Chiu score. (Fig. 2). Histopathological analyses of control animals (Fig. 2A) revealed a normal mucosal pattern. Group I/R animals exhibited severe mucosal damage, including villous edema, vascular congestion, and hemorrhage, compared to the sham group. However, the intestinal mucosa was preserved in group SalA(10)+I/R and SalA(20)+I/R animals, and the injury was much less severe compared to the I/R animals. The difference in Chiu score was significantly better in the SalA(10)+I/R and SalA(20)+I/R groups than the I/R group ($P<0.05$) (Fig. 2B).

Tissue malondialdehyde, myeloperoxidase and glutathione-peroxidase

The levels of small intestinal MDA and GPx are good indicators of lipid peroxidation and ischemic damage. MPO activity is an inflammatory marker of leukocyte infiltration. Intestinal tissue MDA, MPO and GPx levels in the I/R group increased significantly compared
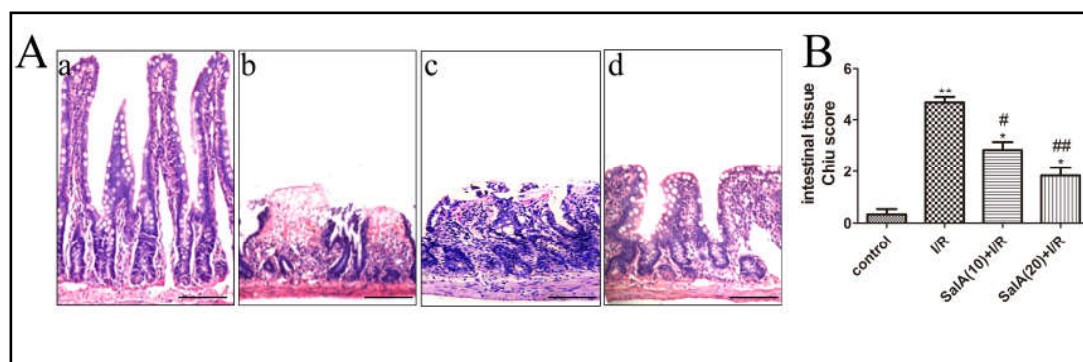

Fig. 2. Histopathological changes in the intestinal mucosa of control, I/R, SalA(10)+I/R and SalA(20)+I/R groups. (A) Tissue sections of small intestinal mucosa stained with H&E in different groups. a, Control group; b, I/R group; c, SalA(10)+I/R; d, SalA(20)+I/R. (original magnification ×200). (B) Chiu scores of the intestinal mucosa of the control, I/R, SalA(10)+I/R and SalA(20)+I/R groups. The results are representative of five separate experiments; data are the means ± SD. H&E, hematoxylin and eosin; I/R, ischemia/reperfusion. *P<0.05 compared to the control group; **P<0.01 compared to the control group; #P<0.05 compared to the I/R group; ##P<0.01 compared to the I/R group.
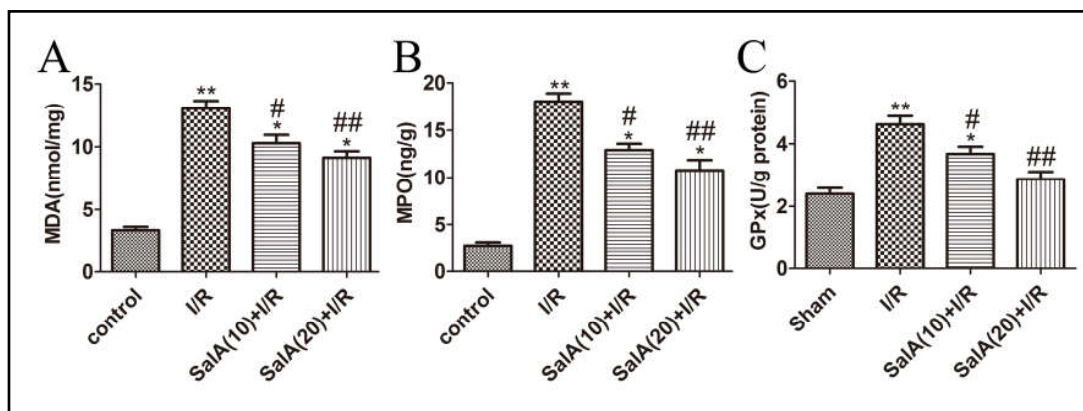
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Fig. 3. Effects of SalA on tissue MDA, MPO and GPx in the ileum after I/R. MDA, MPO and GPx levels in the intestinal tissue were elevated significantly after 6 h of reperfusion in the I/R groups compared to sham rats, and SalA significantly reduced MDA, MPO and GPx levels (A-C, respectively). The results are representative of five separate experiments; data are the means ± SD. *$P<0.05$ compared to the control group; **$P<0.01$ compared to the control group; #$P<0.05$ compared to the I/R group; ##$P<0.01$ compared to the I/R group.
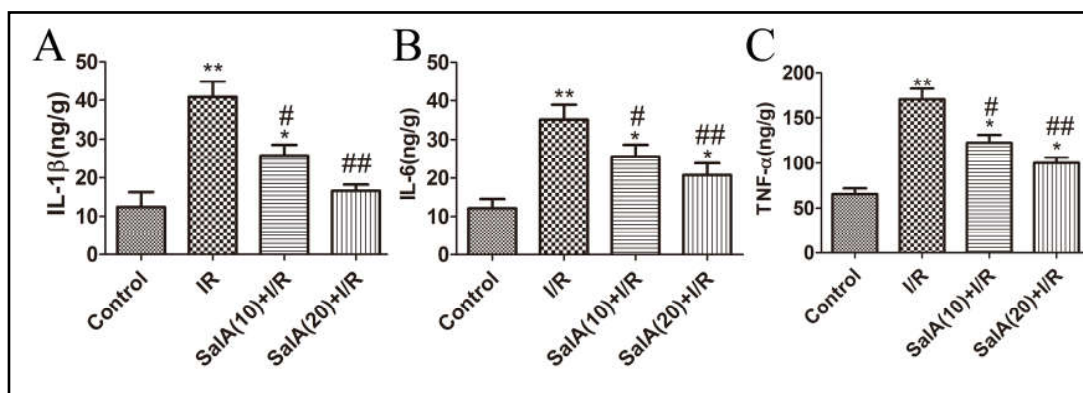

Fig. 4. Effects of SalA on cytokine levels in the ileum after I/R. TNF-α, IL-1β, and IL-6 levels in intestinal tissue were elevated significantly after 6 h of reperfusion in the I/R groups compared to sham rats (A-C, respectively), and SalA significantly reduced TNF-α, IL-1β, and IL-6 levels (A-C, respectively). The results are representative of five separate experiments; data are the means ± SD. *$P<0.05$ compared to the control group; **$P<0.01$ compared to the control group; #$P<0.05$ compared to the I/R group; ##$P<0.01$ compared to the I/R group.

to the control group (Fig. 3). SalA markedly decreased intestinal tissue MDA, MPO and GPx to levels that were comparable with the I/R group.

Tissue cytokine levels

TNF-α, IL-1β, and IL-6 levels in the intestinal tissue of the I/R group increased significantly compared to the control group, and SalA treatment abrogated these increases (Fig. 4). SalA obviously decreased TNF-α, IL-6 and IL-1β levels in intestinal tissue compared to the I/R group.

Intestinal tissue caspase-3, Bax, and Bcl-2 protein and TUNEL

TUNEL staining evaluated the antiapoptotic effect of SalA (Fig. 5A). I/R significantly increased the number of TUNEL-positive cells in rat intestines compared to the Control group ($P<0.05$). SalA treatment reduced the number of TUNEL-positive cells in the intestines of rat subjected to I/R ($P<0.05$).

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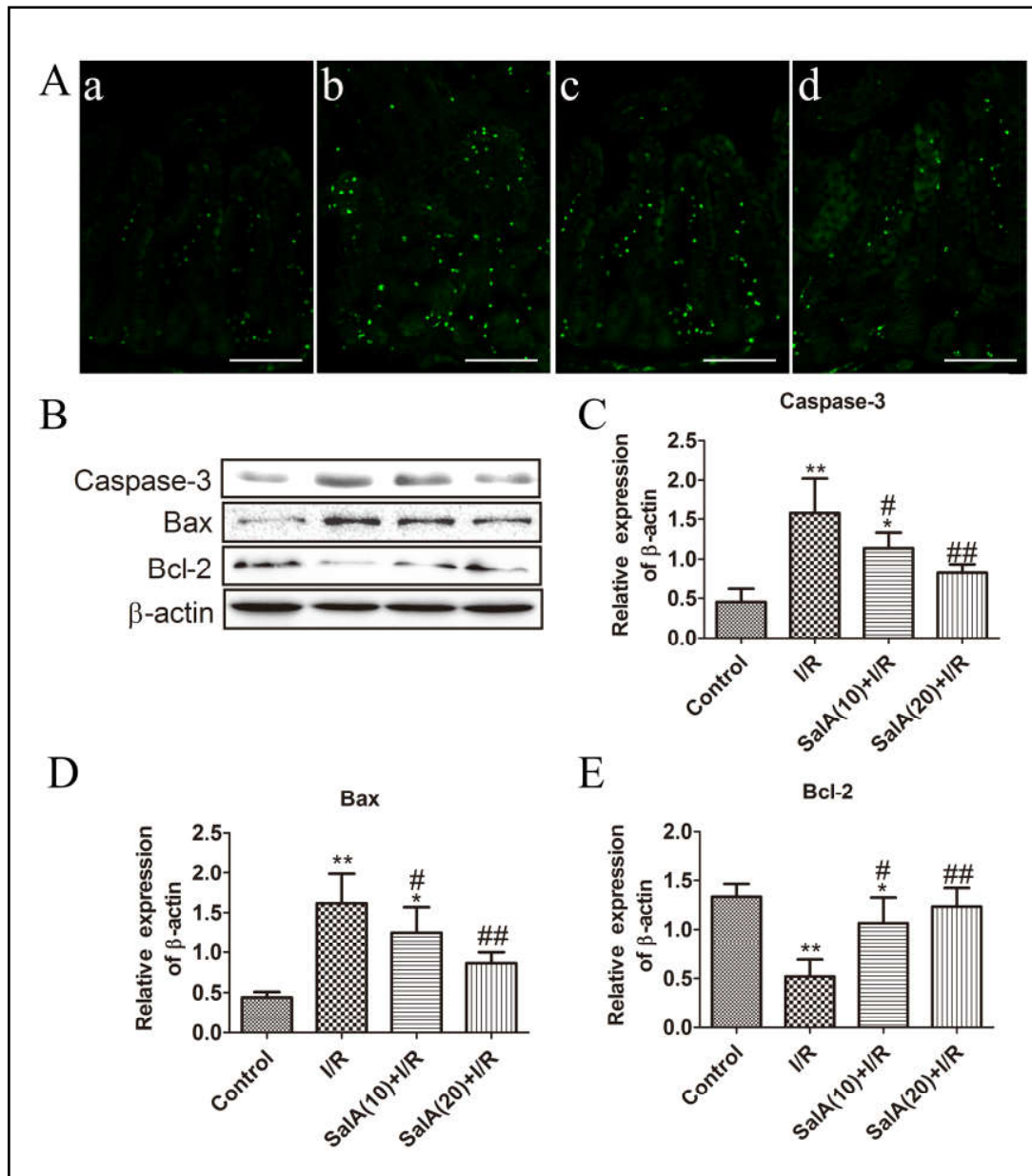Zu et al.: Salvianolic Acid A Protects Intestinal Ischemia-Reperfusion Injury

Fig. 5. TUNEL-positive cells and protein expression of caspase-3, Bax, Bcl-2 in rat intestinal tissue. (A) TUNEL-positive cells were observed in intestinal epithelial cells. a, Control group; b, I/R group; c, SalA(10)+I/R; d, SalA(20)+I/R (original magnification ×200). (B-E) Protein expression of caspase-3, Bax and Bcl-2 were analyzed using Western blot. The results are representative of three separate experiments; data are the means ± SD. *P<0.05 compared to the control group; **P<0.01 compared to the control group; #P<0.05 compared to the I/R group; ##P<0.01 compared to the I/R group.

Western blot analysis revealed that caspase-3 and Bax protein expression in the I/R group were higher compared to the control group (P<0.01), and Bcl-2 protein expression in the I/R group was lower than the control group (Fig. 5B-E). SalA attenuated the increase in caspase-3 and Bax protein expression compared to the I/R group (P<0.05). SalA significantly increased Bcl-2 protein expression compared to the I/R group (P<0.05).

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Fig. 6. Quantitative qPCR (A) and Western blot (B) analysis of Nrf2/HO-1 mRNA expression. Significantly lower Nrf2/HO-1 mRNA expression was observed in the I/R group. Nrf2/HO-1 expression were lower in the SalA(10)+I/R and SalA(20)+I/R groups compared to the control group, and it was stronger compared to the I/R group. The results are
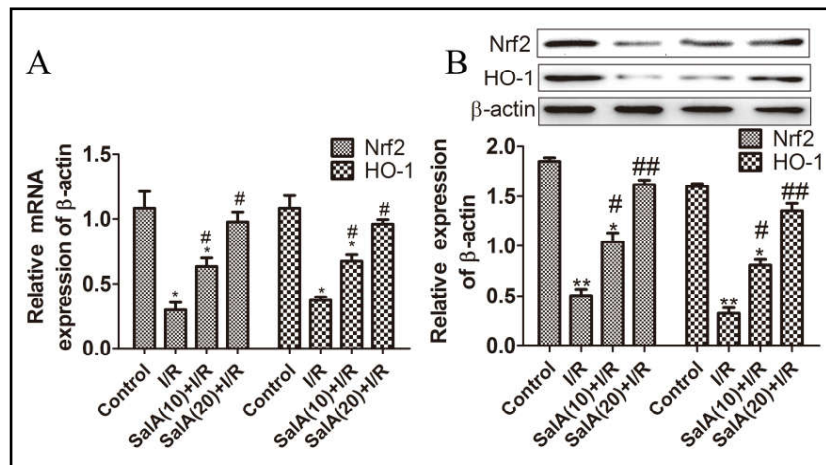
representative of three separate experiments; data are the means ± SD. *P<0.05 compared to the control group; **P<0.01 compared to the control group; #P<0.05 compared to the I/R group; ##P<0.01 compared to the I/R group.
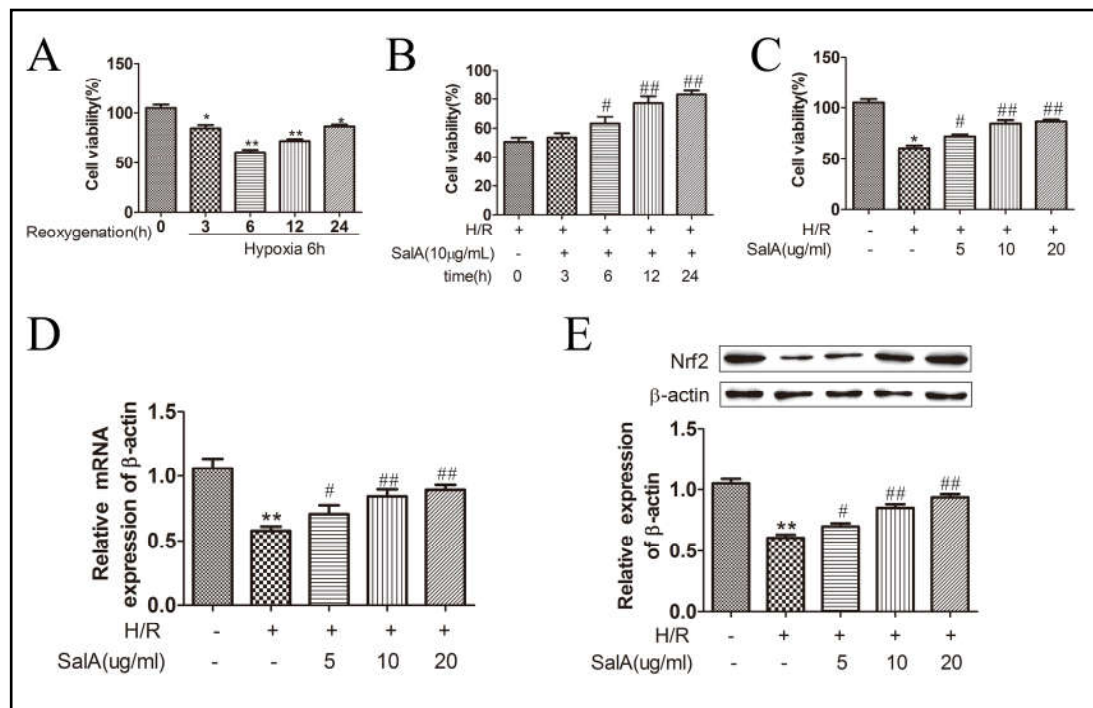

Fig. 7. SalA promotes survival of IEC-6 cells under H/R conditions in vitro. (A) IEC-6 cells were exposed to hypoxic environment for 6 h and reoxygenated for 3, 6, 12, or 24 h. The viable cells were determined using the CCK-8 assay. (B) IEC-6 cells were exposed to H/R and pretreated with SalA(10 µg/ml) for 3, 6, 12, or 24 h. Viable cells were determined using the CCK-8 assay. (C) IEC-6 cells were pretreated with SalA (5, 10, and 20 µg/mL) for 6 h, exposed to hypoxia 6 h followed by reoxygenation for 6 h. Viable cells were determined using the CCK-8 assay. The results are representative of eight separate experiments; data are the means ± SD. (D-E) Representative mRNA and protein expression levels of Nrf2 in IEC-6 cells pretreated with SalA (5, 10, and 20 µg/mL) for 6 h, exposed to hypoxia 6 h followed by reoxygenation for 6 h. The results are representative of three separate experiments; data are the means ± SD. *P<0.05 versus control group; **P<0.01 versus control group; #P<0.05 versus H/R group; ##P<0.01 versus H/R group.

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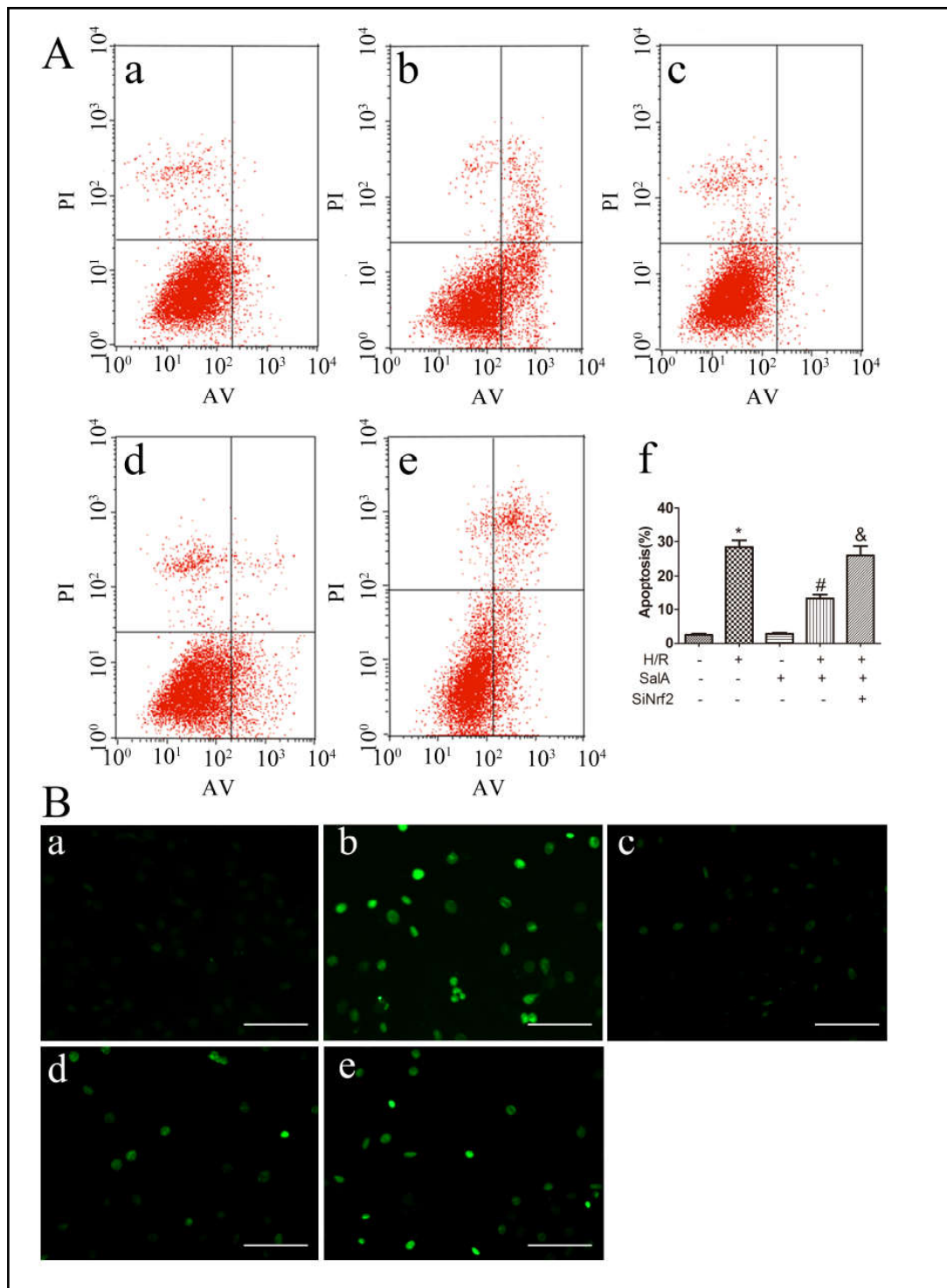Zu et al.: Salvianolic Acid A Protects Intestinal Ischemia-Reperfusion Injury

Fig. 8. Nrf2 activation protects IEC-6 cells against H/R-induced apoptosis and oxidative stress. (A) Apoptosis detection of IEC-6 cells using flow cytometry in different groups (a, Control; b, H/R; c, Control+SalA; d, H/R+SalA; e, H/R+SalA+SiNrf2; f, the results are representative of three separate apoptosis detection using flow cytometry; data are the means ± SD.). (B) ROS determination in each group (a, Control; b, H/R; c, Control+SalA; d, H/R+SalA; e, H/R+SalA+SiNrf2). All data were presented as the means ± SD, n=3. *P<0.05 versus control group; #P<0.05 versus H/R group; &P<0.05 versus H/R+SalA group.

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Expression levels in the Nrf2/HO-1 pathway

The I/R group exhibited lower expression levels of Nrf2/HO-1 on qPCR and Western blots compared to the control group (Fig. 6). Nrf2/HO-1 expression on qPCR and Western blots were higher in the SalA(10)+I/R and SalA(20)+I/R groups compared to the I/R group. There was no obvious difference in Nrf2/HO-1 expression between the SalA(10)+I/R and SalA(20)+I/R groups.

SalA-induced Nrf2 expression under H/R conditions in vitro

An H/R model of IEC-6 cells was established to further investigate the mechanisms underlying the protective effects of SalA. SalA pretreatment effectively suppressed H/R-triggered apoptosis in a time- and concentration-dependent manner (Fig. 7B and 7C). SalA administration induced the Nrf2 mRNA and protein expression in IEC-6 cells in a concentration-dependent manner (Fig. 7D and 7E), which is consistent with the *in vivo* results.

Protective effects of SalA against H/R-induced apoptosis and ROS in vitro

IEC-6 cells were treated with SalA only or SalA and Nrf2 siRNA for 6 h prior to exposure to H/R to examine whether Nrf2 mediated the antioxidant and antiapoptotic activities of SalA. Increased cellular apoptosis and ROS were observed after H/R. SalA pretreatment diminished H/R-induced apoptosis and ROS in IEC-6 cells. However, the antioxidant and antiapoptotic effects of SalA were abrogated in the presence of Nrf2 siRNA (Fig. 8). These results indicate that Nrf2 mediates the protective effects of SalA against H/R-induced oxidative stress and apoptosis.

Discussion

The deleterious effect of reperfusion on the intestinal mucosa after ischemia is the result of multiple complex factors that damage the mucosa. ROS and apoptosis are the major mechanisms of I/R injury. The use of antioxidants and antiapoptosis agents decreased the effects of reperfusion on the intestinal mucosa [5, 16].

SalA is a water-soluble phenolic acid extracted from the dried root and rhizome of Salvia miltiorrhiza, which is commonly called "Danshen" in China [17]. Modern medical research describes the many beneficial effects of SalA. SalA scavenges lipid free radicals and inhibits lipid peroxidation [18]. Notably, SalA exhibits a variety of pharmacological functions, including anti-inflammation, antioxidant, and antiplatelet effects. However, little is known about the function of SalA in protecting intestinal epithelium cells from I/R injury-induced apoptosis and ROS.

Previous findings reported that MDA and GPx levels increased significantly after intestinal I/R, which indicates that lipid peroxidation and inflammatory markers of leukocyte infiltration play important roles in the pathogenesis of I/R injury [19-21]. Pro-inflammatory factors, such as TNF-α, IL-1β, and IL-6, are potent activators of neutrophils and induce tissue damage. These factors are significant contributors to the increased microvascular permeability and perivascular and interstitial edema in I/R injury [22]. Our study measured intestinal tissue MDA, MPO and GPx levels as indexes of oxidative burst and inflammatory markers of leukocyte infiltration and TNF-α, IL-1β, and IL-6 as pro-inflammatory molecules. We demonstrated that SalA ameliorated the acute inflammatory response and systemic oxidative stress of I/R-induced intestinal injuries.

Apoptosis is implicated in numerous physiological and pathological conditions, including intestinal I/R. Numerous regulatory molecules control this process, which mediated by apoptotic signaling. The ratio of Bcl-2/Bax protein determines cell survival or death after I/R [23, 24]. The cleavage of caspase-3 is the final step of the process that initiates the apoptotic signaling pathway. The results of the present study demonstrated that SalA attenuated the

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increase in caspase-3 protein expression and Bcl-2/Bax ratio compared to the I/R group. These results indicate that SalA exerts its intestinal protective effect via the upregulation of Bcl-2 protein expression and downregulation of Bax and caspase-3 protein expression, which inhibit apoptosis in the intestinal epithelium.

Nrf2 adjusts regularly evolving cellular procedures, such as migration, growth, and apoptosis [25]. Nrf2 is a crucial modulator of HO-1 expression [26]. Nrf2/HO-1 signaling is a vital path in the apoptosis process [27]. HO-1 is a stress-responsive enzyme that produces antioxidants, anti-inflammatory agents, and regulators of apoptosis [28]. HO-1 is upregulated and protects cells from apoptosis in the rat myocardium in response to I/R injury [29]. Liu et al. found that apoptosis negatively correlated with Nrf2 and HO-1 expression, which ultimately affected the expression of cleaved caspase-3 [30]. A previous study indicated that reduced activity of the Nrf2/HO-1 pathway significantly promoted cytoprotection in the intestine [31]. SalA was an effective Nrf2/HO-1 pathway activator in our study. SalA exhibited potent antioxidant and antiapoptotic properties via the promotion of Nrf2/HO-1 pathway expression by down-regulating cytokines and inflammatory and oxidative factors. These protective effects of SalA support SalA as a novel Nrf2/HO-1 pathway agent to combat intestinal I/R injury.

Conclusion

In conclusion, the present study demonstrated that SalA ameliorated ROS, pro-inflammatory cytokine production and apoptosis in I/R-induced intestinal injury. SalA may provide a novel prophylactic strategy for the treatment of I/R-induced intestinal injury *in vivo and in vitro*, which partially occurred via activation of the Nrf2/HO-1 signaling pathway *in vitro*. SalA is a promising therapy for I/R-induced intestinal injury with striking efficacy and safety, but it requires further investigation.

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Disclosure Statement

The authors declare no conflicts of interest.

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