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ORIGINAL ARTICLE

Sulforaphane protects liver injury induced by intestinal ischemia reperfusion through Nrf2-ARE pathway

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Abstract

AIM: To investigate the effect of sulforaphane (SFN) on regulation of NF-E2-related factor-2 (Nrf2)-antioxidant response element (ARE) pathway in liver injury induced by intestinal ischemia/reperfusion (I/R).

METHODS: Rats were divided randomly into four experimental groups: control, SFN control, intestinal I/R and SFN pretreatment groups (n = 8 in each group). The intestinal I/R model was established by clamping the superior mesenteric artery for 1 h and 2 h reperfusion. In the SFN pretreatment group, surgery was performed as in the intestinal I/R group, with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation. Intestine and liver histology was investigated. Serum levels of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured. Liver tissue superoxide dismutase (SOD), myeloperoxidase (MPO), glutathione (GSH) and glutathione peroxidase (GSH-Px) activity were assayed. The liver transcription factor Nrf2 and heme oxygenase-1 (HO-1) were determined by immunohistochemical analysis and Western blotting analysis.

RESULTS: Intestinal I/R induced intestinal and liver injury, characterized by histological changes as well as a significant increase in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$), all of which were reduced by pretreatment with SFN, respectively (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$). The activity of SOD in the liver tissue decreased after intestinal I/R ($P < 0.01$), which was enhanced by SFN pretreatment ($P < 0.05$). In addition, compared with the control group, SFN markedly reduced liver tissue MPO activity ($P < 0.05$) and elevated liver tissue GSH and GSH-Px activity ($P < 0.05$, $P < 0.05$), which was in parallel with the increased level of liver Nrf2 and HO-1 expression.

CONCLUSION: SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

Key words: Sulforaphane; Liver injury; Intestinal ischemia reperfusion; NF-E2-related factor-2; Antioxidant response element

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INTRODUCTION

Intestinal ischemia/reperfusion (I/R) is considered to be a grave and triggering event in development of local and distant organ dysfunction[1-4], which occurs in many clinical settings including acute mesenteric ischemia, hemorrhagic, traumatic or septic shock, severe burns, resuscitation, small bowel transplantation and thoracoabdominal aortic aneurysm repair[5,6]. The mechanism of liver injury induced by intestinal I/R is complicated, and it has not been fully elucidated although many studies have been done to mimic the pathophysiologic process by both cell culture and animal models in the past a few years[7-10].

Traditionally, decreased basement membrane integrity and barrier function of the intestine, which facilitate bacterial translocation and local production of inflammatory cytokines[11], promotes the systemic inflammatory response syndrome and multiple organ dysfunction syndrome[12,13]. Recent studies showed that reactive oxygen species (ROS) generated during tissue reperfusion, play an important role in intestinal I/R injury[14], which can initiate lipid peroxidation, oxidize proteins to inactive states and cause DNA strand breaks, and initiation of apoptotic and necrotic cascades[15]. Additionally, ROS also has a function as a second messenger to modulate the secretion of pro-inflammation cytokines and chemokines that can destroy the intestinal barrier, which is a critical junction point to amplify the inflammation reaction described above[11-13]. The administration of antioxidants has been shown to exert beneficial effects in the prevention of ischemia-reperfusion injury[16].

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound[17]. The cytoprotective effect of this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinoine oxidoreductase 1, glutathione reductase, and glutathione peroxidase (GSH-Px)[18-20]. Phase 2 enzymes play a major role in the detoxification of ROS during ischemia/reperfusion[21,22]. Nrf2 is held in the cytoplasm by a cytoskeletal-associated specific inhibitory protein (Kelch-like ECH associated protein 1, Keap1) under circumstances of normal cellular quiescent state. Upon stimulation of oxidative stress, cysteine residues within the hinge region of Keap1 can be modified and cause a conformational change in KEAP1 with the loss of Nrf2 binding, then Nrf2 translocated into the nucleus, where it heterodimerizes with members of the maf protein family, and coordinates upregulation of cytoprotective genes[20]. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes[23,24]. Studies suggest that Nrf2 activation by SFN results in effective protection from cancers[25] and renal I/R[26] by upregulating ARE-related detoxification enzymes. These studies have focused on the chemoprevention by SFN, and to our knowledge, no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

In this study, we investigated the effect of SFN on liver injury following intestinal I/R and explored the mechanism of its protective action through Nrf2-ARE pathway.

MATERIALS AND METHODS

Animal and experimental design

Male Sprague-Dawley rats weighing 190-220 g (from the Animal center of Dalian Medical University, Dalian, China) were used in this study, which was approved by the Institutional Ethics Committee. All rats were provided with standard laboratory chow and water and were housed in accordance with institutional animal care policies.

The rats were divided into four experimental groups randomly: control (A), SFN control (B), intestinal I/R (C) and SFN pretreatment group (D) (n = 8 in each group). The rats in the control group underwent surgical preparation including isolation of the superior mesenteric artery (SMA) without occlusion; the rats in the SFN control group underwent surgery as in the control group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation (SFN was purchased from Sigma Chemical Company and was dissolved in 10% dimethylsulfoxide before administration); the rats in intestinal I/R group were subjected to 1 h intestinal ischemia and 2 h reperfusion after the SMA was isolated and occluded[27]; the rats in the SFN pretreatment group underwent surgery as in the intestinal I/R group with intraperitoneal administration of 3 mg/kg SFN 1 h before the operation.

The dose of SFN administration was determined according to the previous studies with modification from preliminary experiments. The rats in the control and intestinal I/R groups were treated with an equal volume of 10% dimethylsulfoxide. Two hours after reperfusion, blood, intestine and liver tissue samples were obtained for further analysis.

Intestine and liver morphological analysis

The isolated intestine and liver tissues were harvested and fixed in 10% formalin. After being embedded in paraffin, the tissues were stained with hematoxylin and eosin for light microscopy.

Measurement of serum aspartate aminotransferase and alanine aminotransferase

The serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured with an OLYMPUS AU1000 automatic analyzer (AusBio Laboratories Co., Ltd. Beijing, China).

Liver superoxide dismutase, myeloperoxidase, GSH and GSH-Px activity assay

The liver tissues were harvested and homogenized im-

mediately on ice in 5 volumes of normal saline. The homogenates were centrifuged at 1200 r/min for 10 min to remove debris. The superoxide dismutase (SOD), myeloperoxidase (MPO), GSH and GSH-Px activity in the supernatant were determined using an assay kit (Nanjing Jiancheng Corp., China), following the manufacturer's recommendations.

The SOD activity was determined by hydroxylamine assay developed from xanthine oxidase assay. One unit of SOD sample inhibited the reaction by approximately 50% of the initially measured xanthine oxidase reaction at 37℃. SOD activity in the liver tissues was expressed as units per milligram protein (U/mgprot).

One unit of MPO activity is defined as degrading 1 μmol of hydrogen peroxide at 37℃ and MPO activity of tissue was expressed as units per gram (U/g).

Concentrations of GSH were determined by the 5,5' dithio-bis 2-nitrobenzoic acid (DTNB)-GSSG reductase-recycling assay. The amount of GSH was expressed as milligrams per gram protein (mg/gprot).

GSH-Px was measured by the enzymatic reaction which was initiated by addition of H_2O_2 to the reaction mixture containing reduced glutathione, NADPH and glutathione reductase, and the change in the absorbance at 340 nm was monitored by spectrophotometer. Activity was given in units per milligram protein (U/mgprot).

Liver Nrf2 and HO-1 immunohistochemical analysis

Paraffin-embedded tissue sections, 4 μm in thickness, were stained with SP immunohistochemical technique for Nrf2 and HO-1. The immunohistochemical experiments were performed according to the manufacturer's recommendations. After being dewaxed or washed in PBS, tissue sections were cultured in 3% hydrogen peroxide to eliminate intrinsic peroxidase, and quenched in normal goat serum for 30 min. The sections were incubated overnight at 4℃ with polyclonal rabbit anti-rat Nrf2 antibody (Santa Cruz Corp., Ltd.), followed by the addition of the anti-rabbit immunoglobulin and streptavidin conjugated to horseradish peroxides. Finally, 3,3'-diaminobenzidine was used for color development, and hematoxylin was used for counter staining. The brown or dark brown staining in cytoplasm and/or nucleus was considered to be positive. The results were evaluated semi-quantitatively according to percentage of positive cells in five fields at a 400 multiple signal magnification. The protein expression in tissue sections was graded as 0: less than 5%; 1: from 6% to 25%; 2: from 26% to 50%; 3: from 51% to 75%; and 4: more than 75%[28].

Western blotting analysis of liver Nrf2 and HO-1

Nrf2 is a nuclear transcriptional factor that binds to the ARE in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes such as HO-1 and GSH-Px. The level of Nrf2 in the nucleus was examined by Western blotting to assess Nrf2 activation.

Cellular plasma and nuclear protein were extracted from frozen liver tissues with a protein extraction kit (Pierce, Meridian Road, Rockford, IL, USA) for HO-1 and Nrf2 measurement separately. The protein was separated by 10% SDS-PAGE gel electrophoresis. The protein was electroblotted onto NC membranes (Millipore, Bedford, MA, USA) at 9 V for 30 min. The transferred membranes were then incubated overnight at 4℃ with rabbit polyclonal antibody HO-1, Nrf2, GAPDH and PCNA (Santa Cruz Corp., Ltd.) against rat in TBS-T (10 mmol/L Tris-HC1, pH 7.5, 150 mmol/L NaC1, 0.1% Tween-20) containing 5% skim milk. After washing three times in TBS-T, the membranes were incubated for 1 h at 37℃ with an anti-rabbit IgG conjugated with horseradish peroxidase. The signals were visualized using the DAB assay kit (Fuzhou Maixin Biological Technology Co., Ltd, Fuzhou, China) and documented with a gel imaging system (UVP Bioimaging System). The signals were analyzed with software Gel-Pro Analyzer 4.0.

Statistical analysis

All data, which expressed as the mean ± SD, were compared using the paired Student's *t* test with the SigmaStat 3.5 statistical software package. One-way analysis of variance (ANOVA) was used to determine significant differences in antioxidant enzyme activities between the groups. A value of $P < 0.05$ was considered significant.

RESULTS

Changes of liver histology and serum AST and ALT levels

Intestinal I/R induced apparent intestine and liver injury at 2 h after reperfusion, manifested as histological changes in the intestine and liver with edema, hemorrhage and neutrophil infiltration (Figure 1), as well as a significant increase in serum AST and ALT level (AST: 260.13 ± 40.17 U/L *vs* 186.00 ± 24.21 U/L, $P < 0.01$; ALT: 139.63 ± 11.35 U/L *vs* 48.38 ± 10.73 U/L, $P < 0.01$, Figure 2) when compared with the control group. Compared with the intestinal I/R group, the intestinal and liver pathological damage was improved in the SFN pretreatment group. In addition, there was a significant difference in the liver function between the intestinal I/R and SFN pretreatment group in serum AST and ALT levels (AST: 260.13 ± 40.17 U/L *vs* 216.63 ± 22.65 U/L, $P < 0.05$; ALT: 139.63 ± 11.35 U/L *vs* 97.63 ± 15.56 U/L, $P < 0.01$, Figure 2), which indicates that SFN significantly attenuated the intestinal I/R-induced liver injury. There was no significant difference between the SFN control group and the control group in liver pathological damage (Figure 1) and serum AST (AST: 193.38 ± 34.63 U/L *vs* 186.00 ± 24.21 U/L, $P > 0.05$, Figure 2) and ALT (ALT: 48.00 ± 8.52 U/L *vs* 48.38 ± 10.73 U/L, $P > 0.05$, Figure 2).

Changes of liver SOD and MPO activity

SOD is the major enzyme for scavenging ROS, and its activity can reflect its functional status. The liver homogenate SOD activity in the intestinal I/R group decreased significantly at 2 h of reperfusion in comparison with the control group ($P < 0.01$, Figure 3). SOD activity was elevated markedly after SFN pretreatment compared

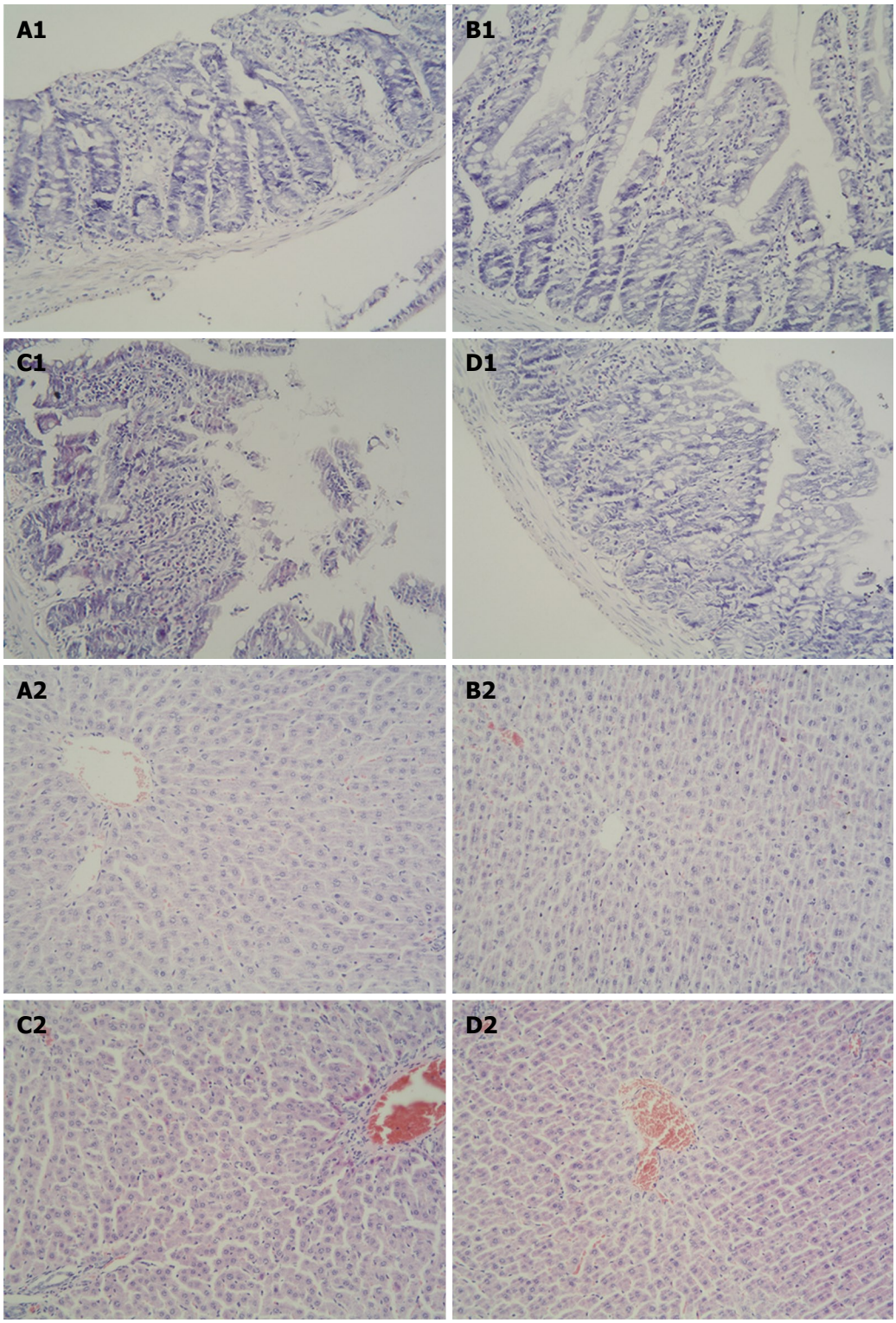

Figure 1 Changes in histology of intestine (A1-D1) and liver (A2-D2) tissues (HE stain, × 200) in the control (A), sulforaphane (SFN) control (B), intestinal ischemia/reperfusion (I/R) (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. A, B: Normal structure of intestine and liver; C: Edema, hemorrhage and neutrophil infiltration were observed in intestinal mucosa and liver tissue; D: Relatively normal histology of intestine and liver with less inflammatory cell infiltration.

with the intestinal I/R group ($P < 0.05$, Figure 3).

MPO activity in the liver homogenate was measured for estimating the leukocyte recruitment to liver tissues. The liver tissue MPO activity increased significantly after intestinal I/R at 2 h of reperfusion compared with the control group ($P < 0.05$, Figure 3). The administration of SFN reduced the MPO activity in liver tissues significantly in comparison with the intestinal I/R group ($P < 0.05$, Figure 3), thus indicating that SFN alleviated the leukocyte recruitment to liver tissues.

GSH is a kind of antioxidant which can scavenge ROS. Phase 2 enzyme GSH-Px present in the cell can

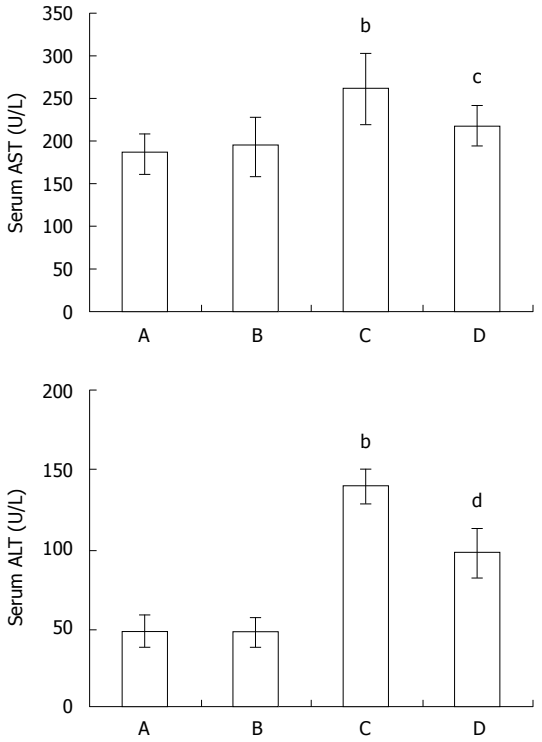

Figure 2 Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (mean ± SD, U/L) levels in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

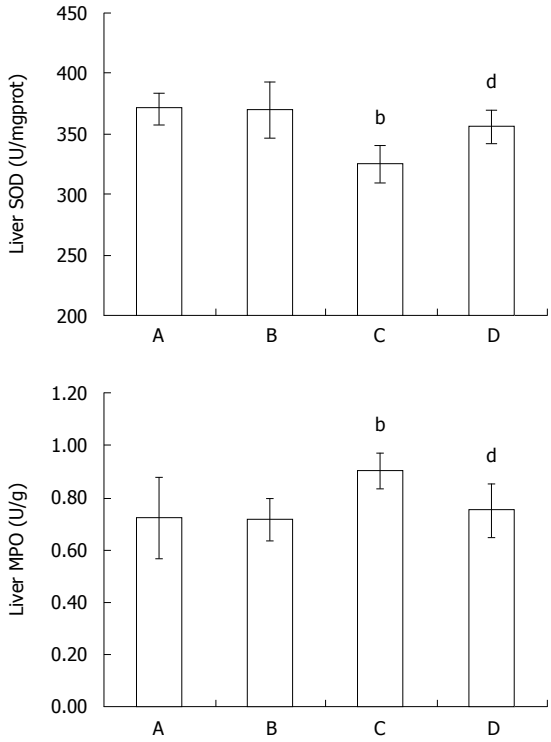

Figure 3 Liver tissue superoxide dismutase (SOD) (mean ± SD, U/mgprot) and myeloperoxidase (MPO) (mean ± SD, U/g) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

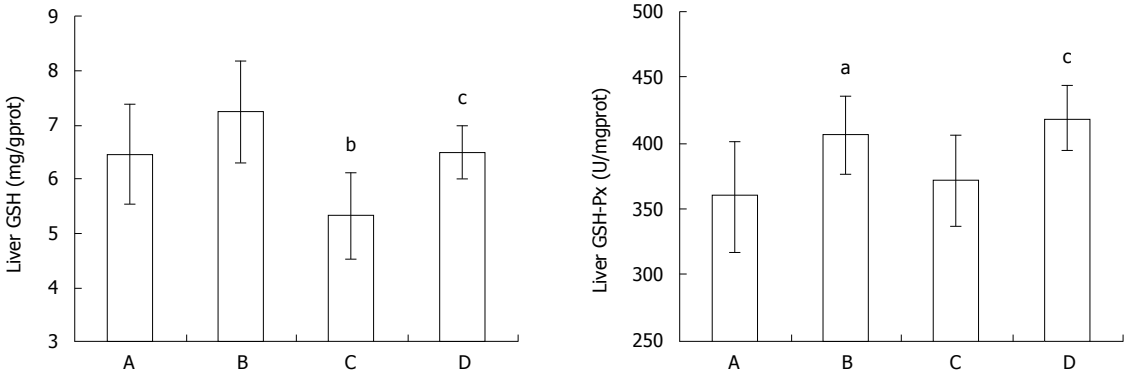

Figure 4 Liver tissue glutathione (GSH) and glutathione peroxidase (GSH-Px) (mean ± SD, U/gprot) level in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$ *vs* intestinal I/R group.

catalyze this reaction. The liver homogenate GSH activity in the intestinal I/R group decreased significantly at 2h of reperfusion as compared with the control group ($P < 0.01$, Figure 4). GSH activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4).

The increased activity of the liver tissue GSH-Px was significant after SFN pretreatment as against the control group ($P < 0.05$, Figure 4). GSH-Px activity was elevated markedly after SFN pretreatment in comparison with the intestinal I/R group ($P < 0.05$, Figure 4), thus supporting the hepatoprotective effect of SFN.

Immunohistochemical expression of liver Nrf2 and HO-1

The expression of Nrf2 in control group showed light brown immunostaining in cytoplasm and no staining in the nuclei. The significantly positive expressions of Nrf2 as strong brown staining in cytoplasm and nuclei were observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 5 and 6). Compared with the intestinal I/R group, the positive rates of Nrf2 expression increased significantly in SFN pretreatment group ($P < 0.01$, Figures 5 and 6).

The expression of HO-1 in the control group showed little brown immunostaining in cytoplasm while signifi-

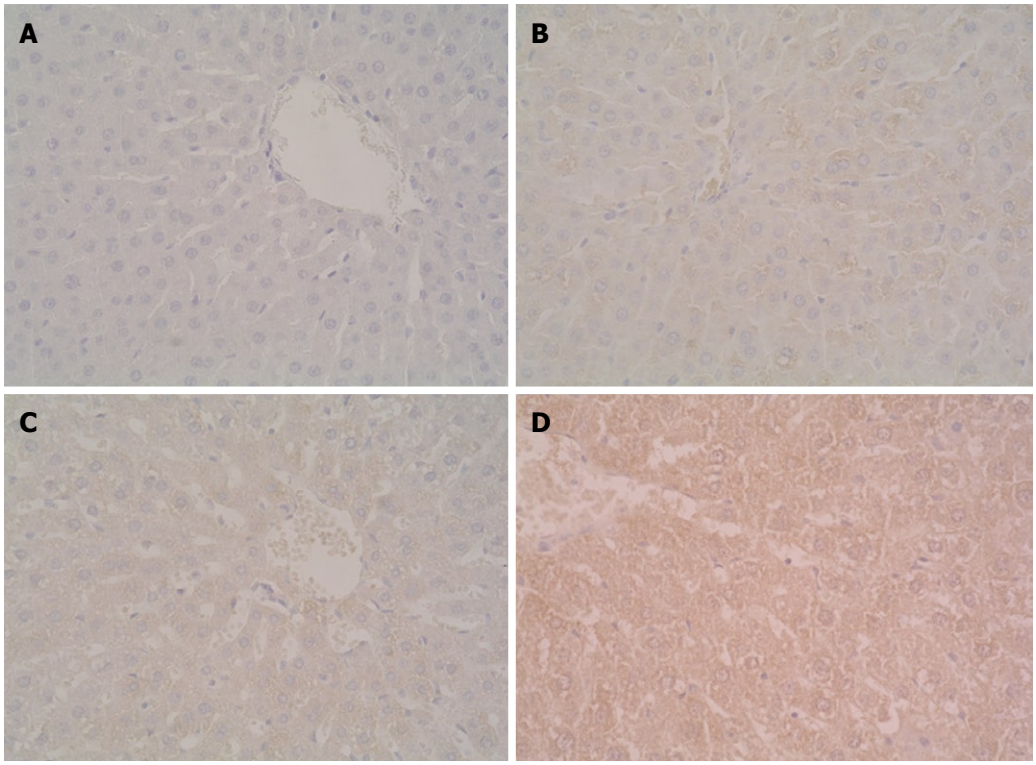

Figure 5 Immunohistochemical staining of liver NF-E2-related factor-2 (Nrf2) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

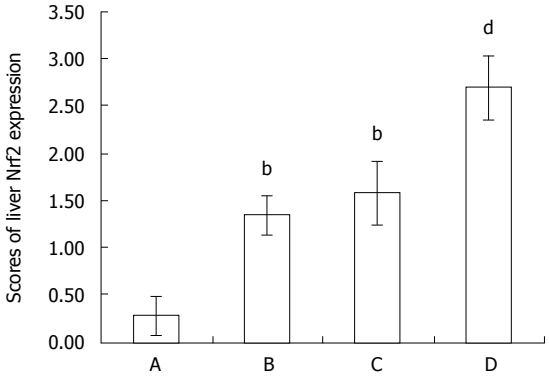

Figure 6 Immunohistochemical results (semi-quantitative analysis) of liver Nrf2 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

cant positive expression of HO-1 as brown staining in cytoplasm was observed in the intestinal I/R group and SFN control groups ($P < 0.01$, $P < 0.01$, Figures 7 and 8). Compared with the intestinal I/R group, the positive rates of HO-1 expression increased significantly in cytoplasm in SFN pretreatment group ($P < 0.01$, Figures 7 and 8).

Western blotting analysis of liver Nrf2 and HO-1 expression

The expression of nuclear Nrf2 of the liver tissues in animals with 2-h reperfusion is shown in Figure 9. The results showed a weak Nrf2 positive signal in the liver of the control group. However, a strong Nrf2 protein signal was found in the intestinal I/R group ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), thereby indicating that SFN could increase the Nrf2 activation in liver tissues.

Western blotting showed weak positive staining for HO-1 in the liver in the control group. However, a significant HO-1 signal was observed in the intestinal I/R group. Compared with the control group, IOD level of HO-1 increased markedly ($P < 0.01$, Figure 9). The signal enhanced significantly in the SFN pretreatment group in comparison with the intestinal I/R group ($P < 0.01$, Figure 9), hinting that HO-1 was activated in liver tissues caused by SFN.

DISCUSSION

Intestinal I/R is not only necessary to the intestine itself, but involves severe distant organ dysfunction. The liver is the first distant organ involved in the severe attack from intestinal I/R[8] due to its vasculature being coupled in series with that of the intestine[29]. During the reperfusion, ROS formed as participants in the I/R-induced leukocyte-mediated liver inflammation responses[30-32], and many clinical and experimental studies showed that ROS plays a crucial role in the pathogenesis of I/R[7,33].

ROS can be restrained by endogenous free radical scavengers such as SOD, catalase, the glutathione peroxidase/glutathione/glutathione reductase system and the thioredoxin peroxidase/thioredoxin/thioredoxin reduc-

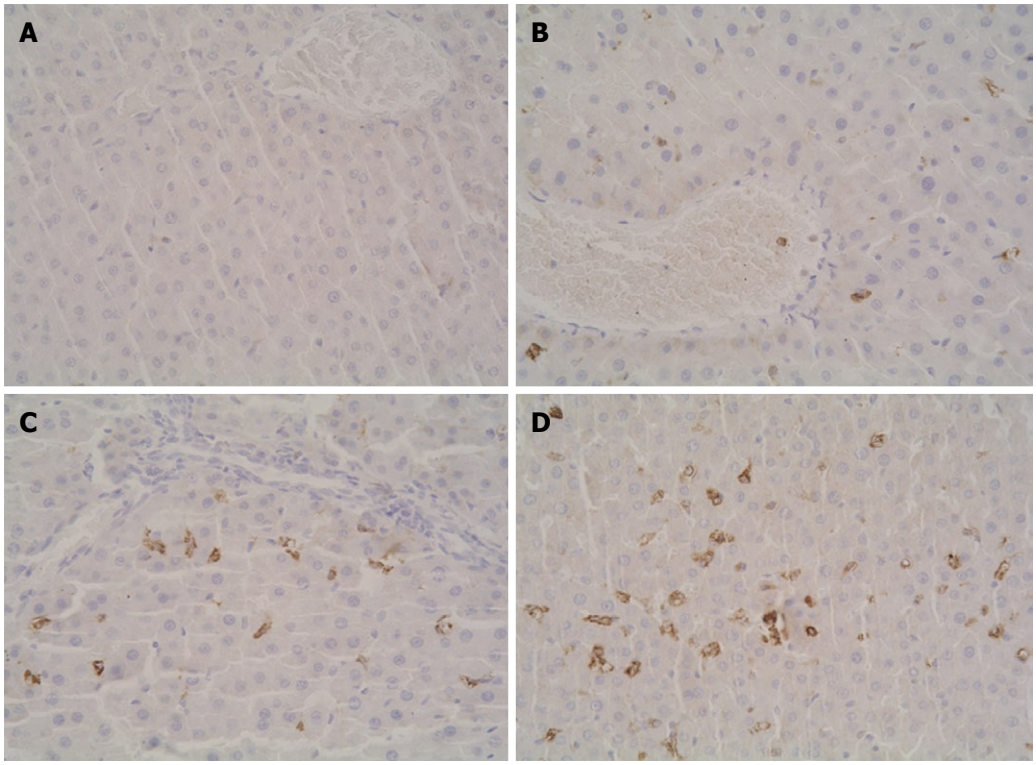

Figure 7 Immunohistochemical staining of liver heme oxygenase-1 (HO-1) expression (original magnification × 400) in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups.

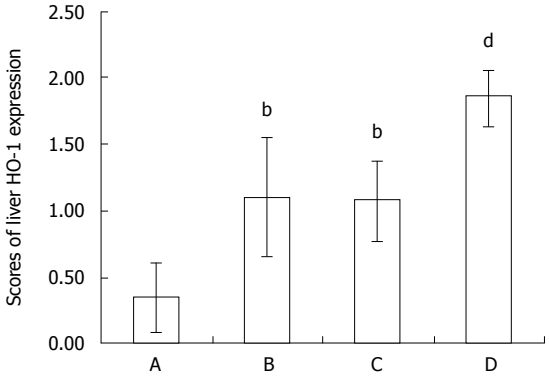

Figure 8 Immunohistochemical results (semi-quantitative analysis) of liver HO-1 expression in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. Data are presented as mean ± SD of eight animals. $^{b}P < 0.01$ *vs* control group; $^{d}P < 0.01$ *vs* intestinal I/R group.

tase system[34]. SOD catalyses the dismutation of the superoxide anion ($O_2^{\cdot}$) into H_2O_2, which can be transformed into H_2O and O_2 by catalase (CAT). GSH as a kind of endogenous free radical scavenger can be regulated by GSH-Px. Ros also oxidizes proteins, induces lipid peroxidation and initiates DNA stand breaks, which can be blocked by the above-mentioned antioxidative agents.

ARE is an enhancer element that initiates the transcription of a battery of genes encoding phase 2 enzymes[35]. Activation of gene transcription through the ARE is mediated primarily by Nrf2[36]. Under basal conditions, Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes[37]. Recent studies have implied that the activation of Nrf2/ARE pathway attenuate ischemia/reperfusion injury of the heart, brain and kidney[26,38,39]. And SFN induces Nrf2-driven phase 2 enzyme expression by modulating the activation in kidney ischemia reperfusion injury[40].

In this study, we evaluated the effect of SFN on regulating Nrf2/ARE in liver injury of the animal intestinal I/R model. We found that Nrf2 activation by sulforaphane pretreatment protected liver against injury induced by intestinal I/R, which was characterized by improved alternation in liver tissue pathology and liver function, and an enhanced antioxidant capacity, being consistent with the Nrf2 expression and content changes.

GSH-Px and HO-1 are two kinds of phase 2 enzymes. HO-1 is a ubiquitous heat shock protein (HSP32) that is highly induced by diverse stress-related conditions[41]. It is upregulated in response to oxidative stress in many tissues, providing generalized endogenous protection against oxidative stress[42]. Our results indicated that GSH-Px and HO-1 can be upregulated by SFN pretreatment through Nrf2-ARE pathway. Therefore, development of a kind of strategy to reduce oxidative stress by inducing endogenous phase 2 enzymes is attractive, which can be regulated by Nrf2. In order to clarify the mechanism comprehensively, further researches should determine the indexes of cell death such as apoptosis and inflammation in this pathophysiology process.

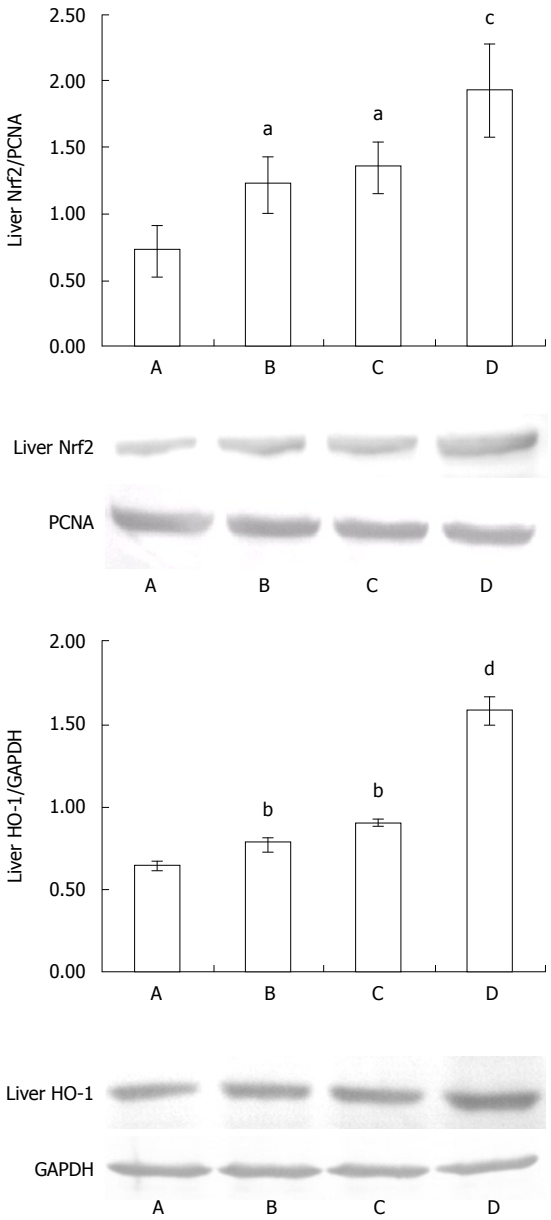

Figure 9 Western blotting analysis of expression of liver Nrf2 and HO-1 in the control (A), SFN control (B), intestinal I/R (C) (1 h ischemia and 2 h reperfusion) and SFN pretreatment (D) groups. PCNA and GAPDH were as the internal control respectively. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* intestinal I/R group.

In conclusion, ROS plays an important role in the pathogenesis of liver injury induced by intestinal I/R. SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

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COMMENTS

Background

Sulforaphane (SFN) is a natural product derived from isothiocyanate which is present in cruciferous vegetables such as broccoli that has been used as a chemopreventive compound. The cytoprotective effect exerted by this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which binds to the antioxidant response element (ARE) in the promoter region of a number of genes, encoding for antioxidative and phase 2 enzymes. SFN can disrupt the Nrf2-Keap1 complex, and permit Nrf2 to translocate into the nucleus to activate the ARE-driven genes.

Research frontiers

Studies suggest that Nrf2 activation by SFN results in effective protection from cancers and renal ischemia/reperfusion (I/R), but no one has evaluated SFN to determine if it can protect liver injury induced by intestinal I/R.

Innovations and breakthroughs

The study for the fist time showed that SFN exerted the protective effect on the liver injury induced by intestinal I/R, attributable to the antioxidant effect through Nrf2-ARE pathway.

Applications

This study has indicated that SFN pretreatment attenuates liver injury induced by intestinal I/R in rats, attributable to the antioxidant effect through Nrf2-ARE pathway.

Terminology

Nrf2 is sequestered in the cytoplasm by Keap1, and upon exposure of cells to inducers such as oxidative stress and certain chemopreventive agents, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and transactivates phase 2 detoxifying and antioxidant genes. The activation of Nrf2/ARE pathway attenuates ischemia/reperfusion injury.

Peer review

The paper is well-written and data are convincing. All data confirm the hypothesis and adequately discussed.

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