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Sodium nitroprusside decreased leukotriene C4 generation by inhibiting leukotriene C4 synthase expression and activity in hepatic ischemia-reperfusion injured rats

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ABSTRACT

The effects of NO on LTC4 generation during hepatic ischemia-reperfusion (I/R) are largely unclear. Sprague–Dawley rats were divided into control, I/R and sodium nitroprusside (SNP, 2.5, 5 and 10 μg/kg/min) + I/R groups. Liver was subjected to I/R injury, saline or SNP administered intravenously. The protein expressions of LTC4 synthesis enzymes including LTC4 synthase (LTC4S), microsomal glutathione-S-transferase (mGST)2 and mGST3 were detected with immunoblotting, the LTC4 synthesis enzymes' activities and LTC4 content were measured by RP-HPLC, the mRNA expressions of inducible nitric oxide synthase (iNOS) and endogenous nitric oxide synthase (eNOS) in liver were measured by RT-PCR. Tissue injuries were assessed by serum ALT and AST and histological changes. Serum NO_2^- and liver tissue GSH were also examined. Compared with I/R group, SNP markedly decreased LTC4 content, LTC4S protein and iNOS mRNA levels, and the LTC4 synthesis enzymes' activities ($P < 0.05$), but significantly enhanced eNOS mRNA expression in liver ($P < 0.05$). The decline in serum ALT, AST and NO_2^- levels ($P < 0.05$) together with hepatic GSH elevation ($P < 0.05$) in SNP + I/R groups were also observed. LTC4S expression in hepatocytes and sinusoidal endothelial cells in SNP + I/R groups was lower than that in I/R group. But no significant differences in the protein expressions of mGST3 and mGST2 existed between control, I/R and SNP + I/R groups ($P > 0.05$). These results demonstrated that the decline in LTC4 production by SNP treatment during hepatic I/R could be partially resulted from SNP down-regulating the protein expression of LTC4S rather than mGST2 or mGST3 and its inhibiting the LTC4 synthesis enzymes' activities.

1. Introduction

Nitric oxide (NO) has become the subject of both intense research as well as heated debate over its role in various biological and pathophysiological processes since its discovery [1]. NO is enzymatically synthesized from L-arginine by three known NO synthase isoforms: constitutively expressed endothelial NO synthase (eNOS) and neuronal NO synthase

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Abbreviations: SNP, sodium nitroprusside; NO, nitric oxide; eNOS, constitutively expressed endothelial NO synthase; iNOS, inducible NO synthase; I/R, ischemia and reperfusion; LT, leukotriene; 5-LO, 5-lipoxygenase; LTC4S, leukotriene C4 synthase; mGST, microsomal glutathione-S-transferase; LPS, lipopolysaccharides; RT-PCR, reverse-transcriptase polymerase chain reaction; RP-HPLC, reversed phase high-performance liquid chromatography; HTMP, 4-hydroxy-2,2,6,6-tetramethylpiperidine-l-o; PAGE, SDS-polyacrylamide gel electrophoresis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, reduced glutathione

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(nNOS) and inducible NO synthase (iNOS) [1,2]. Under normal conditions, only the constitutive eNOS is present in the liver and the low level of NO produced by eNOS regulates hepatic functions. iNOS, however, is readily upregulated in the liver under a number of conditions, including endotoxemia, hemorrhagic shock, I/R, sepsis, infection, hepatitis, ozon exposure and liver regeneration [2,3]. NO may be released from the hepatic vascular endothelium, platelets, nerve endings, mast cells, and Kupffer cells as a response to various stimuli such as endotoxemia, I/R injury, and circulatory shock.

Hepatic injury secondary to warm ischemia and reperfusion (I/R) is an important clinical issue. It has been implicated in the pathogenesis of a variety of clinical conditions including trauma, thermal injury, hypovolemic and endotoxin shock, reconstructive vascular surgery, liver transplantation, and liver resectional surgery [4–9]. However, the role of NO during hepatic I/R is still largely contradictory [10,11].

The cysteinyl leukotriene (LT)s mediate a wide variety of biologic responses including enhanced vascular permeability, smooth muscle contraction, mucus hypersecretion and bronchial hyperreactivity [12]. That the LTC4 synthase (LTC4S), microsomal glutathione-S-transferase (mGST)2 and mGST3 catalyzed LTA4 and reduced glutathione (GSH) to generate LTC4 is the first committed step in the synthesis of the cysteinyl LTs, LTC4, LTD4, and LTE4 (Fig. 1) [13–17]. LTs were related to cholestasis, hepatic inflammation, portal hypertension, hepatorenal syndrome, fulminant hepatic failure and primary graft nonfunction following liver transplantation [13,18,19]. There were only a few studies about the roles of LTs in hepatic I/R injury [21–24]. Recent study reported that the cysteinyl LTs content in the hepatic tissue after 12 and 24 h reperfusion was increased 4- to 5-fold compared to controls and accompanied by the enhancement of hepatic edema and plasma ALT elevation [25].

Some relationships were shown to exist in NO and cysteinyl LTs [26–28]. Larfars et al. first demonstrated that the cysteinyl LTs LTC4 and LTD4, as well as LTB4, activate NO release from human PMN by surface receptor, G-protein and $[Ca^{2+}]$i-dependent mechanisms [26]. The addition of NO via the infusion of sodium nitroprusside (SNP, 0.05 mM, 1 mM) reduced the effect of leukotriene D4 on portal flow, bile flow and bile acid secretion whereas the leukotriene D4 effects on hepatic glucose output remained unaffected. Correlation coefficient between decrease in portal flow and reduction of bile flow by infusing leukotriene D4 was higher than that while in the presence of SNP [27]. LTB4 decreased hepatocyte NO synthesis in a concentration-dependent manner when the cells were stimulated with a combination of cytokines or IL-1 alone [28]. Reduced nitrite (NO_2^-) synthesis was associated with reduced iNOS mRNA levels suggesting that the induction of iNOS was inhibited. These findings demonstrate that eicosanoids can regulate hepatocyte NO synthesis in vitro. However, to this day there is no study associated with the effects of NO on the LTC4 production, the expressions and activities of the LTC4 synthesis enzymes (LTC4, mGST2 and mGST3) in vivo.

In his study we investigated the effects of SNP, a NO donor on the formation of LTC4, the protein expressions of the LTC4 synthesis enzymes and the LTC4 synthesis enzymes' activities after hepatic I/R in rats.

2. Materials and Methods

2.1. *Reagents*

Male Sprague–Dawley rats, weight 230–250 g, were obtained from the Experimental Animal Center, Zhejiang University (Hangzhou, China). Sodium nitroprusside (SNP) was purchased from Shuanghe Medicine Technic Co. (Beijing, China). TRIzol Reagent and MmuLV reverse transcriptase were from GIBCO BRL (Gaithersburg, MD), reduced glutathione and *Taq* DNA polymerase were from Sangon (Shanghai, China). cDNA probes for rat iNOS and eNOS were synthesized by Sangon (Shanghai, China). LTA4 methyl ester, LTC4, and prostaglandin B2 (PGB2) were purchased from Cayman Chemical Co. (Ann Arbor, MI). LTC4S rabbit polyclonal antibody was from Santa Cruz (California, USA). Mouse antibody for mGST2 and

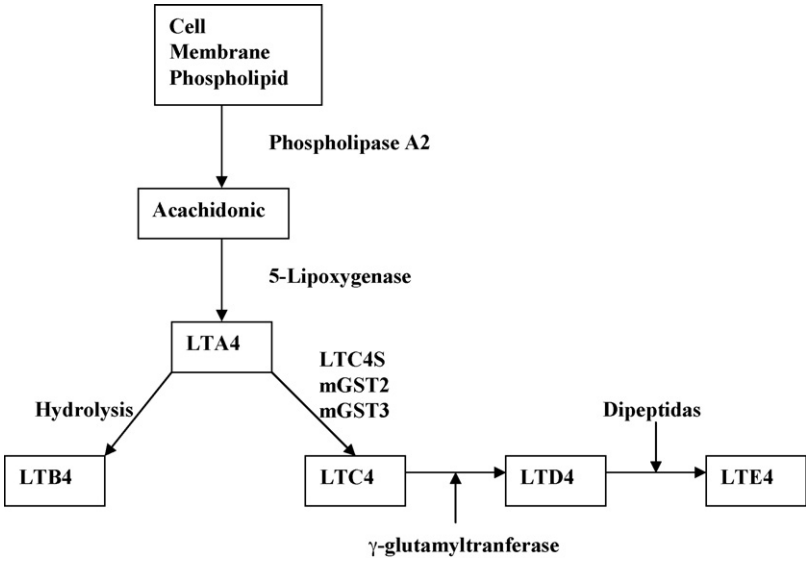

Fig. 1 – Pathway of leukotrienes biosynthesis (revised from Farzaneh-Faret and Moore [20]).

mGST3 were purchased from Abnova (Taipei, Taiwan, China). Enhanced chemiluminescence detection kit for HRP was from Biological Industries (Kibbuz Beit Haemek, Israel). PVDF membranes were from Millipore (MA, USA). Polymer Detection System for immunohistological staining, DAB kit, horseradish peroxidase-linked goat anti-rabbit and goat anti-mouse antibody were from Zhongshan Biological Co. (Beijing, China). ALT, AST and GSH kit were the products of Nanjing Jiancheng (Nanjing, China). All other chemicals were of the highest purity commercially available.

2.2. Animal model of hepatic ischemia and reperfusion injury

The rats were housed and treated in accordance with the Guidelines for the Care and Use of the Experimental Animal Center of Zhejiang University (Hangzhou, China). The study was approved by the Local Animal Ethics Committee. Animals were fasted for 12 h but allowed to drink water prior to the operation and randomized into five groups: I/R group, animals were anesthetized with pentobarbital 50 mg/kg intraperitoneally, the external jugular vein catheter was created using a polyethylene tube of 0.9 mm inner diameter (Becton Dickinson Medical Devices, Suzhou, China), then the rats subjected to midline laparotomy, the liver was exposed, and the left lateral and median lobes were rendered ischemic by clamping the hepatic arterial and portal venous blood supply using a microaneurysm clamp. Following 60 min of hepatic ischemia (or sham), livers were reperfused for 5 h by removing the clamp and the peritoneal cavity was sutured closed. Saline solution (3 mL/kg/min) was intravenously injected by external jugular vein at 15 min before the start of ischemia through reperfusion period. Sham group (Control), surgeries were performed on anesthetized rats in which hepatic blood flow was not occluded. SNP (2.5, 5 and 10 μg/kg/min) + I/R groups, surgeries were performed on anesthetized rat as I/R group, and SNP (2.5, 5 and 10 μg/kg/min) was intravenously injected by external jugular vein at 15 min before the start of ischemia through 5 h reperfusion, respectively. Serum was collected from each animal in the time point of ischemia 1 h, reperfusion 1 h, 3 h and 5 h for liver enzyme and nitrite determinations. Following 5 h of reperfusion, livers were removed, the middle lobe prepared for microsomes and fixed in 10% Formalin for immunohistochemistry or staining with Hematoxylin and Eosin, the left lobule prepared for 10% tissue homogenate and snap frozen in liquid nitrogen then stored at −70 °C for RT-PCR and RP-HPLC detection.

2.3. Immunoblot analysis

Immunoblot analysis was performed as described previously but with a slight modification [29]. Briefly, 100 μg membrane protein in loading buffer were separated by SDS-polyacrylamide gel electrophoresis (PAGE) through a 15% Tris–HCl, precasted, linear gradient gel and electroblotted onto PVDF membranes (Millipore, MA, USA). The transfer efficiency was visualized by using prestained molecular weight protein standards (Fermentas, Sangon). Membranes were then soaked for 1 h at 25 °C in Tris-buffered saline (100 mM Tris–HCl, pH 7.5, 150 mM NaCl) and 5% (w/v) nonfat dried milk. The membranes were subsequently incubated overnight at 4 °C with a polyclonal or monoclonal antiserum raised against a peptide of human LTC4S (residues 51–150) or mGST2 (residues 1–148) or GST3 (residues 28–85), used at dilutions 1:500, 1:3000 and 1:3000, respectively. After washing, the blot was incubated for 1 h at 25 °C with a horseradish peroxidase-linked goat anti-rabbit or goat anti-mouse antibody (1:2, 500 dilution) in 0.1% TTBS and 2% (w/v) nonfat dried milk. The washing steps were repeated, and subsequently enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Kibbuz Beit Haemek). SDS-PAGE immunoblots were quantitated using a Bio-Imaging Analyzer (Bio-Rad) with the Quantity One Version 4.2.2 software (Bio-Rad).

2.4. Immunohistochemistry

The indirect immunoperoxidase method was used to localize the LTC4 synthesis enzymes in paraffin-embedded liver sections from control and I/R rats and carried out using the Polymer Detection System for immunohistological staining and DAB kit (Zhong Shan) according to manufacturer's guidelines. When the sections were deparaffinized and rehydrated, endogenous peroxidase was quenched by incubation of the sections in 3% H_2O_2 in methanol for 20 min. After antigen retrieval, the sections were blocked for nonspecific binding of antibody with PBS containing 10% normal calf serum for 30 min and then overnight incubated at 4 °C with rabbit LTC4S polyclonal antibody (Santa Cruz) in 0.5% bovine serum albumin in phosphate-buffered saline. After three rinses with PBS, the sections were incubated for 1 h in a solution containing goat anti-rabbit or mouse IgG-HRP polymer. The sections were rinsed, stained with diaminobenzidine, and counterstained with hematoxylin.

2.5. Measurement of LTC4 content

LTC4 content in liver tissue was measured by high-performance liquid chromatography as described previously [30]. Briefly, 180–310 mg frozen liver tissue were chopped into small pieces and homogenized in 100 μL of PBS containing internal standard PGB2 (pH 7.4). During these procedures the liver tissue were cooled by liquid nitrogen and ice. The biological samples were made 80% of aqueous methanol, containing 1 mM HTMP, stored at −20 °C for at least 3 h, centrifuged at 8000 × *g* for 10 min, and the supernatant was evaporated to dryness under a gentle stream of nitrogen. The dried samples were again resuspended in 80% cold methanol with HTMP, stored at −20 °C, recentrifuged, and the supernatant evaporated. This procedure was repeated up to two times until a supernatant without visible protein pellet was obtained. The samples were then evaporated again and resuspended in 30% aqueous methanol. HPLC was performed on a C18-Eurospher-100 column (4.6 cm × 250 mm, 5 μm particles, Knauer, Berlin, Germany). The mobile phase consisted of methanol, water, and acetic acid (70:30:0.1, v/v/v) pH 5.6 adjusted with ammonium hydroxide. EDTA (1 mM) was present in all of the mobile phases. The flow rate was 1 mL/min. Authentic standards were used to identify peaks by retention time. The detection limit of this method was approximately 1 ng/mL of sample. The LTC4 in liver tissue was quantitated from the ratio

of the LTC4 peak to that of the internal standard PGB2. LTC4 content was expressed as nanogram of LTC4 per 100 mL of wet tissue.

2.6. Analysis of the LTC4 synthesis enzymes' activities

The activities of the LTC4 synthesis enzymes were examined according to Shimada et al. [31] with a slight modification. Briefly, after 5 h reperfusion, liver tissue was homogenated in 20 mmol/L Tris–HCl (pH 8.0) containing 0.15 mol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid, and sonicated on ice. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C, and the resulting supernatant was further centrifuged at 100,000 × *g* for 1 h at 4 °C. The pellet (microsomal fraction) was resuspended in the same buffer and used as the enzyme solution. Protein concentration was determined by the Coomassie protein assay. The reaction mixture containing 100 mmol/L Tris–HCl buffer (pH 8.0), 10 mmol/L reduced glutathione, 2 mg/mL bovine serum albumin, and the enzyme solution in a total volume of 50 μL was preincubated for 1 min at 37 °C. Incubation was initiated by adding 60 μmol/L LTA4 from freshly hydrolyzed LTA4 methyl ester. After incubation at 37 °C for 5 min, the reaction was terminated by the addition of 100 μL of acetonitrile/methanol/acetic acid (150/50/0.3, v/v) and 729 pmol of PGB2 as an internal standard. The mixture was kept for 30 min at −20 °C, followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. Supernatant was assayed by HPLC (Knauer, Berlin, Germany) with a reverse-phase column (4.6 cm × 250 mm) packed with Zorba SB-C18, 5-μm particles (Agilent, Wilmington, USA) using an isocratic mobile phase (methanol/water/acetic acid, 70/30/0.1, v/v) at a flow rate of 0.8 mL/min, monitoring at 280 nm. Authentic standards were used to identify peaks by retention time. The LTC4 formed was quantitated from the ratio of the LTC4 peak to that of the internal standard PGB2. LTC4S activity was expressed as nanomoles of LTC4 formed per milligram of protein per 5 min.

2.7. Reverse-transcriptase polymerase chain reaction

Total RNA was isolated from whole liver tissue using TRIzol Reagent according to the manufacturer's instructions and quantified by measurement of ultraviolet absorption at 260 nm. One microgram of total RNA from each sample was reverse-transcribed to synthesize the single-stranded cDNA using an antisense specific primer and 200 units of MmuLV reverse transcriptase (Gibco BRL). Sequences of PCR primers for rat β-actin, iNOS (N-terminal, 5′-ATGTCCGAAGCAAACATCAC-3′ and C-terminal, 5′-TAATGTCCAGGAAGTAGGTG-3′) and eNOS (N-terminal, 5′-GCAGAAGAGTCCAGCGAACA-3′ and C-terminal, 5′-GGCAGCCAAACACCAAAGTC-3′) were derived from published sequences [7,32]. Aliquots of the synthesized cDNA (1.5 μL) were amplified with proper cycle using each primer and 1.5 U of *Taq* DNA polymerase in a Mastercycler gradient (Eppendorf, Germany). The reactants were cycled at 95 °C for 45 s, 55.8 °C/58 °C for 45 s, and 72 °C for 45 s. The PCR products were separated by electrophoresis using 1.5% ethidium bromide-stained agarose gel and visualized by ultraviolet transillumination. The intensity of each band was measured by a Bio-Imaging Analyzer (Bio-Rad) and quantified using Quantity One Version 4.2.2 software (Bio-Rad). Using amplification of β-actin as a control, the degree of expression of the mRNA of these products was compared.

2.8. Measurement of NO formation

NO formation was estimated by measuring the stable end-product of NO in aqueous solutions, nitrite (NO_2^-), by using Griess reagent (0.1% *N*-(1-naphthyl)-ethylenediamine and 1% sufanilamide in H_3PO_4) [33]. The nitrite concentration was determined using a curve calibrated on sodium nitrite standard.

2.9. Determination of ALT, AST and GSH levels

ALT and AST levels in serum and GSH content in liver tissue were measured spectrophotometrically using kits according to manufacturer instructions. Briefly, serum ALT was measured by enzymatic reduction of pyruvate to lactare with oxidation of β-nicotinamide adeninine dinucleotide reduced form, serum AST was measured by enzymatic reduction of oxalacetate to lactate with oxidation of β-nicotinamide adeninine dinucleotide reduced form, followed spectrophotometrically by the rate of absorption loss at 340 nm. Total GSH (the sum of oxidized and reduced GSH) in liver homogenates was determined by the enzymatic recycling procedure by using GSH reductase and 5,5′-dithiobis 2-nitrobenzoic acid. Values were normalized for protein content of the samples.

2.10. Histology

Liver was fixed in 10% Formalin at room temperature and embedded in paraffin. Sections of 5 mm were stained with Hematoxylin and Eosin (H&E) by Ms. Yu Jing-ying from Department of Pathology, College of Medicine, Zhejiang University. To determine the severity of hepatic injury, H&E-stained sections were evaluated by a point-counting method on an ordinal scale [34].

2.11. Statistical analysis

Data were expressed as means ± S.E. Kruskal–Wallis test was used to compare the three groups. The Student's *t*-test was used for the comparison of two groups. $P < 0.05$ was considered to be significant.

3. Results

3.1. SNP decreased LTC4 content during hepatic I/R in rats

NO were shown to be associated with cysteinyl LTs, thus we investigated the effects of exogenous NO donor SNP on LTC4 content in liver tissue after 5 h reperfusion. One representative experiment of RP-HPLC chromatograms demonstrating LTC4 content in liver tissue is shown in Fig. 2. Compared with control group, hepatic LTC4 content in I/R and SNP (2.5 μg/kg/min) + I/R groups increased markedly (Fig. 3) ($P < 0.05$). Except for SNP (2.5 μg/kg/min) + I/R group, hepatic LTC4 content in

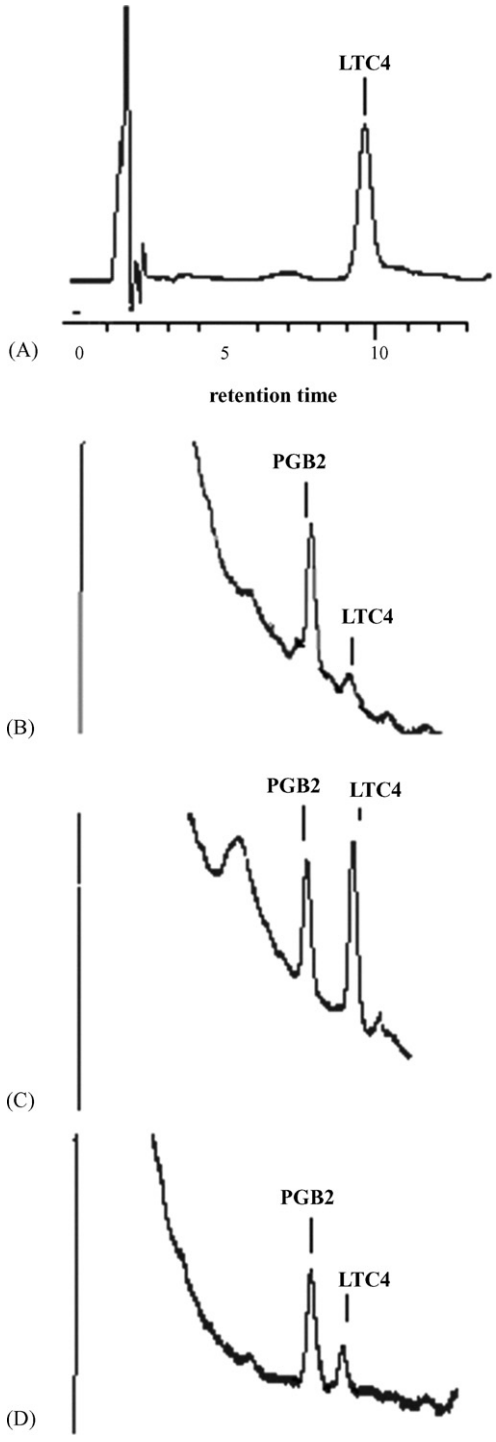

Fig. 2 – HPLC chromatograms demonstrating LTC4 content in rat liver tissue. Rats were subjected to hepatic ischemia 60 min and 5 h reperfusion injury as described in Section 2. (A) LTC4 standard, (B) Control, (C) I/R and (D) SNP (5 μg/kg/min) + I/R group. Absorbance was measured at 280 nm. The retention times of LTC4 standard are indicated. One representative experiment out of six is shown.

SNP (5 and 10 μg/kg/min) + I/R groups were lower than that in I/R group ($P < 0.05$).

3.2. *The effects of SNP on the protein expressions of the LTC4 synthesis enzymes in hepatic I/R injury rats*

The microsomal glutathione-S-transferase super family member, LTC4S, mGST2 and mGST3, were all found to catalyze the production of LTC4 [16,17], here we examined the protein expressions of these enzymes in the control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R groups rats with immunoblot analysis. As shown in Fig. 4, panel A indicates a representative of hepatic protein expressions of LTC4S and mGST3, panel B display densitometric analysis of LTC4S and mGST3 protein levels in control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. The result of mGST2 protein expression was not shown because we cannot repeat it three times for the low purity of its antibody. The LTC4S protein level in I/R and SNP (2.5 μg/kg/min) + I/R groups was higher than that in control group ($P < 0.05$), whereas its protein level in SNP (5 and 10 μg/kg/min) + I/R groups was significantly reduced when compared with the values of I/R group ($P < 0.01$). But the protein levels of mGST3 and mGST2 (data not shown) did not present significant changes between control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R groups ($P > 0.05$). It suggested that during hepatic I/R the decrease of LTC4 level by SNP treatment could be due to its down-regulating the protein expression of LTC4S rather than mGST3 or mGST2.

3.3. *Immunohistochemical localization of LTC4 synthase in rat liver tissue*

To clarify what cell types increased LTC4S expression in an I/R-sensitive manner, we performed immunohistochemical staining in paraffin-embedded liver sections from control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats (Fig. 5). In normal liver, hepatocytes and endothelial cells exhibited low immunological staining for LTC4S (Fig. 5A and D). In I/R liver, LTC4S was detected in endothelial cells and most hepatocytes which were strongly stained, exhibiting a heterogeneous intralobular distribution (Fig. 5B and E), and a few deposited erythrocytes and platelets that adhered to the vascular wall were also positive. The rat liver in SNP (5 μg/kg/min) + I/R group displayed a slight staining for LTC4S (Fig. 5C and F).

3.4. *SNP decreased the LTC4 synthesis enzymes' activities during hepatic I/R in rats*

The above results demonstrated that during I/R SNP (5 and 10 μg/kg/min) reduced LTC4 products and LTC4S protein expression in rat liver. The LTC4 synthesis enzymes including LTC4S, mGST2 and mGST3, are capable of conjugating LTA4 and glutathione to generated LTC4, and all expressed in rat liver at both the mRNA and protein levels [29]. To explore whether declined LTC4 production after SNP injection concerned with the LTC4 synthesis enzymes' activities, we examined the activity in liver microsomes with RP-HPLC. As shown in Fig. 6, compared with control group, the activities of the LTC4 synthesis enzymes were markedly increased in I/R and SNP (2.5 μg/kg/min) + I/R groups; the LTC4 synthesis

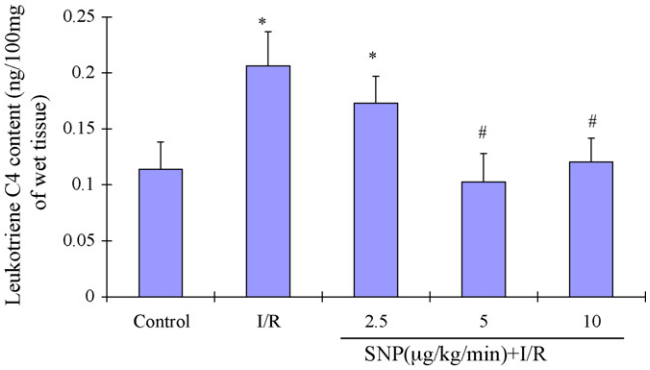

Fig. 3 – Leukotriene C4 content in control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. Levels of LTC4 were determined by RP-HPLC. Each value represents the mean obtained from duplicate experiments with liver tissue from six rats in all groups. Error bars indicate S.E. Compared with control. $^{*}P < 0.05$; compared with I/R, $^{\#}P < 0.05$.

enzymes' activities of liver microsomal part in SNP (5 and 10 μg/kg/min) + I/R groups were lower than that in hepatic I/R rats ($P < 0.05$). It demonstrated that SNP treatment reduced LTC4 generation by its inhibition to the LTC4 synthesis enzymes' activities during hepatic I/R.

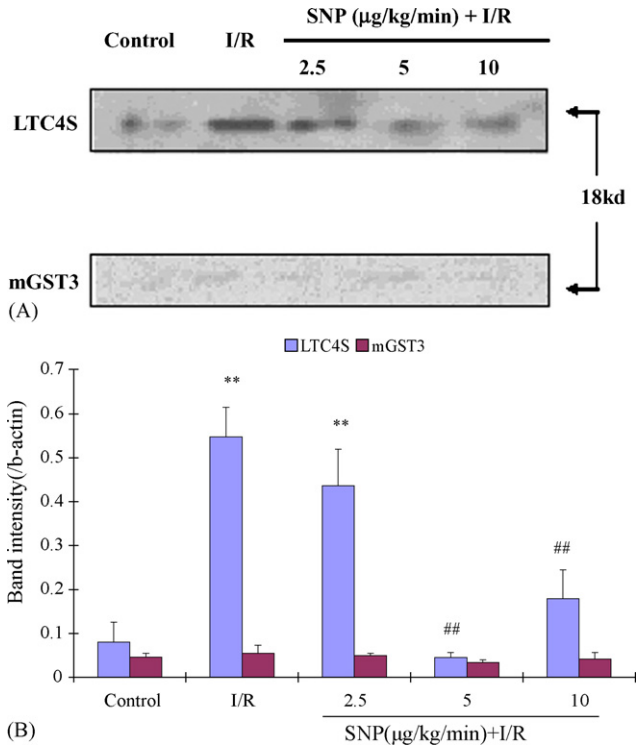

Fig. 4 – Hepatic protein expression of the LTC4 synthesis enzymes in rats after I/R injury. A total of 100 μg of protein was subjected to immunoblot analysis as described in Section 2. (A) Three series of immunoreactive bands corresponding to LTC4S, and mGST3. (B) A histogram obtained by densitometric analysis of several immunoblots for LTC4S. Integrated density values of each blot were calculated for control and I/R rat liver tissues. Bars depict mean values ± S.E. of three determinations. Compared with control, $^{**}P < 0.01$, Compared with I/R, $^{\#\#}P < 0.01$.

3.5. *The effects of SNP on serum NO formation and hepatic GSH content during hepatic I/R in rats*

The relationships between NO and cysteinyl LTs were previously reported [26–28]. Thus, we measured the oxidation products of NO, nitrite (NO_2^-) in serum. As displayed in Table 1, serum NO_2^- content in I/R and SNP (2.5 μg/kg/min) + I/R groups were higher than that in control group ($P < 0.05$), but there were no significant differences in serum NO_2^- levels between control, SNP (5 and 10 μg/kg/min) + I/R groups; the serum NO_2^- levels in SNP (5 and 10 μg/kg/min) + I/R groups markedly decreased compared to the values of I/R group ($P < 0.05$). In addition, in terms of the alteration of the LTC4S synthesis enzymes' activities, we examined GSH content in liver tissue. Liver tissue GSH content in I/R group was lower than that in control group. Except for SNP (2.5 μg/kg/min) + I/R group, hepatic GSH concentration in SNP (5 and 10 μg/kg/min) + I/R groups was higher than that in I/R group.

3.6. *The effects of SNP on the hepatic mRNA expressions of iNOS and eNOS during hepatic I/R in rats*

To explore whether the enhanced NO formation during hepatic I/R injury was relevant to iNOS or eNOS expression alteration, and whether SNP treatment influenced NO formation by its regulating the expressions of NOS, we

Table 1 – Nitrite level in Serum and GSH level of liver tissue from Control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats ($n = 6$)

Groups	Liver GSH (mg/g prot)	Serum nitrite (μmol/L)
Control	98.06 ± 6.89	6.07±1.46
I/R	74.0 ± 6.96[a]	10.5 ± 1.62[a]
SNP (2.5 μg/kg/min)	84.61 ± 4.21	10.23 ± 1.67[a]
SNP (5 μg/kg/min)	91.12 ± 2.41[b]	6.38 ± 1.23[b]
SNP (10 μg/kg/min)	94.09 ± 3.29[b]	7.80 ± 1.13[b]

Compared with control.
[a] $P < 0.05$; compared with I/R.
[b] $P < 0.05$.

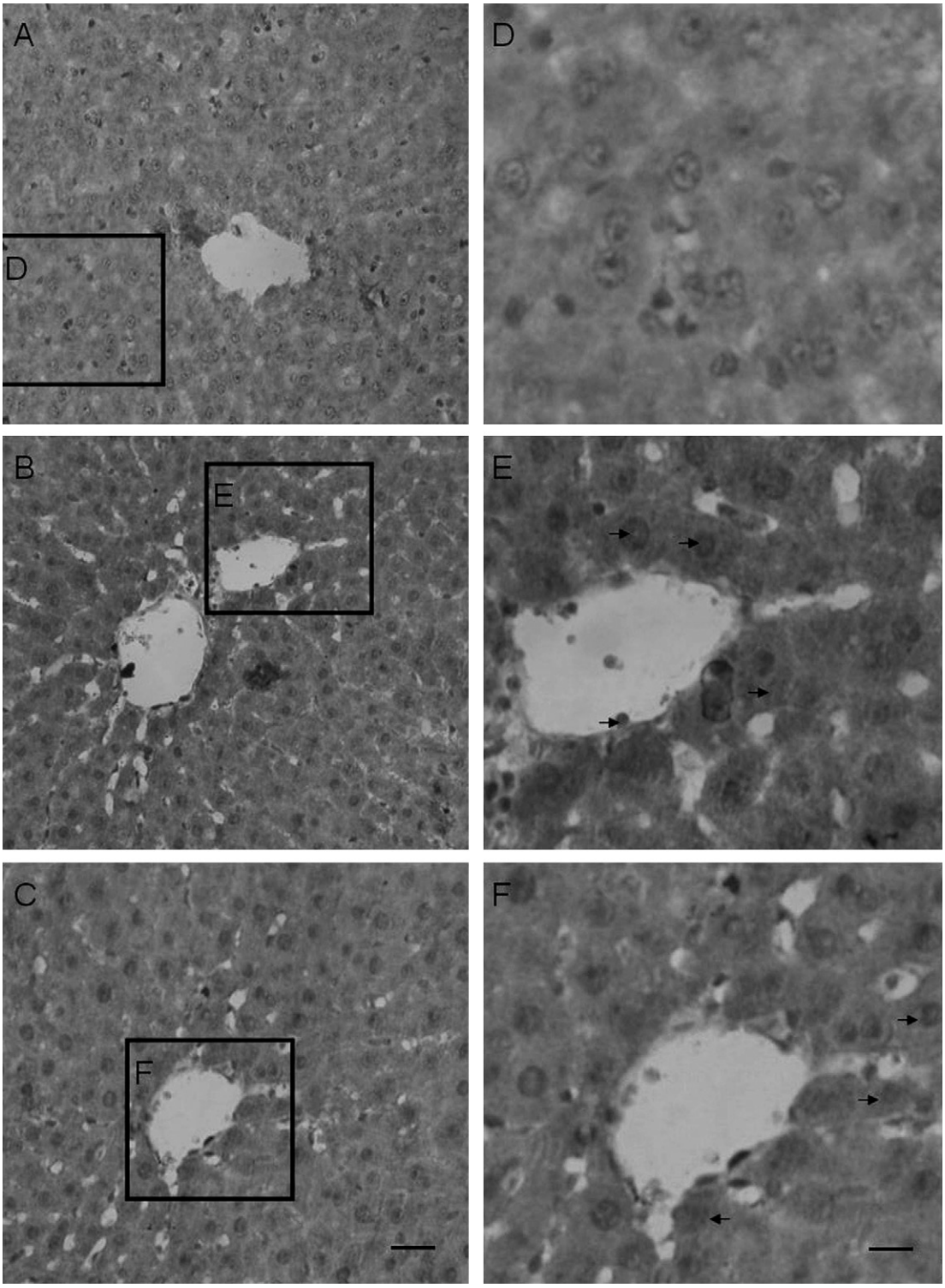

Fig. 5 – Immunohistochemical Localization of LTC4S in control, I/R and SNP (5 μg/kg/min) + I/R rat liver tissue. Immunohistochemical staining in paraffin-embedded liver sections from control, I/R and SNP (5 μg/kg/min) + I/R rats was examined as described in Section 2. (A and D) Immunohistochemical staining for LTC4S in normal rat liver tissues; (B and E) immunohistochemical staining for LTC4S in I/R rat liver tissues (arrows); (C and F) immunohistochemical staining for LTC4S in SNP (5 μg/kg/min) + I/R rat liver tissues (arrows) (Bar = 100 μm (A–C), 50 μm (D–F).

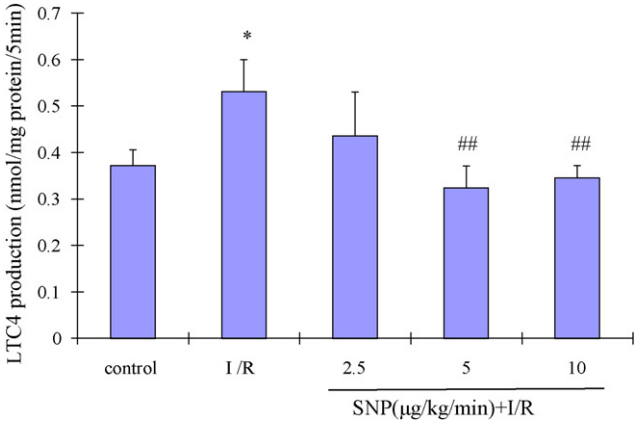

Fig. 6 – LTC4 synthase activity of liver microsomes in Control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. Rats were subjected to hepatic ischemia 60 min and 5 h reperfusion injury as described in Section 2. Microsomes fractions in Control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R groups were incubated with LTA4 (40 μmol/L) and glutathione (10 mmol/L) in the presence of 5 mg/mL BSA at 25 °C for 5 min. Level of LTC4 was determined by RP-HPLC. Each value represents the mean obtained from duplicate experiments with liver tissue from six rats in all groups. Error bars indicate S.E. Compared with control, $^{*}P < 0.05$, compared with I/R, $^{\#\#}P < 0.01$.

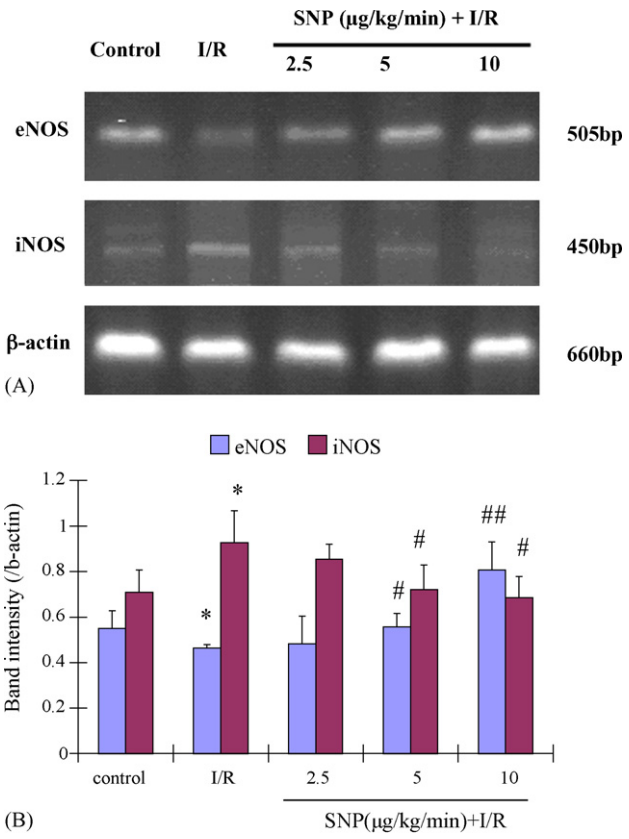

Fig. 7 – The hepatic mRNA expressions of eNOS and iNOS in the control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. (A) A representative of hepatic mRNA expressions of eNOS and iNOS in control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. Single-stranded cDNA synthesized from total RNA (1 μg) was used for PCR amplification with optimal cycles for eNOS, iNOS and β-actin. The PCR products were electrophoresed on 1.5% agarose gel. (B) Densitometric analysis of PCR products of eNOS and iNOS in the control, I/R and SNP (2.5, 5 and 10 μg/kg/min) + I/R rats. The intensity of each band was normalized to that of the corresponding band of β-actin and calculated as the ratio to the value in the control. Values represent the mean ± S.E. ($n = 3$). Compared with Control, $^{*}P < 0.05$; compared with I/R, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

evaluated the hepatic mRNA expressions of iNOS and eNOS after 5 h reperfusion in rats. Our results showed that iNOS mRNA was higher but eNOS mRNA was lower in I/R group than that in control group ($P < 0.05$); expect for SNP (2.5 μg/kg/min) + I/R group, the iNOS mRNA decrease and eNOS mRNA increase in SNP (5 and 10 μg/kg/min) + I/R groups were significant when compared with I/R group ($P < 0.05$) (see Fig. 7).

3.7. *The effects of SNP on the time course of serum ALT and AST levels during hepatic I/R in rats*

As shown in Fig. 8, in the time point of 1, 3 and 5 h hepatic reperfusion SNP (5 μg/kg/min) markedly decreased the levels of serum ALT (Fig. 8A) and AST (Fig. 8B) ($P < 0.01$), but there were no significant differences of ALT or AST levels presented in control, I/R and SNP (5 μg/kg/min) + I/R groups during hepatic ischemia alone ($P > 0.05$; $n = 6$). This demonstrated that present model of hepatic ischemia 60 min following 5 h reperfusion caused a severe damage to liver function whereas NO donor, SNP protected liver from I/R injury.

3.8. *Histological findings*

In the liver, structure damage and some necrosis were observed after 5 h reperfusion, the rat liver tissue in SNP (5 μg/kg/min) + I/R group displayed little structure damage and the control showed normal architecture of the liver (Fig. 9).

4. Discussion

Research over the past 20 years has identified endogenous NO as a key messenger molecule in the cardiovascular, nervous and immune systems [35]. However, the role of NO in hepatic I/R injury is still contradictory [10,11]. LTs are ubiquitous mediators of a wide variety of physiologic and immunologic effects in liver function and disease [36–38]. A growing body of evidences implicates LTs in the pathogenesis of the hepatic I/R injury [21,25,39]. There were some reports connected cysteinyl LTs with NO [26–28]. However, to our knowledge, no studies associated LTs with NO in hepatic I/R injury model, the effects of NO on the protein expressions and activities of the LTC4 synthesis enzymes as well as LTC4 generation during hepatic I/R injury are largely unclear.

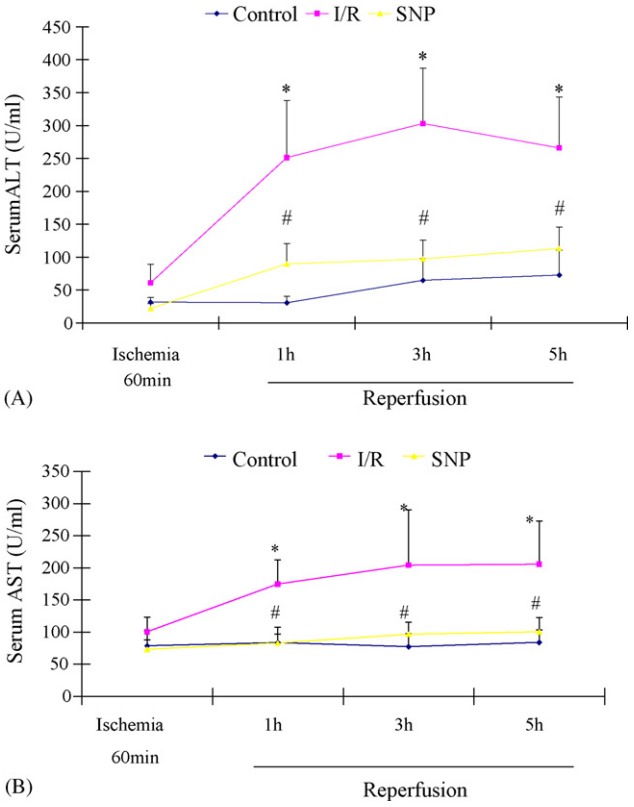

Fig. 8 – Time course of serum ALT and AST levels in control, I/R and SNP (5 μg/kg/min) + I/R rats. (A and B) The level of serum ALT and AST during hepatic I/R injury, respectively. Each value represents the mean obtained from duplicate experiments with serum from six rats in all groups. Error bars indicate S.E. Compared with control, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with I/R, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

In the present study, increased LTC4 production in I/R rat liver tissue (Figs. 2 and 3) agreed with previous reports [25,37,38]. In addition, we observed that during hepatic I/R injury NO donor SNP (5 and 10 μg/kg/min) reduced LTC4 production obviously in rat liver.

LTC4S mRNA was detected in whole liver, hepatocytes, and sinusoidal endothelial cells, but not in Kupffer cells, LPS increased LTC4S expression in hepatocytes, and LTC4S enzyme activity in the microsomal fraction of hepatocytes was also increased [31]. Recent experiments indicated there were no significant changes in the mRNA expression of LTC4S in liver tissues after 3, 12 and 24 h reperfusion [25]. In order to elucidate the mechanism of SNP decreasing LTC4 production during hepatic I/R injury, we investigated the effects of SNP on the protein expressions of the LTC4 synthesis enzymes. Up to now, the investigation that studied the influences of NO on the expressions of the LTC4 synthesis enzymes has not been found in hepatic I/R injury rats. This study first demonstrated that SNP (5 and 10 μg/kg/min) injection down-regulated LTC4S protein expression, but did not affect the protein levels of mGST3 and mGST2 (data not shown) during hepatic I/R injury. In addition, immunohistochemical staining revealed that LTC4S was strongly expressed in endothelial cells and most hepatocytes of I/R rat liver, but SNP (5 μg/kg/min) + I/R rat liver only displayed a slight staining for LTC4S in few endothelial cells and hepatocytes. It suggested that during hepatic I/R SNP reducing LTC4 generation may be due to its down-regulating the protein expressions of LTC4S rather than mGST3 or mGST2 expressions.

The LTC4 synthesis enzymes, including LTC4S, mGST2 and mGST3, are capable of conjugating LTA4 and glutathione to generated LTC4, and all expressed in rat liver at both the mRNA and protein levels [29]. To explore whether declined LTC4 production by SNP injection related to the decrease of the LTC4 synthesis enzymes' activities, we examined it and observed that SNP (5 and 10 μg/kg/min) treatment reduced obviously the LTC4 synthesis enzymes' activities of liver microsomal part in rats during hepatic I/R. It demonstrated that declined LTC4 generation by SNP injection during hepatic I/R be because of its repression to the LTC4 synthesis enzymes' activities.

It was reported that mGST2 has a high activity for formation of LTC4 from LTA4 and mGST3 possesses less activity to form LTC4 [16]. However, to this day the relative contributions of mGST2, mGST3 and LTC4S to cysteinyl LTs formation in the liver have not been fully known. The present data show SNP injection significantly decreased the leukotriene C4 synthesis enzymes' activities in microsomal fraction of liver subjected to I/R, in terms of the above results of the protein expressions and immunohistochemistry of the LTC4 synthesis enzymes, the declined LTC4 synthesis enzymes' activities by SNP treatment may be primarily resulted from its repressing LTC4S rather than mGST3 or mGST2 in rats. This conclusion supported that the LTC4 synthase is predominant in vivo source of cysteinyl LT synthesis [40].

To elucidate whether these effects of SNP on LTC4 generation were associated with NOS and NO productions, we detected the SNP action on NO formation and the mRNA expressions of iNOS and eNOS. We observed that during hepatic I/R except that SNP (2.5 μg/kg/min) could not enfluence serum NO_2^- levels and NOS, SNP (5 and 10 μg/kg/min) markedly decreased serum NO_2^- levels and iNOS mRNA expression, but increased eNOS mRNA level in rats. It suggest that exogenous NO donor SNP could regulate NO production dose-dependently by inhibiting iNOS excessive expression and enhancing eNOS expression in hepatic I/R injury rats, then the suitable production of NO because of SNP injection presented its effects on LTC4 generation and the LTC4 synthesis enzymes.

NO exhibitis a remarkably broad spectrum of functions, including roles in neurotransmission and memory formation, prevention of blood clotting, regulation of blood pressure, and mediation of bactericidal and tumoricidal activity of macrophages [41]. In the early phase of the reperfusion period, SNP injection increased hepatic microcirculation and reduced hepatocyte damage, suggesting that the administration of SNP may help protect ischemia-reperfusion injuries [42]. The addition of NO via the infusion of sodium nitroprusside (SNP, 0.05 mM, 1 mM) reduced the effect of leukotriene D4 on portal flow, bile flow and bile acid secretion [27]. Endogenous, but not exogenous, NO decreases P-selectin and ICAM-1 mRNA expression, thereby reducing polymorphonuclear neutrophil-dependent reperfusion tissue injury [43]. In the present studies, appropriate NO production by SNP injection could

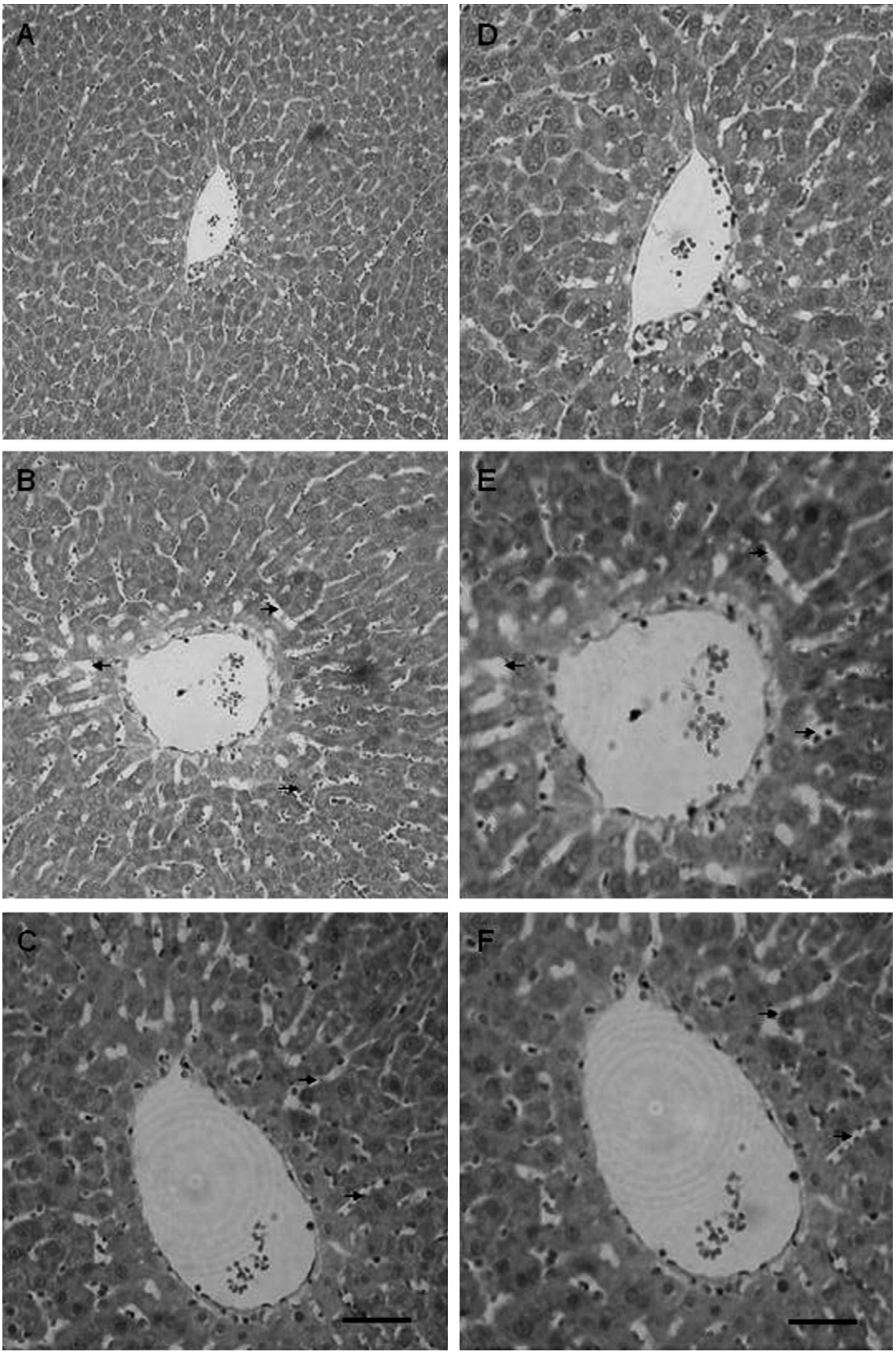

Fig. 9 – Histology of the liver in the control, I/R and SNP (5 μg/kg/min) + I/R rats after 5 h reperfusion following 60 min of hepatic ischemia. The control (A and D) showed normal architecture of the liver, the I/R group (B and E) showed some structure damage and necrosis (arrows), and the SNP (5 μg/kg/min) + I/R group (C and F) exhibited little structure damage and necrosis in the liver (arrows) (bar = 100 μm (A–C), 50 μm (D–F).

reduce polymorphonuclear neutrophil and platelets accumulation, resulting in the decrease in 5-LO and LTC4S expression. In addition, our immunohistochemical results was shown that most hepatocytes and sinusoidal endothelial cells in I/R liver intensively expressed LTC4S, but LTC4S only slightly expressed in few hepatocytes and sinusoidal endothelial cells in SNP + I/R rats. Moreover, the LTC4 synthesis enzymes' activities were repressed by SNP injection in our experiments. Thus, decreased LTC4 synthesis enzymes' activities fail to induce LTA4 to synthesize LTC4 promptly, then reducing LTC4 synthesis in liver after 5 h reperfusion in SNP treated rats. On the other hand, increased GSH level in SNP + I/R groups further manifested that GSH used for LTC4 synthesis by weakened LTC4 synthesis enzymes might be declined (Table 1).

The liver has emerged as the major organ participating in the degradation and elimination of arachidonate products of systemic origin [18]. Cysteinyl LTs and LTB4 are degradated by ω-oxidation and β-oxidation in the liver and this degradation pathway is modulated under several conditions such as liver cirrhosis and hepatorenal syndrome [25,44]. In the isolated perfused rat liver, hypoxia did not influence the uptake of ^{3}H-LTC4 and ^{3}H-LTE4 but biliary elimination was reduced and the metabolite pattern in bile was also significantly changed with a decrease of omega-oxidation products. Following reoxygenation larger amounts of LTs were excreted from the liver into the bile [45]. In this study, we did not examine these possibilities, but raised LTC4 content accompanied by serum ALT, AST increase, liver tissue GSH decrease as well as histological damage, it suggests that LTC4 might be involved in hepatic I/R injury, and hepatic I/R could cause some modifications to the mechanisms of degradation and/or elimination of LTs. This was partly supported by previous report in which LTs may be involved in the direct damage of liver cells under pathological conditions associated with enhanced LTs formation [45]. SNP (5 μg/kg/min) injection reduced serum ALT and AST levels during hepatic reperfusion, diminished histological damage, and increased liver tissue GSH level, suggesting that exogenous NO donor SNP could improve liver function and structure and antagonize oxidation stress state. Thus, during hepatic I/R injury SNP may decrease LTC4 accumulation by modifying the liver function of excreting LTC4 in rats.

Our results demonstrated that during I/R injury SNP reduced the activities of the LTC4 synthesis enzymes, down-regulated the protein expression of LTC4S and decreased hepatic LTC4 levels, but did not affect hepatic protein levels of mGST3 and mGST2 (data not shown) in rats. It suggest that declined LTC4 by SNP treatment during hepatic I/R could be partially resulted from SNP down-regulating protein expression of LTC4S rather than mGST3 or mGST3 and its inhibiting the LTC4 synthesis enzymes' activities, as well as its improvement to damaged liver function and histological structure caused by hepatic I/R injury. These effects of SNP may be relevant to its regulation of iNOS and iNOS expressions and NO production.

Acknowledgements

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