V-PYRRO/NO downregulates mRNA expression levels of leukotriene C4 synthase during hepatic ischemia reperfusion injury in rats via inhibition of the nuclear factor-κB activation pathway

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Abstract. The aim of the present study was to explore the mechanism underlying the effects of a selective liver nitric oxide (NO) donor, O^2-vinyl1-(pyrrolidin-1-yl)-diazen-1-ium-1,2-diolate (V-PYRRO/NO), on the gene expression of leukotriene C4 synthase (*LTC4S*) during hepatic ischemia/reperfusion (I/R). Adult male Sprague-Dawley rats were divided into 3 groups: Sham (control), I/R and V-PYRRO/NO + I/R groups. The liver was subjected to 1 h of partial hepatic ischemia followed by 5 h of reperfusion, saline or V-PYRRO/NO (1.06 μmol/kg/h) administered intravenously. The mRNA expression levels of *LTC4S* in rat liver tissue were examined by the reverse transcription-polymerase chain reaction method, the protein expression levels of nuclear factor-κB (NF-κB) p65, p50 and IκBα in liver cell lysates and nuclear extracts were detected by western blot analysis. Hepatic mRNA expression of *LTC4S* was lower in V-PYRRO/NO + I/R group compared to the I/R group. In addition, the protein expression levels of NF-κB p65 and p50 in the nucleus extract were lower in the V-PYRRO/NO + I/R group when compared with the I/R group. However, the IκBα protein in the 3 groups was not changed. Immunohistochemistry staining revealed that the I/R liver exhibited strong cytoplasmic and nuclear staining for NF-κB p65; however, the V-PYRRO/NO + I/R group liver presented slight cytoplasmic and nuclear staining. In conclusion, V-PYRRO/NO may downregulate *LTC4S* mRNA expression by inhibiting NF-κB activation independent of IκBα during hepatic I/R injury.

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Introduction

Hepatic ischemia/reperfusion (I/R) injury has been indicated in the pathogenesis of a variety of clinical conditions, including trauma, reconstructive vascular surgery, liver transplantation and liver resection surgery (1-4). Accumulating evidence has shown that cysteinyl leukotrienes (LTs) were associated with hepatic I/R injury. LTC4 synthesis enzymes, including leukotriene C4 synthase (LTC4S), microsomal glutathione S-transferase (mGST) 2 and mGST3, can conjugate LTA4 and reduce glutathione to form LTC4, which is the first synthesis step of the cysteinyl LTs, LTC4, LTD4 and LTE4. A pivotal inflammatory transcription factor, nuclear factor-κB (NF-κB), appears to have a central role in the cascade of inflammatory mediators induced during I/R injury (5). NF-κB activation has been shown to occur in models of warm and cold I/R injury. LPS downregulates cysteinyl LT release and LTC4 synthase gene expression in mononuclear phagocytes by an NF-κB-mediated mechanism (6). Nitric oxide (NO) is enzymatically synthesized from L-arginine by three known NO synthase (NOS) isoforms: Constitutively expressed endothelial NOS, neuronal NOS and the inducible NOS (iNOS) (7,8). The association between cysteinyl LTs and NO has been shown in previous studies (9-11). When cells were stimulated with a combination of cytokines or with interleukin-1, LTB4 decreased hepatocyte NO synthesis in a concentration-dependent manner (9). Reduced synthesis of NO_2^- 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*. Numerous studies have suggested that NO is associated with NF-κB in hepatic I/R injury (12-17). Our previous study has suggested that the NO donor sodium nitroprusside (SNP) downregulated the mRNA expression of *LTC4S* by inhibiting NF-κB activation in an IκBα-independent manner (12). Recently, we reported that a selective liver NO

Table I. Oligonucleotide primer used for the analysis of *LTC4S* and β-actin genes by RT-PCR.

Genes	Sense and antisense	PCR product, bp	PCR cycles	Annealing temperature˚C
LTC4S	5'-CGAGTACTTTCCGCTGTTC-3' 5'-TAGTGTGCCAGGGAGGAAG-3'	237	35	55.8
β-actin	5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3' 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'	660	25	58.0

RT-PCR, reverse transcription-polymerase chain reaction; *LTC4S*, leukotriene C4 synthase.

donor, O^2-vinyl1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO), downregulated the mRNA expression of *LTC4S* (18). However, whether the underlying influence on *LTC4S* mRNA expression levels is involved in NF-κB activation remains to be elucidated.

Materials and methods

Materials. In total, 18 male Sprague-Dawley rats, weighing 230-250 g, were obtained from the Experimental Animal Center, Nanchang University (Nanchang, China). V-PYRRO/NO was purchased from Cayman Chemical Company, Inc. (Ann Arbor, MI, USA). TRIzol reagent and MmuLV reverse transcription (RT) were from Gibco-BRL (Gaithersburg, MD, USA), and reduced glutathione and Taq DNA polymerase were from Sangon Biotech Co., Ltd. (Shanghai, China). cDNA probes for rat *LTC4S* were synthesized by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). NF-κB p50, IκBα and β-actin rabbit polyclonal antibodies together with NF-κB p65 mouse monoclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The enhanced chemiluminescence detection kit for horseradish peroxidase (HRP) was from Biological Industries (Biological Industries, Kibbutz Beit-Haemek, Israel). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Billerica, MA, USA). The Polymer Detection system for immunohistological staining, DAB kit, HRP-linked goat anti-rabbit (#ZB-2301) and goat anti-mouse antibody (#ZB-2305) were from Zhongshan Biological Co. (Beijing, China). All other chemicals were of the highest purity commercially available.

Animal model of hepatic I/R injury. The rats were housed and treated in accordance with the Guidelines for the Care and Use of the Experimental Animals Center of Nanchang University (Nanchang, 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 surgery, and were randomized into 3 groups consisting of 6 animals. In the I/R group, animals were anesthetized with 50 mg/kg pentobarbital intraperitoneally, the external jugular vein catheter was created using a polyethylene tube of 0.9 mm inner diameter (BD Biosciences Medical Devices Co. Ltd., Suzhou, China) and was 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 for 5 h. Saline solution (3 ml/kg/min) was intravenously injected by external jugular vein at 15 min before the start of ischemia through 5 h reperfusion. In the sham group (control), surgeries were performed on anesthetized rats in which hepatic blood flow was not occluded. In the V-PYRRO/NO (1.06 μmol/kg/h) + I/R group, surgeries were performed on anesthetized rat as for the I/R group, and V-PYRRO/NO (1.06 μmol/kg/h) was intravenously injected through the external jugular vein catheter using a micro-injector (19) at 15 min before the start of ischemia through 5 h reperfusion, respectively. Following 5 h of reperfusion, the livers were removed, medium lobe fixed in 10% formalin for immunohistochemistry, and the left lobule was snap frozen in liquid nitrogen and subsequently stored at -80˚C for RNA determination and western blot analysis.

RT-polymerase chain reaction (PCR). The mRNA expression levels of *LTC4S* were detected as described in our previous studies (2,10,11). Briefly, total RNA was isolated from whole liver tissue using TRIzol reagent, according to the manufacturer's protocol, and quantified by measurement of ultraviolet absorption at 260 nm. A total of 1 μg of total RNA from each sample was RT to synthesize the single-stranded cDNA using an antisense specific primer and 200 units of MmuLV RT (Gibco-BRL). Sequences of the PCR primers for rat β-actin and *LTC4S* were derived from published sequences (10,11) (Table I). Aliquots of the synthesized cDNA (1.5 μl) were amplified with a proper cycle using each primer and 1.5 units of Taq DNA polymerase in a Mastercycler gradient (Eppendorf, Hamburg, Germany). The reactants were cycled at 95˚C for 45 sec, 55.8/58˚C for 45 sec and 72˚C for 45 sec. The PCR products were separated by electrophoresis using a 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, Berkeley, CA, USA) 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.

Western blot analysis. The protein expression levels of NF-κB p50, p65 or IκBα were performed as described in our previous study (12). Deep-frozen liver samples were lysed in 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 7.5), 1% NP-40, 0.25 deoxycholate, 0.1% SDS supplemented with the protease inhibitor phenylmethanesulfonyl fluoride, pepstatin, leupeptin and aprotinin. The protein concentration was determined as described by Lowry *et al* (20). Nuclear extracts were prepared

from liver tissue as described by Deryckere and Gannon (21). Equal amounts of liver lysates (100 μg) or nuclear extracts (50 μg) were loaded on an SDS-PAGE gel (12%), and electroblotted onto PVDF membranes. The transfer efficiency was visualized using prestained molecular weight protein standards (Fermentas, Sangon). Membranes were subsequently soaked for 1 h at 25˚C in 5% (w/v) non-fat dried milk. The PVDF membranes were subsequently incubated overnight at 4˚C with specific rat polyclonal or monoclonal antibodies raised against a peptide of human NF-κB p50 (#SC-114), p65 (#SC-8008) or IκBα (#SC-371) and β-actin (#SC-1616), used at dilutions of 1:500 or 1:1,000. After washing, the blot was incubated for 1 h at 25˚C with a HRP-linked goat anti-rabbit or goat anti-mouse antibody (1:5,000 dilution) in 0.1% phosphate-buffer solution with Tween-20 and 5% (w/v) non-fat dried milk. The washing steps were repeated and subsequently enhanced chemiluminescence detection was performed according to the manufacturer's protocols (Biological Industries).

Immunohistochemistry. The indirect immunoperoxidase method was used to localize NF-κB p65 in paraffin-embedded sections from the control, I/R and V-PYRRO/NO + I/R group rats and was performed using the Polymer Detection System for immunohistological staining and DAB kit (Zhongshan Biological Co.), according to the manufacturer's protocols. 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. Following antigen retrieval, the sections were blocked for nonspecific binding of the antibody with phosphate-buffered saline (PBS) containing 10% normal calf serum for 30 min and subsequently incubated overnight at 4˚C with mouse NF-κB p65 monoclonal antibody (Santa Cruz Biotechnology, Inc.) at a dilution of 1:100 in 0.5% bovine serum albumin in PBS. After three washes with PBS, the sections were incubated for 1 h in a solution containing goat anti-mouse immunoglobulin G-HRP polymer. The sections were washed, stained with diaminobenzidine and counterstained with hematoxylin.

Statistical analysis. Data are expressed as mean ± standard deviation. Kruskal-Wallis test was used to compare the 3 groups. The Student's t-test was used for the comparison of two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

RT-PCR analysis of hepatic mRNA expression levels of LTC4S in the control, I/R and V-PYRRO/NO + I/R group rats. A representation of the hepatic mRNA expression levels of *LTC4S* is shown in Fig. 1A and B, exhibited as densitometric analysis of the *LTC4S* PCR products in the control, I/R and V-PYRRO/NO (1.06 μmol/kg/h) + I/R group rats. The mRNA expression of *LTC4S* in the I/R group was significantly higher compared with the control groups (P<0.05). Compared with the I/R group, the mRNA expression of *LTC4S* in the liver tissue was significantly decreased after 5 h reperfusion in the V-PYRRO/NO (1.06 μmol/kg/h) + I/R group (P<0.05).

Immunoblot analysis of hepatic protein expression of NF-κB p-50, p-65 and IκB in control, I/R and V-PYRRO/NO + I/R group rats. NO was demonstrated to be associated with NF-κB in hepatic I/R injury (12-17). The present study examined the protein expression levels of NF-κB p-50, p-65 and IκBα in nuclear extracts and whole liver lysates with western blot analysis. As indicated in Fig. 2, the nuclear NF-κB p65 and p50 protein expression levels in the I/R group were significantly increased compared to the control group, whereas the

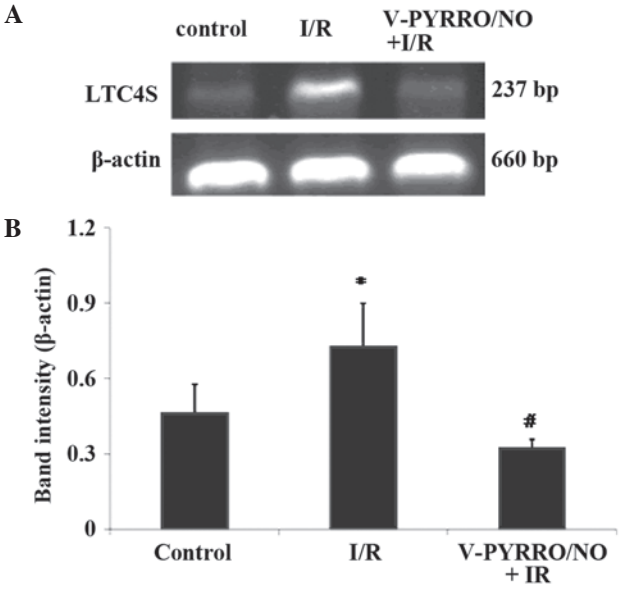

Figure 1. Hepatic mRNA expression of leukotriene C4 synthase (*LTC4S*) in the control, ischemia/reperfusion (I/R) and O^2-vinyl1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolate (V-PYRRO/NO) + I/R groups rats. (A) A representation of the hepatic mRNA expression of *LTC4S* in the control, I/R and V-PYRRO/NO + I/R group rats. (B) Densitometric analysis of polymerase chain reaction products of *LTC4S* in the control, I/R and V-PYRRO/NO + I/R group 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 ± standard deviation (n=3). *P<0.05 compared with control, #P<0.05 compared with I/R.

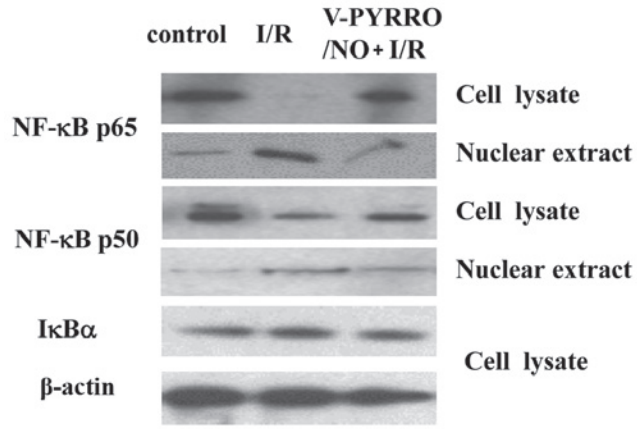

Figure 2. Protein levels of nuclear factor-κB (NF-κB) subunits and IκBα in the rat livers in the control, ischemia/reperfusion (I/R) and O^2-vinyl1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolate (V-PYRRO/NO) + I/R groups. Livers were subjected to 60 min of ischemia and 5 h of reperfusion. Nuclear extracts and whole liver lysates were prepared as described in the Materials and methods and equal amounts were loaded onto SDS-PAGE. Western blot analysis of NF-κB p-50, p-65 and IκBα was performed as described in the Materials and methods. The image is one representative blot from two completely independent experiments with almost equal results.

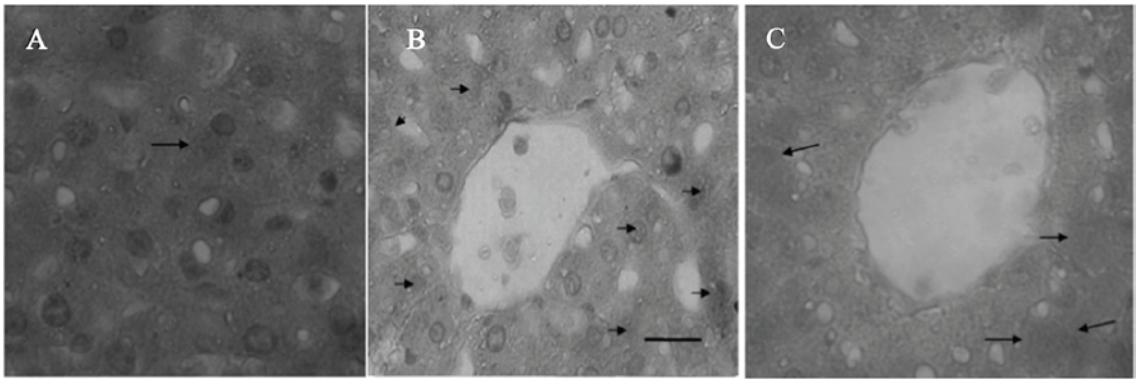

Figure 3. Cytoplasmic and nuclear staining for nuclear factor-κB (NF-κB) p65 in the control, ischemia/reperfusion (I/R) and O^2-vinyl1-(pyrrolidin-1-yl) diazen-1-ium-1,2-diolate (V-PYRRO/NO) + I/R group rat livers. The immunohistochemical staining in the paraffin-embedded liver sections was performed as described in the Materials and methods. (A) Immunohistochemical staining for NF-κB p65 in the (A) normal liver with slight cytoplasmic and nuclei staining; (B) I/R liver tissues with strong cytoplasmic and nuclei staining (arrows); and (C) V-PYRRO/NO + I/R rat liver tissues with slight cytoplasmic and nuclei staining (arrows). Bar, 50 μm.

protein levels of cytoplasmic NF-κB p65 and p50 in the I/R group were markedly lower compared to in the control group; V-PYRRO/NO (1.06 μmol/kg/h) decreased the protein levels of NF-κB p65 and p50 in the nuclear extracts while it increased the protein levels of NF-κB p65 and p50 in the liver lysates during hepatic I/R in rats. However, there was no difference in the IκBα protein level in all the groups.

Immunohistochemical staining for NF-κB p65 in the liver sections in the control, I/R and V-PYRRO/NO + I/R group rats. To further examine NF-κB translocation in rat liver tissue, immunohistochemical staining was performed for NF-κB p65 to detect the cytoplasmic and nuclear staining in paraffin-embedded liver sections from the control, I/R and V-PYRRO/NO (1.06 μmol/kg/h) + I/R group rats. The cytoplasmic and nuclei staining for NF-κB p65 was slight in the normal (Fig. 3A) and V-PYRRO/NO (1.06 μmol/kg/h) + I/R (Fig. 3C) group liver tissues, and was strong in the I/R liver tissues (Fig. 3B).

Discussion

Numerous studies have indicated LTs in the pathogenesis of the hepatic I/R injury (2,22,23). The biosynthesis of cysteinyl LTs (LTC4, LTD4 and LTE4) is catalyzed by LTC4S, mGST2 and mGST3 (24,25). A previous study demonstrated that *LTC4S* mRNA was detected in whole liver, hepatocytes and sinusoidal endothelial cells, but not in Kupffer cells (26). Endogenous NO has also been identified as a key messenger molecule in the cardiovascular, nervous and immune systems (27). Our previous study and others studies have reported the association that exists between cysteinyl LTs and NO (8,10,28,29). The present study further elucidates whether a selective liver NO donor, V-PYRRO/NO, could regulate the gene expression of *LTC4S* in rats. The results revealed that V-PYRRO/NO completely reveresd the upregulation of *LTC4S* gene expression in hepatic I/R rats.

Whether NO can activate the NF-κB signaling pathway remains to be elucidated (7). LPS has been reported to downregulate cysteinyl LT release and *LTC4S* gene expression in mononuclear phagocytes by an NF-κB-mediated mechanism (6). The major pathway for NF-κB activation is well known to depend on the activation of the IκK complex, which leads to the phosphorylation of serine residues of IκB and the degradation of IκB via the ubiquitin-proteasome system (30). Our previous study suggested that SNP downregulated the mRNA expression of *LTC4S* by inhibiting NF-κB activation in an IκBα-independent manner (11). In order to investigate whether a selective liver NO donor, V-PYRRO/NO, can regulate the gene expression of *LTC4S* via NF-κB signaling pathway in rats, the protein levels of NF-κB p-50, p-65 and IκBα were examined in nuclear extracts and whole liver lysates with western blotting analysis. V-PYRRO/NO clearly decreased the protein levels of NF-κB p65 and p50 in the nuclear extracts but increased the protein levels of NF-κB p65 and p50 in the liver lysates during hepatic I/R in rats (Fig. 2); but the IκBα protein expression presented no differences in all the groups. To further evaluate the alterations of NF-κB translocation in the liver tissue, immunohistochemical staining was performed for NF-κB p65 to detect the cytoplasmic and nuclear staining in paraffin-embedded liver sections. The data showed slight cytoplasmic and nuclei positive staining for NF-κB p65 in the normal and V-PYRRO/NO + I/R group liver tissues, and the I/R liver tissue exhibited strong cytoplasmic and nuclei positive staining. These results suggest that an exogenous NO donor, V-PYRRO/NO, inhibited the NF-κB activation in a manner independent of IκBα degradation during hepatic I/R injury in rats. Considering the above result of *LTC4S* gene expression levels, V-PYRRO/NO evidently downregulated the mRNA expression of *LTC4S* by inhibiting NF-κB activation independent of IκBα degradation. This result was in accordance with a previous study, which suggested that NF-κB is activated by c-Src dependent tyrosine phosphorylation of IκBα but not IκBβ during I/R injury, and this process occurs in the absence of IκBα ubiquitin-dependent degradation (11,31). However, whether V-PYRRO/NO can regulate *LTC4S* gene expression via NF-κB signaling pathway by c-Src dependent tyrosine phosphorylation of IκBα remains to be elucidated.

In conclusion, the present findings demonstrated that a selective liver NO donor, V-PYRRO/NO, may downregulate the mRNA expression of *LTC4S* by inhibiting NF-κB activation in an IκBα-independent manner.

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