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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Received August 10, 2015; Accepted September 18, 2015

DOI: 10.3892/br.2015.533

Abstract. The aim of the present study was to explore the mechanism underlying the effects of a selective liver nitric oxide (NO) donor, O2-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.

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 cysteinyll 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, LTD4, LTE4 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; 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 donor, O2-vinyl1-(pyrrolidin-1-yl)-diazen-1-ium-1,2-diolate, regulates leukotriene C4 synthase in rat liver cells by targeting NF-κB activation (13).

Key words: nitric oxide donor, O2-vinyl1-(pyrrolidin-1-yl)-diazen-1-ium-1,2-diolate, nuclear factor-κB, leukotriene C4 synthase, ischemia reperfusion injury, liver

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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). NF-κB p50, 65 or IκBα were derived from published sequences (10,11).

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 polymerase in a Mastercycler gradient (Eppendorf, Arbor, MI, USA).

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sense and antisense</th>
<th>PCR product, bp</th>
<th>PCR cycles</th>
<th>Annealing temperature °C</th>
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| LTC4S   | 5'-CGAGTACTTTCGCCGTTC-3'  
5'-TAGTGTGCCAGGAGGAAG-3' | 237 | 35 | 55.8 |
| β-actin | 5'-TGACGGGGGTCAACCACTTGCCCATCTA-3'  
5'-CTAGAAGCATTTGCGGAGCATGAGG-3' | 660 | 25 | 58.0 |

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

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 electrophoresed 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 IkBa (#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 immunohistochemical 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% H2O2 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 IkBa 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 IkBa 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
Discussion

Numerous studies have indicated LTs in the pathogenesis of the hepatic I/R injury (2,22,23). The biosynthesis of cysteinyll 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 cysteinyll 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 reversed 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 IκBβ-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 p56 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 IκB 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 in the control, ischemia/reperfusion (I/R) and SNP + 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).

Figure 3. Cytoplasmic and nuclear staining for nuclear factor-κB (NF-κB) p65 in the control, ischemia/reperfusion (I/R) and O2-vinyl1-(pyrrolidin-1-yl) diazen-i-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.
Acknowledgements

The present study was supported by National Natural Science Foundation of China (grant no. 81260504) and Educational Commission of Jiangxi Province of China (grant no. GJJ12073).

References