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HEPATOLOGY

Expression of inducible nitric oxide synthase in the liver is under the control of nuclear factor kappa B in concanavalin A-induced hepatitis

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concanavalin A, inducible nitric oxide synthase (iNOS), inhibitor of kappa B (IκB), liver, nuclear factor kappa B (NF-κB).

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Abstract

Background and Aim: Both nuclear factor kappa B (NF-κB) activation and inducible nitric oxide synthase (iNOS) expression increase in the liver injury, and there are NF-κB binding sites in the iNOS promoter. The aim of this study was to investigate the correlation between iNOS expression and NF-κB activation in hepatitis induced by concanavalin A (con A).

Methods: Eighty-eight male BALB/c mice were randomly divided into three groups: vehicle control group, con A group and pyrrolidine dithiocarbamate (PDTC) plus con A group. In the vehicle control group, the mice were treated with saline (0.3 mL, i.v.). In the con A group, the mice were treated with con A (20 mg/kg, i.v.). In the PDTC + con A group, the mice were pretreated with PDTC (120 mg/kg, i.p.) 30 min before administration of con A (20 mg/kg, i.v.). Blood samples were taken from the retro-orbital venous plexus at 0.5, 1, 4, 8 and 16 h after con A injection and the mice were killed immediately. The plasma alanine aminotransferase (ALT) levels were measured by the standard photometric method. Nitric oxide (NO) levels in the liver homogenate were assayed by spectroscopy. Liver tissues were sectioned and stained with hematoxylin–eosin for histological examination. Activation of NF-κB, degradation of inhibitor of kappa B alpha (IκBα), and expression of iNOS were measured by western blot.

Results: In the con A group, the plasma ALT activity and NO levels in the liver increased significantly at 1 h ($P < 0.05$, $n = 8$) and reached a peak at 4 h after con A injection. The liver injury in this group was characterized by liver necrosis, cell swelling and fatty degeneration. Cytosolic IκBα decreased slightly at 30 min after con A challenge, was undetectable at 1 h and reappeared at 4 h. Correspondingly, the NF-κB level in the nucleus was highest at 1 h. The iNOS expression increased at 30 min after con A injection and reached a maximum at 4 h. Pretreatment with PDTC prevented these changes and attenuated the liver injury.

Conclusion: Con A-induced iNOS expression in the liver is dependent on the activation of NF-κB.

Introduction

Hepatic disease is one of the major causes of human death. Many kinds of liver diseases, such as viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis and liver allograft rejection, are associated with the activation of T cells that infiltrate and progressively destroy the liver parenchyma. Concanavalin A (con A) is a polyclonal mitogen. Upon injection in mice, it can induce T lymphocyte activation[1] and a cytokine secretion syndrome that leads to organ-specific immune liver injury.[2]

It is reported that the nuclear factor kappa B (NF-κB) family plays a major role in this liver injury. The NF-κB family is one of the important dimeric transcription factor families.[3] It consists of NF-κB1 (P50 and its precursor P105), NF-κB2 (P52 and its precursor P100), RelA (P65), c-Rel (Rel) and RelB. RelA–NF-κB1 (P65–P50) is the most common dimer formed.[4] Several lines of evidence indicate that activation, translocation and binding of NF-κB are pivotal steps in the regulation of immune and proinflammatory cytokine genes.[5] Under resting conditions, NF-κB is sequestered in the cytoplasm through interaction with its inhibitor, IκB. When the cell is activated, the IκB protein is phosphorylated and degraded rapidly. NF-κB then undergoes rapid nuclear translocation and participates in the induction of numerous cellular genes.[6]

Recently, inducible nitric oxide synthase (iNOS) was reported to be expressed in con A-induced hepatitis, and iNOS$^{-/-}$ mice were protected from liver damage after con A treatment.[7] Inducible nitric oxide synthase is an inducible member of the three nitric oxide synthase isoforms (endothelial nitric oxide synthase [eNOS], neural nitric oxide synthase [nNOS] and iNOS). They catalyze the oxidation–reduction reaction of L-arginine in the presence of oxygen to form nitric oxide (NO) and L-citrulline.[8] Nitric oxide is a highly reactive oxidant produced by parenchymal and non-parenchymal liver cells.[9,10] Under normal conditions, only the constitutive eNOS is present in the liver, and low levels of NO regulate hepatic perfusion.[11] Under pathological conditions, however, iNOS is strongly upregulated and large amounts of NO are generated in the liver.

There are NF-κB binding sites in the iNOS promoter.[12] NF-κB is important in the regulation of human-inducible nitric oxide synthase (hiNOS) transcription in A549 human lung epithelial cells.[13] Although both iNOS expression and NF-κB activation are increased during the process of con A-induced liver injury, there is no report about the correlation between them. Therefore, we hypothesized that the expression of iNOS and the subsequent production of NO are dependent on the activation of NF-κB. To test this hypothesis, we compared NF-κB activation, iNOS expression and NO production in the liver after con A injection with or without pyrrolidine dithiocarbamate (PDTC) pretreatment.

Methods

Experimental animals

Male BALB/c mice (aged 6–8 weeks, weight range: 18–22 g) were purchased from the Experimental Animal Center of Chinese Academy of Science. All animals received care in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals (Experimental Animal Center of Nanjing Medical University). All mice were fasted overnight before the experiment.

Reagents

Con A and PDTC were purchased from Sigma (St. Louis, MO, USA). Rabbit antimouse P65 and inhibitor of kappa B alpha (IκBα) antibodies were kindly provided by Dr Chuanfu Li (Department of Surgery, Quillen College of Medicine, Johnson City, TN, USA). Rabbit antimouse iNOS antibody and peroxidase-conjugated goat antirabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Experimental protocols

BALB/c mice were randomly divided into three groups: vehicle control group, con A group, and PDTC + con A group. There were eight animals in each group at each time point. In the con A group, 20 mg/kg con A dissolved in 300 μL pyrogen-free phosphate-buffered saline (PBS)[2] was administered to the mice via the tail vein. The vehicle control mice were injected in the same manner with pyrogen-free PBS. In the PDTC + con A group, PDTC (120 mg/kg) dissolved in 300 μL pyrogen-free PBS[14] was administered intraperitoneally 30 min before con A treatment. Blood samples were taken from the retro-orbital venous plexus under ether anesthesia at 0.5, 1, 4, 8 and 16 h after the injection of con A. The mice were killed and their livers were removed immediately.

Assay for plasma aminotransferase activities

Hepatocyte damage was assessed at 0.5, 1, 4, 8 and 16 h after con A injection by measuring plasma alanine aminotransferase (ALT) activity. Plasma ALT was quantified using a diagnostic assay kit (KeXin Biochemical, Shanghai, China).

Measurement of tissue nitric oxide levels

The mice were killed and NO levels in the livers were measured. Briefly, a part of the liver (0.5 g) from every mouse was homogenized in 2.5 mL PBS and centrifuged at 55 *g* for 5 min. Nitric oxide levels were measured in the supernatants as recommended by the supplier (JianCheng Biochemical, Nanjing, China).

Histology study

The left liver lobe from every mouse was fixed in 4% paraformaldehyde in PBS at room temperature, embedded in paraffin, sectioned and stained with hematoxylin–eosin for histological examination.

Extraction of nuclear and cytoplasmic proteins

About 0.1 g of pulverized liver sample was homogenized in 0.8 mL of ice-cold hypotonic buffer (10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] pH 7.9; 10 mmol/L KCl; 0.1 mmol/L ethylenediamine tetraacetic acid [EDTA]; 0.1 mmol/L ethylene glycol tetraacetic acid [EGTA]; 1 mmol/L dithiothreitol [DTT]; protease inhibitors: 0.5 mmol/L phenylmethylsulphonyl fluoride [PMSF], and aprotinin, pepstatin, leupeptin [10 μg/mL each]; phosphatase inhibitors: 50 mmol/L NaF, 30 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, and 20 mmol/L β-nitrophenyl phosphate). The homogenates were centrifuged for 30 s at 55 *g* at 4°C to eliminate any unbroken tissue. The supernatants were incubated in chopped ice for 20 min, vortexed for 30 s after adding 50 μL of 10% Nonidet P-40 in the hypotonic buffer and then centrifuged at 12 000 *g* for 1 min at 4°C. Supernatants containing cytoplasmic proteins were collected and stored at −80°C. The pellets, after a single wash with the hypotonic buffer without Nonidet P-40, were suspended in an ice-cold hypertonic salt buffer (20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT and protease inhibitors and phosphatase inhibitors), then incubated in chopped ice for 30 min, mixed frequently, finally centrifuged at 10 000 *g* for 15 min at 4°C. The supernatants were collected as nuclear extracts and stored at −80°C. The concentration of total protein in the samples was determined by bicinchoninic acid (BCA) assay kit (Pierce Biotechnology, Rockford, IL, USA).

Western blot analysis

Sixty micrograms of cytoplasmic protein extracts at 0.5, 1, 4, 8 and 16 h after the treatment of con A were resolved in 12% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). After electro-

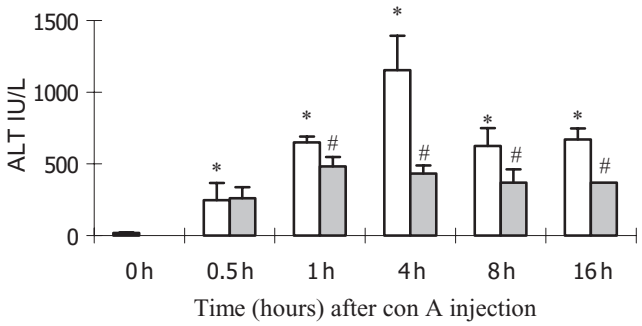

Figure 1 Time-course of the plasma alanine aminotransferase (ALT) concentration in mice of the concanavalin A (con A; □) and pyrrolidine dithiocarbamate + con A (PDTC + con A; ▨) groups. Mice were injected with 20 mg/kg of con A or pretreated with 120 mg/kg PDTC 30 min before con A injection. Plasma ALT was measured at various time points as indicated in the abscissa. Each sample represents a group of eight mice. Data are expressed as the mean ± SEM. *$P < 0.05$, con A group versus vehicle control group. #$P < 0.05$, con A group versus PDTC + con A group.

phoresis, the separated proteins were transferred from the gels onto nitrocellulose membranes and then incubated in blocking solution (5% dry milk in tris-buffered saline [TBS]) for 2 h at room temperature, washed four times in TTBS (TBS, 0.05% Tween-20) and finally incubated with primary IκBα antibody in TTBS overnight at 4°C. The membranes were then washed four times for 10 min each in TTBS and incubated with peroxidase-labeled goat antirabbit IgG for 1 h at room temperature. After three washes with TBS, membrane-bound antibody was visualized with the Amersham (Arlington Heights, IL, USA) enhanced chemiluminescence (ECL) detection reagent or stained with diaminobenzidine-H_2O_2 (DAB-H_2O_2) solution.

Sixty micrograms of nuclear protein extracts at each time point were resolved in 12% SDS-PAGE. The P65 protein levels in the nucleus were measured by the same method.

The levels of iNOS protein in the cytoplasm were assessed using the same method except 10% SDS-PAGE was used instead of 12%.

Statistical analysis

Results were expressed as mean ± SEM. Statistical comparisons were made using the Student–Newman–Keuls' test; $P < 0.05$ was considered to be significant.

Results

Severe hepatic injury in BALB/c mice after con A injection

The ALT levels were measured in plasma at 0.5, 1, 4, 8 and 16 h after the con A injection (Fig. 1). The results showed that the ALT increased rapidly and reached peak level at 4 h, which was significantly higher than that of control mice ($P < 0.05$). Figure 2 shows histological changes in the liver. Four hours after con A injection, liver parenchyma showed spotty necrosis (Fig. 2b). Most hepatocytes showed typical features of cell swelling and fatty degeneration (Fig. 2d).

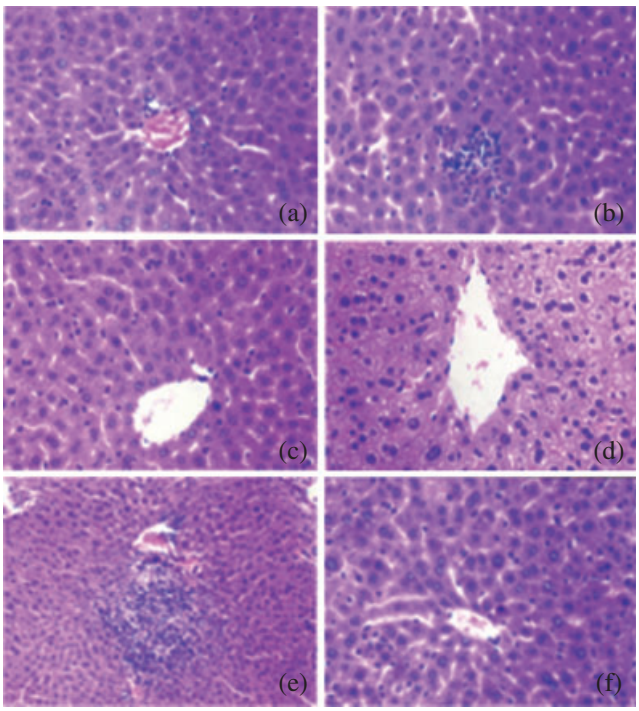

Figure 2 Effects of concanavalin A (con A) administration and pyrrolidine dithiocarbamate (PDTC) pretreatment on liver histology (hematoxylin–eosin staining). (a) The liver histology of control mice (× 400). (b) 4 h after con A was given i.v., spotty necrosis of liver parenchymal cells was observed (× 400). (c) 4 h after con A injection plus PDTC pretreatment, no necrosis was observed (× 400). (d) 8 h after con A was given i.v., cell swellings and fatty degeneration were present (× 400). (e) 8 h after con A was given i.v., spotty necrosis of liver parenchymal cells was observed (× 100). (f) 8 h after con A injection plus PDTC pretreatment, no necrosis, cell swelling or fatty degeneration were observed (× 400).

Pretreatment with PDTC buffered the increase of plasma ALT in mice challenged with con A. At 1, 2, 4 and 8 h after the administration of con A, the ALT in PDTC + con A group was 481.59 ± 77.82, 433.9 ± 67.03, 368.76 ± 54.41 and 368.54 ± 94.42 IU/L, respectively, significantly lower than that of the con A group at the same time points (Fig. 1). Moreover, another sign of alleviation of damage in the liver tissue of the PDTC + con A group was the fact that there was no necrosis in the liver section (Fig. 2f).

Degradation of IκBα and the translocation of P65

The activation and mobilization of NF-κB from the cytoplasm to the nucleus is regulated by IκB.[6] Thus, we studied the degradation of IκBα in the cytoplasm and the translocation of NF-κB to the nucleus at different time points by western blot. The levels of IκBα

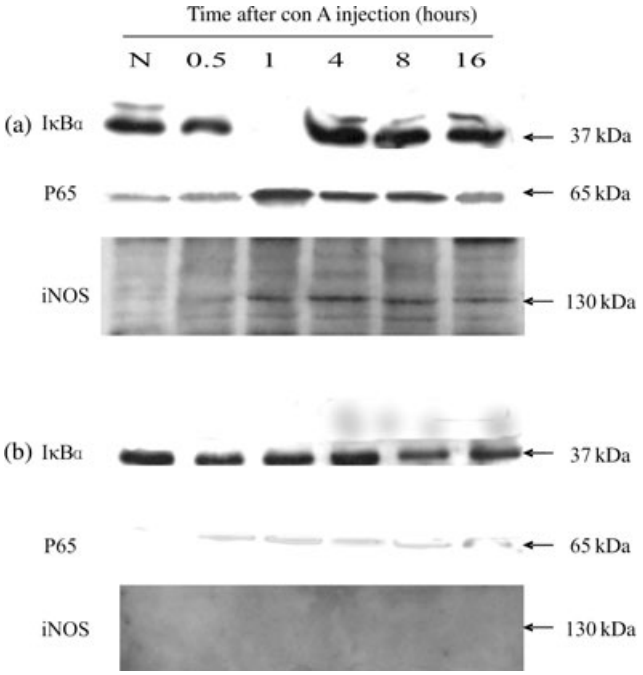

Figure 3 Western blot analysis of inhibitor of kappa B alpha (IκBα), nuclear factor kappa B (NF-κB; P65) and inducible nitric oxide synthase (iNOS) in liver extracts of mice, untreated or pretreated with 120 mg/kg pyrrolidine dithiocarbamate (PDTC) 30 min before challenge with 20 mg/kg concanavalin A (con A). Protein was extracted from livers isolated at the times indicated after con A administration. IκBα and iNOS proteins were analyzed in the cytoplasm and P65 proteins were analyzed in nuclei. (a) Effects of con A administration on IκBα degradation, P65 translocation and iNOS protein expression. (b) Effects of PDTC + con A administration on IκBα degradation, P65 translocation and iNOS protein expression. N, vehicle control group.

detected in vehicle control liver cytosolic extracts were decreased at 30 min after con A challenge, undetectable at 1 h and reappeared at the 4-h time point (Fig. 3a).

Western blot analysis of the nuclear extract showed an increase in nuclear level of P65 at 0.5 h after con A injection and the peak increase at 1 h. Thus, the translocation of NF-κB coincided with the depletion of IκBα. Pretreatment of mice with PDTC significantly prevented the degradation of IκBα in the cytoplasm and reduced the increase of P65 in the nucleus. Therefore, PDTC can prevent NF-κB activation.

Relationship between NF-κB activation and iNOS expression

In the mice of the con A group, the iNOS protein was expressed at 0.5 h after con A treatment and reached the maximal level at 4 h (Fig. 3a). Pretreatment with PDTC completely abolished the expression of iNOS induced by con A (Fig. 3b).

Nitric oxide levels in the liver of the different groups

In the con A group, treatment of con A greatly increased the NO level in the liver. At 4 h after con A administration, the NO level was 0.2617 ± 0.0596 μmol/g protein, significantly higher than the

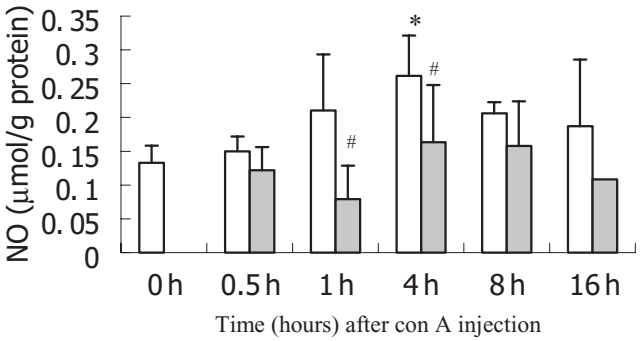

Figure 4 Effects of concanavalin A (con A; □) administration and pyrrolidine dithiocarbamate (PDTC; ▩) pretreatment on nitric oxide generation in the liver at the time points as indicated in the abscissa. Data are expressed as the mean ± SEM. *$P < 0.05$, con A group versus vehicle control group. #$P < 0.05$, con A group versus PDTC + con A group.

NO level prior to the con A administration ($P < 0.05$; Fig. 4). Pretreatment with PDTC significantly decreased the increase in NO induced by con A. At 4 h after con A administration, the NO level in the PDTC + con A group was 0.1635 ± 0.0492 μmol/g protein, significantly lower than that of con A group ($P < 0.05$; Fig. 4).

Discussion

The NF-κB family of transcription factors plays a critical role in cell growth, differentiation, apoptosis and adaptive response to changes in the cellular redox balance.[15] It is reported that NF-κB activation is involved in the pathogenic process of some diseases, including immune liver injury.[6] The acute hepatitis induced by con A is mediated by NF-κB.[16] After con A treatment, iNOS expression increased at 30 min and reached peak level at 4 h. In order to investigate the contribution of NF-κB activation to con A-induced iNOS expression, PDTC, a highly selective inhibitor, was used to block NF-κB activation. PDTC inhibited both the NF-κB translocation and iNOS expression induced by con A. Thus, it is clear that NF-κB is the main regulatory transcription factor in con A-mediated iNOS expression. Furthermore, PDTC decreased the rise of plasma ALT and attenuated the liver injury following treatment with con A. These data were consistent with previous reports by Lauzurica.[14]

There are at least three isoforms of NOS: nNOS, eNOS and iNOS. They differ in function, distribution and regulation, but catalyze the same redox reaction.[17,18] Neural NOS is mainly found in neurons[19] and eNOS is mainly found in the endothelial cells of blood vessels.[20] These two isoforms are constitutively expressed and generate only small amounts of NO. In contrast, iNOS is not constitutively expressed in normal tissues; however, it can be overexpressed and generate large amounts of NO if induced by cytokines, for example, TNF-α and IFNγ.[21,22] Nitric oxide participates in diverse physiological processes, including vasodilation, neurotransmission and non-specific host defenses.[23] Although moderate levels of iNOS-derived NO are principally beneficial, many diseases are caused by overproduction of NO.[24] Nitric oxide can interact with superoxide anion (O_2^-) to form peroxynitrite

($ONOO^-$),[25] a more long-lived cytotoxic oxidant. Both peroxynitrite and other reactive nitrogen species can combine with the iron-sulfur of heme-containing proteins and cause enzyme activation or inhibition.[23] Nitric oxide can also inhibit mitochondrial respiration and DNA synthesis.[23] In this study, the NO levels increased rapidly after the con A injection and reached peak level at 4 h. At 8 h after con A treatment, necrosis was present in liver tissues. Pretreatment with PDTC significantly reduced NO levels and, subsequently, the liver injury. These results indicate that the increase in NO contributes to the development of liver injury after con A treatment.

In conclusion, the present study demonstrates that iNOS expression and overproduction of NO are contributed by the activation of NF-κB induced by con A and overproduction of NO is involved in the pathogenic process of con A-induced acute hepatitis.

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