INHIBITION OF NF-κB ACTIVATION DURING ISCHEMIA REDUCES HEPATIC ISCHEMIA/REPERFUSION INJURY IN RATS

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ABSTRACT — The aim of this study was to determine whether nuclear factor-κB (NF-κB) inhibitors are efficient against hepatic ischemia/reperfusion (I/R) injury. We previously demonstrated that xanthine oxidase-derived reactive oxygen species activate NF-κB during ischemia. However, the role of NF-κB activation during ischemia in post-reperfusion injury remains unclear. Therefore, while we examined the effects of NF-κB inhibitors, sulfasalazine and pyrrolidinedithiocarbamate on hepatic I/R injury using a rat lobar hepatic I/R model, we estimated the relationship between NF-κB activation during ischemia and following hepatic damage caused by reperfusion. The portal vein and the hepatic artery were clamped for 1 hr followed by reperfusion for up to 24 hr. NF-κB activation was determined by Western blot analysis. NF-κB activation was observed in the ischemic lobe of the liver, and the activation was prevented by pre-administration with NF-κB inhibitors. Although the serum ALT level, hepatic MPO activity and BSP clearance, as an index of hepatic injury, were increased after reperfusion, the increase was attenuated by pre-administration with NF-κB inhibitors. These findings suggest that NF-κB activation during ischemia is relevant to hepatic I/R injury. Moreover, we first showed that pre-administration with NF-κB inhibitors is effective against hepatic I/R injury.

KEY WORDS: Ischemia/reperfusion injury, Liver, NF-κB, Sulfasalazine, Pyrrolidinedithiocarbamate

INTRODUCTION

Liver transplantation is one of the most efficacious remedies against end-stage hepatic diseases. The rate of success is increasing due to the development of various factors such as immunosuppressive drugs and preservation solutions. However, the primary non-function or dysfunction of grafts undergoing ischemia/reperfusion (I/R) is also a serious problem (Clavien et al., 1992). Therefore, many studies have attempted to elucidate the mechanisms of I/R-caused hepatic disorders by means of appropriate in vivo models.

It has been demonstrated that hepatic I/R injury occurs from biphasic responses involving the initial phase and subsequent phase (Jaeschke et al., 1990). The event occurring in the initial phase is believed to be characterized by the production of reactive oxygen species (ROS) originating from xanthine oxidase (XOD), whereas the subsequent phase is typified by the accumulation and activation of inflammatory cells. In the initial phase, the post-ischemic organ inflicts hypoxic damage and direct injury to cell membranes through lipid peroxidation by XOD-derived ROS. However, ROS has been thought to cause the generation of both primarily proinflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β) and cytokine-induced neutrophil chemoattractant (CINC) as well as the expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) (Serracino-Inglott et al., 2001). This may be involved in the mechanisms of neutrophil infiltration in ischemic hepatic lobes. In the subsequent phase, the accumulation of neutrophils was markedly observed mainly within 24 hr after reperfusion. The activated neutrophils play a pivotal role in the later phase of hepatic I/R injury (Jaeschke et al., 1990). Thus, ROS production during the initial phase may result in the subsequent phase with serious hepatic inflammation.

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Indeed, Yabe et al. (2001) observed that hepatic disorder due to I/R was diminished by treatment with SOD and/or catalase derivatives with the cancellation of neutrophil chemotaxis. However, the details of the relationship between ROS production during the initial phase and inflammation during the later phase remain unclear.

ROS, especially hydrogen peroxide, is known to activate nuclear factor-κB (NF-κB). The activated NF-κB up-regulates the expression of different genes encoding inflammation-related transcripts such as acute phase proteins, cell adhesion molecules, cell surface receptors, cytokines and chemokines (Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). In a previous report, we demonstrated that both cytokines and chemokines (Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). In a previous report, we demonstrated that both lipid peroxidation and NF-κB activation occur during the warm ischemic period, and found that the pre-administration of allopurinol, a XOD inhibitor (Matsui et al., 2000), prevents all of these incidents. Furthermore, Matsumura et al. reported that pre-treatment with xanthine oxidase inhibitor attenuates both neutrophil accumulation and CINC production following I/R in rat liver (Matsumura et al., 1998). These findings suggest that NF-κB activation during ischemia may be crucial for the construction of late-phase severe inflammation. Therefore, we examined whether treatment with an NF-κB inhibitor is efficient against I/R injury using a rat in vivo model, and considered the role of NF-κB on I/R-induced liver damage.

MATERIALS AND METHODS

Animals

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Male Wistar S/T rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals each weighed 190-250 g and were housed in conventional animal facilities.

Hepatic I/R injury model

Animals were fasted for 12 hr and anesthetized with sodium pentobarbital (50 mg/kg, i.p.: Dainippon Pharmaceutical Co., Ltd.). A rat model of lobar hepatic I/R was performed according to the method previously described (Matsui et al., 2000). Briefly, the abdomen was opened through a midline incision. The portal vein, hepatic artery, and bile duct of the left and middle lobes (ischemic lobes) were occluded with a vascular clamp. The remaining three caudal lobes (control lobes) retained an intact portal and arterial blood supply, in addition to venous outflow, preventing the development of intestinal venous hypertension and the possible leakage of bacteria or bacterial products into the circulation. After 1 hr of liver ischemia, the vascular clamp was removed, the abdomen was closed with silk and the animals were allowed to awaken. Animals were sacrificed at each indicated time after reperfusion for analysis. Sulfasalazine (1-100 mg/kg i.p.: SIGMA-ALDRICH, Inc.) (Frode-Saleh and Calixto, 2000) and pyrrolidinedithiocarbamate (PDTC) (50-200 mg/kg i.p.: SIGMA-ALDRICH, Inc.) (Liu et al., 1999), inhibitors of NF-κB, were administered 1 hr and 3 hr before occlusion, respectively. Each administration time point was set at the time showing the maximal NF-κB inhibition in a preliminary experiment.

NF-κB activation

Samples of the ischemic lobe and the control lobe were obtained from the left lateral lobe and the right lobe of the liver, respectively. Liver tissues were quickly excised and homogenized in ice-cold hypotonic buffer (10 mM HEPES-KOH, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 2 mM phenylmethyl-sulfonly fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, pH 7.8) using a Dounce type homogenizer. After standing in an ice bath for 10 min, the homogenate was centrifuged (400 ×g, 10 min, 4°C) and the supernatant was removed. The precipitate was suspended in ice-cold hypotonic buffer, and then centrifuged (400 ×g, 10 min, 4°C). Finally, the precipitate (nucleus) was suspended in an extraction buffer (50 mM HEPES-KOH, 20% glycerol, 420 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 2 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, pH 7.8), and then gently shaken at 4°C for 30 min. The nuclear extracts were centrifuged (20,000 ×g, 30 min, 4°C), and then the supernatant was collected and kept at −80°C for Western blot analysis, which was performed according to Laemmli’s method (Laemmli, 1970). The protein concentrations in nuclear extracts were measured using the Bradford method, and adjusted to 1 mg/ml. The extracts were heated to 100°C for 3 min in Laemmli sample buffer, then run on 10% SDS-PAGE gels and electroblotted onto cellulose nitrate membranes. An anti-NF-κB p65 antibody (C-20, goat polyclonal IgG, Santa Cruz Biotechnology) was used as the primary antibody, and anti-goat IgG-HRP (Santa Cruz Biotechnology) was used as the secondary antibody. Peroxidase labeling was detected by chemiluminescence using ECL Western Blotting.
Detection System (Amersham Biosciences). The densities of the resultant bands were quantified using densitometry.

**Serum ALT level**

Serum enzymes were measured using a standard clinical assay kit according to the Reitman-Frankel method (Wako Pure Chemical, Japan).

**Hepatic myeloperoxidase (MPO) activity**

MPO activity was measured as a marker enzyme to indicate neutrophil accumulation in liver tissue. Samples of the ischemic lobe and the control lobe were obtained from the left lateral lobe and the right lobe of the liver, respectively. The livers were quickly removed, weighed and homogenized in 10 volumes of 50 mM phosphate-buffered solution (PBS) (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide using a Polytron homogenizer. The homogenate was freeze-thawed, sonicated and then centrifuged (16,000 × g, 10 min, 4°C). The supernatant was then ultracentrifuged (16,000 × g, 10 min, 4°C). It was then ultrafiltrated at 10,000 M.W. using an Ultrafree-MC (MILLIPORE). The precipitate was resuspended in 50 mM PBS (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide for the determination of MPO activity. The reaction mixture consisted of a 100 µl sample solution, 100 µl of 3,3',5,5'-tetramethylbenzidine (final concentration: 0.16 mM) dissolved in dimethyl sulfoxide and 800 µl of H₂O₂ (final concentration: 0.24 mM) diluted in 80 mM PBS (pH 5.4). The mixture was incubated for 5 min at 37°C and the reaction was stopped by adding 10 µl of catalase (final concentration: 13.6 µg/ml) and 4 ml of 200 mM sodium acetate buffer (pH 3.0). MPO activity in each sample was determined by measuring the change in absorbance at 450 nm using a spectrophotometer. The level of MPO activity was defined by the standard curve using human myeloperoxidase (ELASTIN PRODUCTS CO., INC.) as the standard.

**Bromsulfalein (BSP) clearance**

The BSP clearance was measured as an indicator of hepatic function including uptake, conjugation, and biliary excretion. The BSP clearance was measured at 24 hr after reperfusion. Animals were anesthetized with ether. 0.5% BSP (SIGMA-ALDRICH, Inc.) was then injected into the left jugular vein at a dose of 2 ml/kg. Approximately 300 µl of blood was collected from the right jugular vein at 1, 2, 3 and 5 min after injection. The serum concentrations of BSP were measured at 580 nm absorbance using a spectrophotometer. The retention rate of BSP at 3 min after the injection was calculated from the serum concentration.

**Statistical analysis**

Each value is expressed as the mean ± standard error of the mean (S.E.M). Statistical significance was evaluated by the unpaired Student’s t test (Gad and Weil, 1982) with p<0.05 regarded as significant.

**RESULTS**

**Time course of NF-κB activation induced by I/R in rat liver**

Only a single band of approximately 68 kDa was detected in nuclear extracts from the liver by Western blot analysis using the antiNF-κB p65 antibody, and was identified using Hela/TNF-α nuclear extract (Clontech) as a positive control. The intranuclear NF-κB level and index of NF-κB activation increased after 1 hr ischemia in ischemic lobes. After reperfusion, the NF-κB level decreased gradually with time. In the control lobes, no increase of intranuclear NF-κB level during 1 hr ischemia was observed. The time course of the intranuclear NF-κB level in the control lobe was similar to the ischemic lobe in the sham operation group (Fig. 1).

**Effects of drugs on NF-κB activation during ischemia in rat liver**

As shown in Fig. 2, the activation of NF-κB was defined by the ratio of the ischemic lobe to the control lobe of the intranuclear NF-κB level in liver tissue. The ratio of NF-κB activation significantly increased after 1 hr ischemia compared with before ischemia. The activation of NF-κB at 1 hr after ischemia was significantly prevented by the pre-administration of sulfasalazine (1 mg/kg-100 mg/kg) and PDTC (50 mg/kg-200 mg/kg) in a dose-dependent manner.

**Effects of NF-κB inhibitors on the increase in serum ALT level induced by I/R in rats**

The serum ALT level was measured mainly as damage to hepatocytes and significantly increased after reperfusion, peaking at 3 hr after reperfusion (Fig.3). The pre-administration of sulfasalazine and PDTC significantly attenuated the increase in serum ALT level at 3 hr after reperfusion (Fig. 4)
Effects of NF-κB inhibitors on hepatic MPO activity in rats

Hepatic MPO activity was measured as a marker enzyme to indicate neutrophil accumulation in the liver tissue. Hepatic MPO activity significantly increased at 3 hr after reperfusion in the ischemic lobe and this activity peaked at 6 hr after reperfusion (Fig. 5). Hepatic MPO activity was significantly reduced by the pre-administration of sulfasalazine (50 mg/kg-200 mg/kg) and PDTC (50 mg/kg-200 mg/kg) in a dose-dependent manner at 6 hr after reperfusion (Fig. 6).

Effects of NF-κB inhibitors on BSP clearance in rats

The BSP clearance was measured as an indicator of hepatic function including uptake, conjugation and biliary excretion. The retention ratio of BSP at 3 min after injection was significantly higher at 24 hr after reperfusion than that in the sham-operated animals. The increase was significantly prevented by the pre-administration of sulfasalazine (100 mg/kg, i.p.). However, the increase was only weakly reduced by the pre-administration of PDTC (200 mg/kg, i.p.) (Fig. 7).

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**Fig. 1.** Western blot analysis of intranuclear NF-κB p65 subunit amount in liver tissue.

**Fig. 2.** Effect of drugs on the increase in NF-κB activation induced by 1 hr ischemia. The ratio of NF-κB activation was calculated as the ratio of the ischemic lobe and the control lobe on the intranuclear NF-κB level measured by Western blotting analysis. Sulfasalazine was pre-administered i.p. 1 hr before ischemia. PDTC was pre-administered i.p. 3 hr before ischemia. Each value represents the mean ± S.E.M. (n = 4–7). **p < 0.01 versus pre., # p < 0.05 versus the control.
DISCUSSION

In many clinical cases, I/R-induced hepatic dysfunction after liver transplantation is a major problem of rejection. The inflammation associated with neutrophil accumulation is suggested to be especially important in these events during I/R (Jaeschke et al., 1990; Serracino-Inglott et al., 2001). In this study using an I/R model, we observed severe hepatic injury with hepatic enzyme leakage, neutrophil accumulation and

Fig. 3. Change in the serum ALT activity induced by liver ischemia/reperfusion in rat livers. Each value represents the mean ± S.E.M. (n=3-8), ** p < 0.01, * p < 0.05 versus 0 hr.

Fig. 5. Change in the MPO activity induced by liver ischemia/reperfusion in rat livers. Closed square: ischemic lobe, Open square: control-lobe. Each value represents the mean ± S.E.M. (n = 3-8), ** p < 0.01, * p < 0.05 versus the control lobe.

Fig. 4. Effect of drugs on the increase in serum ALT activity induced by 3 hr reperfusion. Sulfasalazine was pre-administered i.p. 1 hr before ischemia. PDTC was pre-administered i.p. 3 hr before ischemia. Each value represents the mean ± S.E.M. (n=3-8), * p < 0.05 versus the control.

Fig. 6. Effect of drugs on the increase in MPO activity in liver tissues induced by 6 hr reperfusion. Sulfasalazine was pre-administered i.p. 1 hr before ischemia. PDTC was pre-administered i.p. 3 hr before ischemia. Each value represents the mean ± S.E.M. (n = 4-6), * p < 0.05 versus control.
decreased BSP clearance signally after reperfusion.

Although it has been indicated that ROS, a well-known NF-κB activator, is generated only during reperfusion, but not during ischemia (Brass et al., 1994), our previous study clearly showed increased lipid peroxide, uric acid and xanthine as by-products of XOD-derived ROS generation during ischemia. Suzuki et al. (1994) and Suematsu et al. (1992) have also reported that ROS generation was induced during hypoxia and was inhibited by XOD inhibitor. These observations strongly indicate that ROS originating from XOD is generated not only in reperfusion but in ischemia. Indeed, NF-κB activated by ROS is markedly activated during ischemia, and the inhibition of XOD by the pre-administration of allopurinol strongly diminished NF-κB activation (Matsui et al., 2000). Some reports have also revealed that NF-κB was activated during hypoxia, but not reoxygenation (Muraoka et al., 1997; Ricciardi et al., 2000). Moreover, we also demonstrated in this experiment that NF-κB was activated during ischemia, but not after reperfusion (Fig. 1). Therefore, we considered that appropriate ROS for NF-κB activation was generated during ischemia in the present warm hepatic I/R model.

NF-κB is the pivotal transcription factor in various types of inflammation. For example, NF-κB plays an essential role in the transcriptional induction of cell adhesion molecules (ICAM-1, E-selectin, and VCAM-1) and chemokines (IL-8, CINC, MIP-1, and MIP-2) (Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). Moreover, it was also reported that NF-κB is involved in the regulation of apoptosis (Wang et al., 1998; Schneider et al., 1999).

Sulfasalazine (Frode-Saleh and Calixto, 2000) and PDTC (Liu et al., 1999) have been widely used as the specific inhibitors of NF-κB activation for in vivo experiments. In addition, in our study, administration of maximum dose of sulfasalazine (200 mg/kg) and PDTC (200 mg/kg) did not induce increases in serum aminotransferases and MPO activity 7 hr after injection in normal rat (data not shown). In this study, we demonstrated that the pre-administration of sulfasalazine and PDTC markedly prevented NF-κB activation in a dose-dependent fashion (Fig. 2). Neutrophil infiltration into the liver following reperfusion evaluated by MPO activity was markedly decreased by the pre-administration of sulfasalazine and PDTC in a dose-dependent manner (Fig. 6). These results suggest that NF-κB, which is actuated by ROS generated during ischemia, may play a key role in neutrophil accumulation in reperfused organs, and neutrophils may result in I/R injury, especially during reperfusion. In respect of neutrophil infiltration into I/R organs, Matsumura et al. (1998) have reported that CINC, a potent neutrophil chemoattractant, is produced by I/R. Some studies have shown that ICAM-1 expression increased after I/R in the liver (Serracino-Inglott et al., 2001; Yadav et al., 1998). Furthermore, NF-κB contributes to the transcriptional regulation of these proinflammatory mediators (Baldwin, 2001; Tak and Firestein, 2001; Yamamoto and Gaynor, 2001). Therefore, we propose the hypothesis that NF-κB activation caused by XOD-derived ROS during ischemia may lead to the production of both proinflammatory mediators and chemokines during reperfusion, and hypothesize that the neutrophils attracted by chemokines may account for the pathophysiology of hepatic disorders. This hypothesis is also supported by a previous report that the XOD inhibitor significantly attenuates the production of CINC following hepatic I/R in in vivo model (Matsumura et al., 1998).

The serum ALT levels significantly increased after reperfusion immediately before the increase of hepatic MPO activity (Fig. 3). The increase of serum ALT level at 3 hr after reperfusion was significantly attenuated by the pre-administration of PDTC or sulfasalazine (Fig. 4). However, the ALT increase was not completely prevented although these drugs were used.

![Fig. 7. Effect of drugs on the increase in the retention rate of BSP induced by 24 hr reperfusion. Sulfasarazine was pre-administered i.p. 1 hr before ischemia. PDTC was pre-administered i.p. 3 hr before ischemia. Each value represents the mean ± S.E.M. (n = 3-12), ** p < 0.01 versus sham, # p < 0.05 versus control.](image-url)
at the maximum dose preventing NF-κB activation. The regulation of transcripts such as proinflammatory mediators may follow an NF-κB activation-mediated inflammatory process causing hepatic damage several hours after reperfusion, because MPO activity maximally increased at 6 hr after reperfusion in this model. This shows that some hepatic damage shown as the rising of serum ALT level at 3 hr after reperfusion can be accounted for by such inflammatory mechanisms. Although I/R-induced hepatic injury is mainly due to NF-κB activation, the damage may be involved with other unknown mechanisms.

BSP clearance is widely used as an index of total hepatic function including hepatic blood flow and hepatocyte ability. In this study, we utilized BSP clearance as the total sum of hepatic damage and neutrophil accumulation, respectively, shown by the serum ALT and the tissue MPO activity 24 hr after reperfusion. The degradation of BSP clearance may be due to decreased hepatic blood flow caused by hepatic microcirculation injury resulting in abundant neutrophil accumulation in the liver. The decline in BSP clearance after reperfusion was significantly prevented by the pre-administration of sulfasalazine, but only partial efficacy was observed when PDTC was pretreated (Fig. 7). However, this may be due to non-specific effects, except NF-κB inhibition by PDTC. Although numerous studies have demonstrated PDTC to be a relatively selective inhibitor of NF-κB activation, it also has been reported that PDTC is able to affect other redox-sensitive factors. For example, the intraperitoneal injection of PDTC (200 mg/kg) induces AP-1 activation in rats (Liu et al., 1999), and the activation of JNK/AP-1 signaling cascade has an injurious role in I/R liver injury (Czaja, 2002). It is supposed that PDTC increases hepatic damage by AP-1 activation, while also attenuating inflammatory injury caused by neutrophil accumulation due to the NF-κB inhibition. This may be one of the reasons for incomplete restoration after hepatic injury by PTDC treatment. Further studies are needed to understand these mechanisms.

In summary, these findings indicated that NF-κB activation during ischemia has a relevant role in I/R-induced hepatic injury. Furthermore, we provide the first evidence that NF-κB activation during ischemia by the pre-administration of NF-κB inhibitors prevents I/R-induced hepatic injury in an in vivo rat model. In addition, these findings suggested that NF-κB inhibitors have potential as therapeutic drugs against I/R injury.

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