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Protective Effect of Neutrophil Elastase Inhibitor (FR136706) in Lethal Acute Liver Failure Induced by D-Galactosamine and Lipopolysaccharide in Rats

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Background/aims. **It has been reported that liver dysfunction with ischemia-reperfusion is improved through selective inhibition of neutrophil elastase (NE) by NE inhibitor. This study was designed to investigate whether NE inhibitor has protective effect in lethal acute liver failure.**

Materials and methods. **Rats were treated with D-galactosamine plus lipopolysaccharide (GalN/LPS) to induce acute liver failure. NE inhibitor (FR136706) was administered intravenously before GalN/LPS injection.**

Results. **NE inhibitor increased the survival rate to approximately 80% compared with less than 10% in GalN/LPS-treated rats. NE inhibitor prevented GalN/LPS-induced increase of enzymes and total bilirubin in serum, which are related to liver injury. Histopathological analysis revealed that NE inhibitor decreased the incidence of hepatic apoptosis and neutrophil infiltration in the liver. NE inhibitor inhibited the increased concentration of proinflammatory cytokines (tumor necrosis factor-alpha, interleukin-6 and interferon-gamma), and chemokines (CINC-1 and MIP-2) in serum or liver caused by GalN/LPS, and enhanced anti-inflammatory cytokine, interleukin-10 concentration. NE inhibitor prevented the activation of the transcription factor, nuclear factor-kappa B, induced by GalN/LPS. NE inhibitor also reduced the induction of inducible nitric oxide synthase mRNA and its protein in GalN/LPS-treated liver, and resulted in a decrease in nitric oxide production.**

Conclusions. **These results indicate that NE inhibitor, FR136706, inhibits the induction of a variety of inflammatory mediators such as cytokines, chemokines, and nitric oxide, in part through the inhibition of nuclear factor-kappa B activation, resulting in the prevention of fulminant liver failure.** **© 2008 Elsevier Inc. All rights reserved.**

Key Words: **acute liver failure; D-galactosamine; lipopolysaccharide; neutrophil elastase inhibitor; proinflammatory cytokine; nitric oxide; inducible nitric oxide synthase; nuclear factor-kappa B.**

INTRODUCTION

Fulminant hepatic failure is defined as severe acute liver failure, complicated by hepatic encephalopathy less than 2 wk after the onset of jaundice, acute impairment of liver function, and the absence of pre-existing liver disease. The most serious characteristic is very high mortality (60% to 80%) due to the effects of a variety of complications and lack of effective therapies [1, 2]. At present, no specific therapy is available other than liver transplantation [3, 4]. Therefore, there is an urgent need for effective therapy for fulminant hepatic failure.

Rodents treated with a combination of D-galactosamine (GalN) plus lipopolysaccharide (LPS) are often used as a liver injury model [5]. Treatment with LPS stimulates tumor necrosis factor-alpha (TNF-α) production *in vivo* and then TNF-α triggers hepatocyte apoptosis. Although rodents are constitutively resistant to LPS or TNF-α, it was reported that GalN is metabolized through D-glucosamine pathways and causes a transcriptional blockade only in the liver [6]. Treatment with GalN in conjunction with LPS resulted in a marked sensitization to LPS and a potentiation of

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TNF-α-induced hepatocyte apoptosis [7–9]. Thus, this is a useful experimental model for human fulminant hepatic failure as reported previously [10].

It is well-known that neutrophils play an important role in ischemia-reperfusion injury. Neutrophil elastase (NE) not only causes tissue damage, but also mediates neutrophil priming. Okajima *et al.* reported that NE has a critical role in the development of ischemia reperfusion-induced liver injury by decreasing endothelial production of nitric oxide (NO) and prostacyclin [11]. NE inhibitor reduces the microvascular dysfunction mediated by TNF-α, interleukin (IL)-1β. and NE [12]. NO radical is implicated to be involved in the pathophysiology of liver ischemia-reperfusion injury [13]. In the liver with inflammation, high levels of NO are generated by inducible NO synthase (iNOS). iNOS can be induced transcriptionally by LPS and/or proinflammatory cytokines such as TNF-α, IL-1β and interferon-gamma (IFN-γ), in liver cells, including both the resident macrophages (Kupffer cells), and parenchymal cells (hepatocytes) [14–17].

Although it has been reported that NE inhibitor has effective roles in the liver ischemia-reperfusion model [18, 19], it is unclear whether NE inhibitor has a protective effect in acute liver failure. The aims of this study are to investigate the protective effect of exogenous NE inhibitor on lethal acute liver failure in rats induced by GalN/LPS, in particular regarding the induction of proinflammatory cytokines and iNOS, and to evaluate the therapeutic potential of NE inhibitor in the management of fulminant liver failure.

MATERIALS AND METHODS

Materials

Neutrophil elastase inhibitor (FR136706) was provided by Astellas Pharmaceutical Co., Osaka, Japan. D-galactosamine hydrochloride and LPS (*Escherichia coli* 0111: B4) were purchased from Wako Pure Chemical (Osaka, Japan).

Experimental Design

Male Sprague-Dawley rats, obtained from Charles River Japan, Inc., were kept at 22°C under a 12-h light-dark cycle and received food and water *ad libitum*. Rats (250 to 300 g, 8 wk old) were anesthetized with ether prior to injection. GalN/LPS (400 mg/kg GalN plus 16 μg/kg LPS) was injected into the penile vein as reported previously [10]. FR136706 (1 to 100 mg/kg) was administered intravenously 30 min before injection of GalN/LPS. The survival was checked during the 48 h following injection of GalN/LPS (n = 7 to 28 in each group). Blood and liver samples were obtained from individual rats at the indicated times. All experimental animals used in this study were treated according to the guidelines set by the Animal Care and Use Committee of Kansai Medical University Animal Center.

Biochemical Analysis

Blood and liver samples were collected 1, 3, and 6 h after injection. Liver samples stored at −80°C were homogenized in four volumes of cell homogenizing buffer (50 mM Tris-HCl, pH 7.4, containing complete protease inhibitor (Roche, Mannheim, Germany) and 1 mM phenylmethylsulfonylfluoride (PMSF) and centrifuged (16,500 × g, 20 min). The supernatant was used for measurement of cytokines and chemokines. Serum levels of aspartate transaminase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and total bilirubin were determined using commercial kits (Wako Pure Chemical). IL-1β, IFN-γ, IL-10, IL-6, MIP-2 (Biosource International, Camarillo, CA), TNF-α, and CINC-1 (Amersham Biosciences Corp., Piscataway, NJ) were measured in serum and/or liver using commercial kits.

Histopathological Analysis

Excised liver, kidney and lung specimens taken 6 h after GalN/LPS treatment were fixed in 10% formalin and embedded in paraffin. Sections (3 to 5 μm) were cut and stained with hematoxylin and eosin. Apoptotic bodies were detected by terminal deoxynucleotidyltransferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL) staining using apoptosis detection kit (Medical and Biological Laboratories Co., Nagoya, Japan). The TUNEL-positive and negative cells were counted in randomly selected fields of view under light microscopy (×100). The mean number of TUNEL-positive cells evaluated as apoptotic index (%) (number of apoptotic cells/total number of cells ×100). Neutrophil infiltration was evaluated by staining using the naphthol AS-D chloroacetate esterase [20]. The number of neutrophils per 20 high-power fields was counted under light microscopy (×400).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared from frozen liver at −80°C [21]. Liver sections (0.1 g) were homogenized with a Dounce homogenizer in 2 mL of buffer A (10 mM HEPES-KOH, pH 7.9, containing 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF and 500 U/mL Trasylol), allowed to swell for 15 min and centrifuged (1100 × g, 5 min). The pellet was suspended in 1 mL of lysis buffer (buffer A supplemented with 0.1% Triton X-100), allowed to stand for 10 min and centrifuged (1100 × g, 10 min). The nuclear pellet was suspended in 80 μL of nuclear extraction buffer (20 mM HEPES-KOH, pH 7.9, containing 0.42 M NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF, 500 U/mL Trasylol, 0.2 mM ethylenediamine tetraacetic acid (EDTA) and 25% (vol/vol) glycerol), incubated for 30 min and centrifuged (16,500 × g, 20 min).

Binding reactions were performed by incubating the nuclear extract in reaction buffer (20 mM HEPES-KOH, pH 7.9, 1 mM EDTA, 60 mM KCl, 10% glycerol, 1 μg of poly(dI-dC)) with the probe (40,000 dpm) for 20 min at room temperature. Products were electrophoresed on a 4.8% polyacrylamide gel in high ionic strength buffer, and dried gels were analyzed by autoradiography. An nuclear factor-kappa B (NF-κB) consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC) from the mouse immunoglobulin light chain was purchased (Promega, Madison, WI) and labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. Protein was measured using the method of Bradford [22].

Serum Nitrite/Nitrate Analysis

The sum of serum nitrite and nitrate (NO_2^- and NO_3^-, stable metabolites of nitric oxide) was measured using the Griess reagent method [23] using a commercial kit (Roche).

Western Blot Analysis

Frozen liver samples were homogenized in five volumes of cell solubilizing buffer (10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% NP-40, 1 mM EGTA, 1 mM EDTA, 150 mM NaCl ,and 1 mM PMSF) and centrifuged (16,500 × g, 15 min). The supernatant was subjected to SDS-PAGE (7.5% gel) and electroblotted onto a polyvinylidene-difluoride membrane (Bio-Rad, Hercules, CA). Immunostaining was performed using an ECL blotting detection agent

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