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JNK mediates hepatic ischemia reperfusion injury

Tetsuya Uehara[1], Brydon Bennett[2], Steve T. Sakata[2], Yoshitaka Satoh[2], Graham K. Bilter[2], John K. Westwick[2], David A. Brenner[3,*]

[1]*Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[2]*Signal Research Division, Celgene Corporation, San Diego, CA, USA*
[3]*Department of Medicine, Columbia University, 630 W. 168th Street, PH 8 E Room 105, New York, NY, USA*

***Background/Aims*: Hepatic ischemia followed by reperfusion (I/R) is a major clinical problem during transplantation, liver resection for tumor, and circulatory shock, producing apoptosis and necrosis. Although several intracellular signal molecules are induced following I/R including NF-κB and c-Jun N terminal kinase (JNK), their roles in I/R injury are largely unknown. The aim of this study is to assess the role of JNK during warm I/R injury using novel selective JNK inhibitors.**

***Methods*: Male Wistar rats (200 ± 25 g) are pretreated with vehicle or with one of three compounds (CC0209766, CC0223105, and CC-401), which are reversible, highly selective, ATP-competitive inhibitors of JNK. In the first study, rats are assessed for survival using a model of ischemia to 70% of the liver for 90 min followed by 30% hepatectomy of the non-ischemic lobes and then reperfusion. In the second study, rats are assessed for liver injury resulting from 60 or 90 min of ischemia followed by reperfusion with analysis over time of hepatic histology, serum ALT, hepatic caspase-3 activation, cytochrome *c* release, and lipid peroxidation.**

***Results*: In the I/R survival model, vehicle-treated rats have a 7-day survival of 20–40%, while rats treated with the three different JNK inhibitors have survival rates of 60–100% ($P < 0.05$). The decrease in mortality correlates with improved hepatic histology and serum ALT levels. Vehicle treated rats have pericentral necrosis, neutrophil infiltration, and some apoptosis in both hepatocytes and sinusoidal endothelial cells, while JNK inhibitors significantly decrease both types of cell death. JNK inhibitors decrease caspase-3 activation, cytochrome *c* release from mitochondria, and lipid peroxidation. JNK inhibition transiently blocks phosphorylation of c-Jun at an early time point after reperfusion, and AP-1 activation is also substantially blocked. JNK inhibition blocks the upregulation of the pro-apoptotic Bak protein and the degradation of Bid.**

***Conclusions*: Thus, JNK inhibitors decrease both necrosis and apoptosis, suggesting that JNK activity induces cell death by both pathways.**

***Keywords*: Apoptosis; Necrosis; Tumor necrosis factor α; c-Jun; Liver**

1. Introduction

Ischemia reperfusion (I/R) injury is an important clinical problem for several organs including brain, heart, kidney, and liver. Hepatic I/R injuries occur during transplantation, liver resection for tumor, and circulatory shock [1]. Possible

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* Corresponding author. Tel.: +1 212 305 5838; fax: +1 212 305 9822.
E-mail address: dab2106@columbia.edu (D.A. Brenner).

Abbreviations: I/R, ischemia reperfusion; JNK, c-Jun N terminal kinase; ALT, serum alanine aminotransferase; AFC, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; HNE, 4-hydroxynonenal-modified proteins; ROS, reactive oxygen species; MPT, mitochondrial permeability transition; NFκB, nuclear factor κB; TNFα, tumor necrosis factor α; PVDF, polyvinylidene fluoride; EMSA, electrophoretic mobility shift assay; ELISA, enzyme-linked immunoassay; IL-8, interleukin-8; ICAM-1, intercellular adhesion molecule-1; ATP, adenosine triphosphate; ASK1, apoptosis stimulating kinase 1.

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consequences of hepatic I/R injury include liver failure and/or multi-organ system failures, resulting in morbidity and mortality [2]. Hepatic I/R injury produces two types of cell death, apoptosis and necrosis in hepatocytes and non-parenchymal cells [3–5]. Several intracellular signaling molecules are activated by I/R including NFκB and c-Jun N terminal kinase (JNK) [6]. However, their role in the molecular pathogenesis of hepatic I/R injury is largely unknown.

JNK is phosphorylated and activated by several types of stresses, including stimulation by cytokines, such as TNFα [7] and IL-1 [8], and environmental stresses such as radiation and oxidant stress [9,10]. Substrates for JNK include the transcription factors c-Jun and ATF-2. Recent studies have proposed that activated JNK may also directly affect mitochondria through undefined substrates leading to apoptosis [11–14]. JNK is strongly induced during warm hepatic I/R injury [8] and during the cold ischemia/warm repetition injury of liver transplantation [6,15].

Using selective JNK inhibitors, we now report that JNK blockade suppresses liver injury in a rat model of hepatic warm I/R. These are the first data from animal studies directly demonstrating that JNK plays a harmful role during liver I/R injury.

2. Materials and methods

2.1. Reagents

Specific JNK inhibitors [16–19], CC0209766, CC0223105, and CC-401 were synthesized by Signal Pharmaceuticals, Inc. Each JNK inhibitor is dissolved in vehicle before injection (5% 1-methyl-2-pyrrolidone, 30% PEG-400, 25% PEG-200, 20% propylene glycol, USP, 20% 0.9% sodium chloride for injection, USP). JNK inhibitors (3–20 mg/kg rat, dissolved in 0.6–8.0 mg/ml vehicle) or equivalent volume of vehicle only are administrated intravenously at 15 min before starting ischemia. Some rats are given JNK inhibitors twice at 15 min before ischemia and at 4 h after reperfusion.

2.2. Animal experiments

Male Wistar rats (Harlan, Indianapolis, IN) weighing 175–225 g are used for all experiments. Rats are allowed free access to rat chow and water before and after surgical procedures. All experiments are conducted in compliance with the University of North Carolina Institutional Animal Care and Use Committee.

2.3. Model of total hepatic ischemia (survival study)

Liver ischemia followed by partial hepatectomy of the non-involved liver is performed by a modification of the technique described by Kohli et al. [3]. Briefly, rats are anesthetized by intramuscular injection of Ketamine (83.3 mg/kg rat) and Acepromazine (0.03 mg/kg rat). After a midline laparotomy, the portal triad is exposed and all structures (hepatic artery, portal vein, and bile duct) to the left and median liver lobes are occluded with a soft vascular clamp (Diethrich Bulldog Clamp, Fine Science Tools). Some rats receive an injection of dye solution into inflow vessels to ensure complete occlusion. Preservation of perfusion of the remaining liver prevents mesenteric venous congestion during ischemia. During ischemia the abdominal wall is closed with sutures. Immediately after reperfusion, the non-ischemic lobes (right and caudate) are removed by hepatectomy. Survival of the animals is dependent solely on the remaining 70% of the liver, which had been subjected to the ischemic injury. This model allows for maintenance of portal decompression while the liver is rendered ischemia and thus avoids both the use of temporary portacaval bypass and production of intestinal congestion. Animals surviving for 7 days after surgery are considered survivors. With this model, survival rates for vehicle treatment are 77.8 ($n=9$), 33.3 ($n=30$), and 0% ($n=7$) with the ischemic period for 60, 90, and 120 min, respectively. The time of 90 min of ischemia was chosen for the survival study.

2.4. Model of partial hepatic ischemia (liver functional study)

A non-lethal model of partial (70%) hepatic ischemia was developed. Under anesthesia using Ketamine and Acepromazine, rats are subjected to 70% ischemia for 60 or 90 min as described above, without resecting the remaining lobes. Rats are sacrificed at different periods after reperfusion for tissue and blood samples. Each JNK inhibitor or vehicle control is intravenously injected at 15 min before the start of ischemia.

2.5. Serum ALT levels after I/R injury

Serum alanine aminotransferase (ALT) levels are measured 6 h after reperfusion by automatic analysis by the Pathology Department, University of North Carolina. A period of 6 h after reperfusion is chosen because it corresponds to peak serum enzyme concentrations after ischemic liver injury in the rat [20].

2.6. Histology and cell counting for apoptosis and necrosis

Livers are fixed in 10% Formalin for 24 h at room temperature, washed with water, stored in 70% ethanol at 4 °C, and embedded in paraffin. Sections of 5 μm are stained with Hematoxylin and Eosin. The number of apoptotic and necrotic cells are counted in 10 high power fields (400×) on each slide and their percentages with respect to all hepatocytes are determined (Olympus IX70). Apoptotic hepatocytes and sinusoidal endothelial cells are identified by standard morphological criteria (cell shrinkage, chromatin condensation, and apoptotic body). Necrotic cells are identified by standard morphological criteria (loss of architecture, vacuolization, karyolysis, and increased eosinophilia). The number of neutrophils is counted as described above.

2.7. Caspase 3 measurement

Caspase-3 activity is measured with an in vitro fluorogenic peptide substrate carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (AFC) using the FluorAce kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacture's instructions. Frozen samples of ischemic lobes obtained at 6 h after reperfusion are homogenized in 200 μl of lysis buffer (10 mM Hepes, pH 7.4, 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 20 μg/ml leupeptin). Samples are centrifuged at 16,000*g* in a microfuge for 10 min at 4 °C. The protein concentrations of supernatants are measured using the Bradford assay (Bio-Rad). Supernatants (250 μg) are incubated with 25 mmol/l z-DEVD-AFC (Enzyme and Systems Products, Livermore, CA) at room temperature. The change in fluorescence (excitation at 370 nm and emission at 490 nm) is monitored after 60 and 120 min of incubation time and expressed as picomoles of AFC release per microgram of protein.

2.8. Western blotting for phospho-cJun

Liver lobes subjected to 60 min of ischemia and then obtained at 0, 15, 30, or 60 min of reperfusion are prepared for whole cell extraction. Liver samples are homogenized in lysis buffer (10 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% NP-40, and 25% glycerol) with protease and phosphatase inhibitors at 4 °C, followed by rotating the tubes for 30 min at 4 °C. After centrifugation, cleared tissue lysates are collected