Activation of c-Jun N-terminal kinase during ischemia and reperfusion in mouse liver

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Abstract We have generated a mouse model for hepatic ischemia in which surgical subcutaneous transposition of the spleen allows hepatic ischemia to be applied without affecting other tissues. Using this mouse model we investigated the relationship between the length of ischemic periods in the liver and subsequent liver function; furthermore, we assayed the activation of c-Jun N-terminal kinase (JNK) during ischemia and reperfusion. Although prior to this study only the activated form of JNK was known to be translocated to the nucleus, we found that JNK translocates to the nucleus during ischemia without activation and is then activated during reperfusion. These results suggest a novel mechanism of JNK activation.

Key words: Ischemia; Signal transduction; Mitogen-activated protein kinase; c-Jun N-terminal kinase; Stress-activated protein kinase

1. Introduction

Prolonged ischemia leads to cell death and irreversible organ dysfunction, while the effects of transient ischemia can be reversed by reperfusion. The length of ischemic periods is, therefore, the key to limiting damage to the affected tissue. In response to the reperfusion of ischemic tissue, immediate early genes (e.g. c-*jun*, c-*fos*, *Egr*-1) are induced [1]. These gene products are involved in a variety of different cell fate-related processes, such as apoptosis [2,3], proliferation [4], and differentiation [5,6]. The transactivation activity of the c-Jun transcription factor is regulated by phosphorylation at Ser^{63} and Ser^{73} within the amino-terminal activation domain [7,8]. c-Jun N-terminal kinase (JNK; also known as stress-activated protein kinase, SAPK), a member of the mitogen-activated protein kinase (MAPK) family, binds to the c-Jun activation domain and phosphorylates it on Ser^{63} and Ser^{73} [9,10]. Transcription of c-*fos* is induced by phosphorylation of the transcription factor TCF (ternary complex factor), which binds to the serum responsible element in the c-*fos* promoter [11]. The phosphorylation of TCF is mediated not only by JNK [12], but also by ERK [13], another member of the MAPK family. MAPKs are activated in response to various extracellular stimuli, including growth factors and environmental stresses [14,15]. In the cytoplasm, MAPKs are activated and subsequently translocated to the nucleus [12,16,17], where the activated MAPKs phosphorylate their substrates, including c-Jun and TCF. Thus, nuclear translocation of MAPKs is a crucial step in signal transduction. Recently JNK was shown to be activated during ischemia and reperfusion in the heart [18] and kidney [19]; however, the mechanism of JNK activation remains unclear.

In the present study we surgically generated a model mouse that allows us to perform hepatic ischemia without affecting other tissues. Using this mouse we investigated the relationship between the length of ischemic periods in liver and its subsequent function. In addition, we examined how ischemia and reperfusion in the liver affect the subcellular localization of JNK and the time course of its activation.

2. Materials and methods

2.1. Model mouse for ischemia-reperfusion

All animal experiments were conducted in accordance with local institutional guidelines for the care and use of laboratory animals. Slc:ddY mice (30–35 g) were fed a standard laboratory diet and water ad libitum before surgery. To prevent mesenteric venous hypertension during hepatic ischemia, subcutaneous transposition of the spleen was performed essentially as described by Bengmark et al. [20], who had applied this surgical treatment to the rat. Total hepatic ischemia of the model mouse was induced by occlusion of the hepatic artery and portal vein with a small vascular clip. Reperfusion was initiated by removing the clip.

2.2. Evaluation of liver function

Blood samples were obtained after 1 h of reperfusion of ischemic livers, and subjected to Normotest [21] to measure the coagulation activity of blood coagulation factors II, VII, and X, whose levels reflect liver function.

2.3. TUNEL method

Specimens were prepared as described previously [22], and apoptotic cells were detected by the TUNEL method [23] using ApopTag Plus (Oncor).

2.4. Protein kinase assay

Liver samples were homogenized in buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, and 20 mM *p*-nitrophenyl phosphate), and immunoprecipitated with anti-JNK antibody G151-333.8 (Pharmingen) or anti-ERK antibody sc-154 (Santa Cruz). Immunocomplex kinase assays were carried out at 30°C for 20 min using 2 μg of substrate, 20 μM ATP and 5 μCi of [γ-^{32}P]ATP in 30 μl of buffer (25 mM HEPES pH 7.6, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, and 2 mM dithiothreitol). Glutathione *S*-transferase (GST)-c-Jun and myelin basic protein (MBP) were used as substrates in the JNK and ERK kinase assays, respectively.

2.5. Immunohistochemistry

Specimens were prepared and immunohistochemical staining of

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Abbreviations: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; GST, glutathione *S*-transferase; MBP, myelin basic protein

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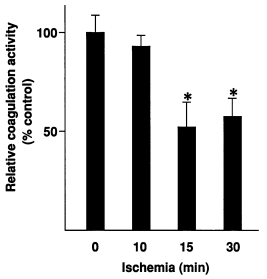

Fig. 1. Effect of the length of ischemic periods on liver function. Livers were subjected to ischemia for the indicated periods of time. Blood samples were obtained after 1 h of reperfusion, and used to measure coagulation activity to evaluate liver function. Bars represent mean ± S.E.M. ($n = 4$). Asterisks indicate a significant difference from control (no ischemia; $P < 0.05$).

JNK was carried out with anti-JNK antibody sc-474-G (Santa Cruz) as described previously [22].

3. Results and discussion

To examine the relationship between the length of ischemic periods in liver and the function of the liver, we generated a model mouse by subcutaneous transposition of the spleen. By 3 weeks after surgery, the mice had developed portasystemic shunts that enabled hepatic ischemia to be applied without affecting other tissues. Liver function was evaluated by measuring the activities of blood coagulation factors II, VII, and X. The mouse was subjected to hepatic ischemia for 10, 15, or 30 min, and the coagulation activities were measured after 1 h of reperfusion. As shown in Fig. 1, ischemia for 10 min had little effect on function, whereas ischemia for 15 and 30 min significantly reduced liver function, to 53 and 58%, respectively, of the function in a control mouse.

We next examined the appearance of apoptotic cells during ischemia (0–15 min) and reperfusion (0–48 h) in the liver. Apoptosis was detected by in situ staining of cells using the TUNEL method. In response to reperfusion after 10 min of ischemia, apoptotic cells appeared around the portal and hepatic veins (Fig. 2A), their number peaked after 6 h of reperfusion (Fig. 2B). By contrast, ischemia for 15 min caused no

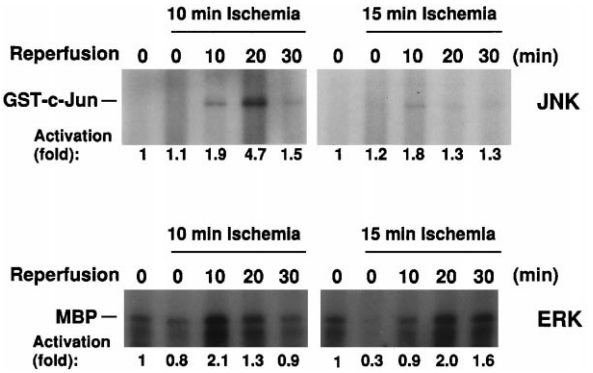

Fig. 3. Activation of JNK and ERK during ischemia and reperfusion. Livers were subjected to ischemia and reperfusion for the indicated periods of time. Homogenates of the liver samples were prepared, and immunocomplex kinase assays were carried out as described in Section 2. GST-c-Jun and MBP were used as substrates for the JNK and ERK kinase assays, respectively. The level of activation relative to the control (no ischemia) is indicated. Experiments were repeated at least twice with similar results.

or a very small number of apoptotic cells during reperfusion (Fig. 2B). Necrotic cells were detected in livers subjected to ischemia for 30 min, but not for shorter periods (10 or 15 min) (data not shown). Taking into account the effect of the length of ischemic periods on the liver function (Fig. 1), these results may suggest that apoptosis plays a protective role in tissue subjected to ischemia and reperfusion.

JNK activity during reperfusion (0–30 min) after hepatic ischemia for 10 or 15 min was investigated using GST-c-Jun as the substrate (Fig. 3). JNK was not activated by 10 min of ischemia alone; however, reperfusion of the ischemic liver increased JNK activity with a peak at 20 min (4.7-fold increase over control). The activity almost completely disappeared after 30 min of reperfusion. No or very little activation of JNK was observed during reperfusion after 15 min of ischemia. Recently, Bendinelli et al. [24] reported JNK activation during reperfusion for 30 min to 1 h after partial hepatic ischemia. The difference in time course from our result is most likely due to the different degrees of hepatic ischemia. Our

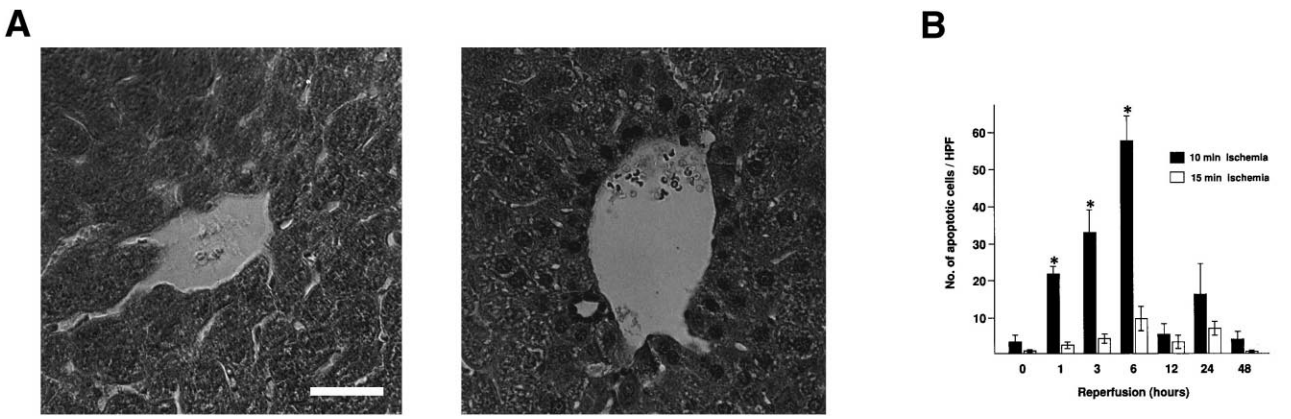

Fig. 2. Apoptosis induced by ischemia and reperfusion. A: Livers were subjected to ischemia for 0 min (left panel) or reperfusion for 1 h after 10 min ischemia (right panel). Specimens were prepared and apoptotic cells were detected by the TUNEL method as described in Section 2. The right panel shows TUNEL-positive nuclei around the hepatic vein. Scale bar is 50 μm. B: After 10 or 15 min of hepatic ischemia, reperfusion was carried out for the indicated periods of time. 30–50 high-power fields (HPF; 200–300 hepatocytes per field) were scored for TUNEL-positive nuclei. Bars represent mean ± S.E.M. ($n = 5$). Asterisks indicate a significant difference from control (10 min ischemia alone; $P < 0.05$).

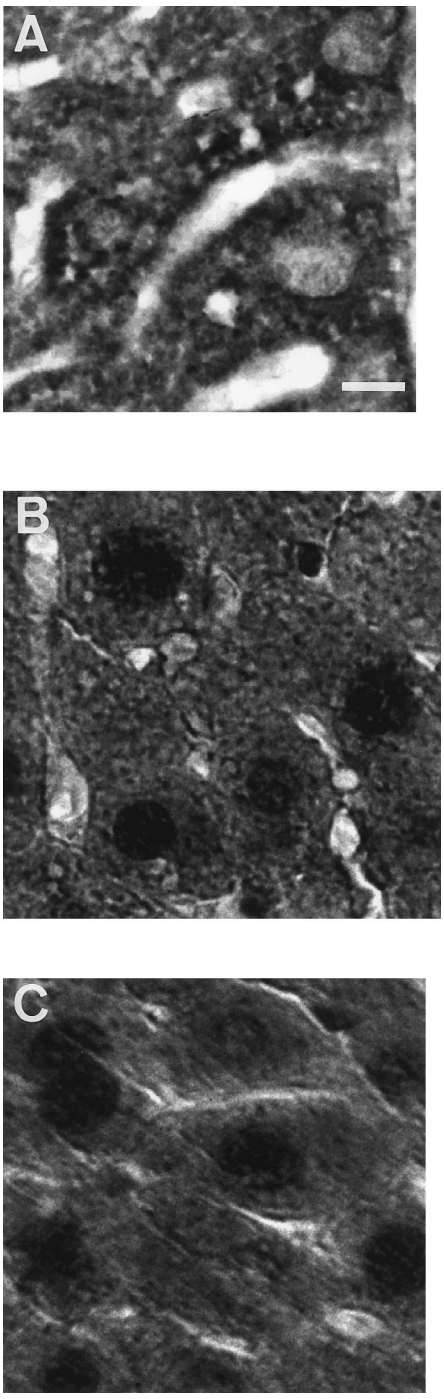

Fig. 4. Immunohistochemical localization of JNK in mouse liver during ischemia and reperfusion. Livers were subjected to ischemia for 0 min (A), or 10 min (B), or to reperfusion for 20 min after 10 min ischemia (C). Specimens were prepared, and immunohistochemical staining of JNK with anti-JNK antibody was carried out as described previously [22]. Scale bar is 3.5 μm.

mouse model permits complete hepatic ischemia; reperfusion following the higher degree of hepatic ischemia could cause the quicker JNK response.

We also examined whether the JNK-related kinase, ERK, would respond to ischemia and reperfusion in the same manner as JNK. ERK was moderately activated during postischemic reperfusion after ischemia was maintained for 10 or 15 min (Fig. 3). These results indicate that the mechanism of JNK activation during postischemic reperfusion is different from that of ERK activation, as suggested by Knight and Buxton [18].

An immunohistochemical study using anti-JNK antibody was carried out to examine the subcellular localization of JNK during ischemia and reperfusion in the liver (Fig. 4). Unexpectedly, ischemia alone for 10 min induced the translocation of JNK to the nucleus (Fig. 4B), even though we observed no activation of JNK at that time (see Fig. 3). JNK remained in the nucleus during postischemic reperfusion for at least 20 min (Fig. 4C). Taken together with the time course of JNK activation shown in Fig. 3, these results suggest that JNK translocates to the nucleus during ischemia without activation and is subsequently activated during reperfusion. Because MAPKs, including JNK, are known to be primarily activated in the cytoplasm in response to stimuli and then translocated to the nucleus, our data suggest a novel mechanism of JNK activation. During the course of our study, Mizukami et al. [25] reported similar results using perfused rat heart. This novel scheme for JNK activation, therefore, seems not to be restricted to the liver, but common to a number of tissues.

JNK can be activated by a dual-specificity threonine/tyrosine kinase SEK1 (also known as MKK4/JNKK) [26–28]. However, stimuli such as UV light activate JNK in a SEK1-independent manner [29]. At present we do not know what signaling molecules are involved in JNK activation during postischemic reperfusion. The technique described here can be applied to transgenic mice expressing altered signaling molecules, such as a dominant inhibitory mutant of SEK1. Use of these model mice should provide important clues to understanding the signaling pathway leading to JNK activation during ischemia and reperfusion.

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