Heat shock protein 72 normothermic ischemia, and the impact of congested portal blood reperfusion on rat liver

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INTRODUCTION

From the technical aspect of liver surgery, control of bleeding during hepatic parenchymal resection is one of the most important procedures in hepatectomy. Pringle's maneuver, a temporary cross-clamping of the hepatoduodenal ligament, has often been used for this purpose[1]. This is the simplest and useful technique to reduce intraoperative blood loss. Unfortunately, this method has resulted in normothermic ischemiareperfusion injury of the liver. Many clinical observations indicate that prolonged intraoperative normothermic ischemia and succeeding reperfusion of the liver are the most significant disadvantages of Pringle's manever, and may cause postoperative functional disorder of the liver[2-6].

Although the process of ischemia reperfusion injury of the liver is complicated, it consists of three pathologic components: hepatic ischemia, portal congestion, and reinflow of arterial and congested portal blood. Ischemia-reperfusion injury is not merely an ischemic injury but is also related to reperfusion of blood. Moreover, it has been revealed that reactive oxygen radicals and some chemical mediators activated during recirculation play an important role in the development of liver injury after ischemia[7-11]. Because portal congestion is an extremely harmful factor that can induce bacterial translocation, generation of chemical mediators, and reactive oxygen radicals in the portal flow, it is conceivable that reinflow of congested portal blood containing activated pathogens may play a role in the development of ischemia-reperfusion injury of the liver. However, the influence of portal congestion on the development of liver injury during ischemia-reperfusion injury has not been well researched. Only a few authors have described the significance of the reperfusion of congested portal blood[12].

Heat shock protein 72 (HSP72) is produced in the liver after normothermic ischemia and reperfusion [13-15]. HSP72 is now being intensively investigated as one of the most fundamental stress proteins. The protein is produced in response to a variety of stress-induced stimuli and is considered to be an important factor which helps to maintain cellular homeostasis and increase the chance of pateint survival[16-23].

In the present study, we investigated the induction of HSP72 in the liver tissue after 15-minute Pringle's maneuver with or without portosystemic shunt in the rat model, to determine the influence of congested portal blood on the development of ischemia-reperfusion injury in liver.

MATERIALS AND METHODS

Male Wistar rats weighing 200 g to 300 g were housed in chip-bedded cages in a climate-controlled room ($24°C \pm 1°C$) under a 12-hour light-dark cycle. The rats were provided with ordinary rat chow and water *ad libitum*- according to the animal protection guidelines of Kyoto University. Rats were premedicated with an intramuscular injection of atropine sulfate (0.05 mg) and anesthetized with an intraperitoneal injection of pentobarbital sodium (40 mg/kg rats weight) before all operative procedures. They were divided into two groups. In the first group (Group P), the abdomen was opened through a median laparotomy and the liver was detached from its ligaments. Total hepatic ischemia with portal congestion was produced by clamping

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the hepatoduodenal ligament for 15 minutes with a microvascular clamp. In the second group (Group S), the abdomen was opened in the same way and the liver was also detached from its ligaments. Extracorporeal portosystemic shunt was achieved from the cecal branch of the superior mesenteric vein to the right external jugular vein, using a polyethylene tube (0.58 mm inner diameter×200 mm long, Dural Plastics Co., Australia) filled with 0.04 mL heparinized saline solution (250U/mL). The liver was subjected to warm ischemia by cross-clamping the hepatoduodenal ligament for 15 minutes with a microvascular clamp. However, portal congestion was prevented by the extracorporeal shunt. During these interventions, the abdomen was temporarily closed and an electric heater was placed under the animal to keep the rectal temperature at 36℃ ± 1℃. Then, declamping allowed reperfusion until sampling after specified periods.

Liver tissue was removed at 48 hours after each intervention and Western blot analysis was performed to detect HSP72 (monoclonal SPA-810, StressGen Biotechnologies Corp., Canada)[13]. To measure adenosine nucleotide concentration, liver tissue was sampled by freeze-clamping at 1 ($n = 6$), 3 ($n = 6$) and 48 hours ($n = 6$) after reperfusion. Enzymatic assay was performed to measure the concentration of adenine nucleotides in the liver tissue [24,25]. The energy charge potential (EC) was calculated according to the formula proposed by Atkinson: (ATP+0.5 ADP)/(ATP+ADP+AMP)[26], where ATP, ADP and AMP are adenosine tri, *di*, and monophosphate, respectively. Blood samples were taken from the aorta at 1 ($n = 6$), 3 ($n = 6$), and 48 hours ($n = 6$) after the end of each intervention. The serum concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactic dehydrogenase (LDH) were measured at a biochemical laboratory. After sampling, rats were killed.

The values were expressed as mean ± standard deviation (SD). For the statistical analysis, Student's *t* test was performed for the biochemical parameters. A *P* value <0.05 was considered to be significant.

RESULTS

Comparing these two groups, HSP72 was markedly overexpressed in group P but its accumulation in group S was very slight (Figure 1).

In the comparison between AST, ALT and LDH levels in group P and group S (Table 1). There was a trend towards higher transaminase levels in group P, but the difference was not statistically significant(*P*>0.05).
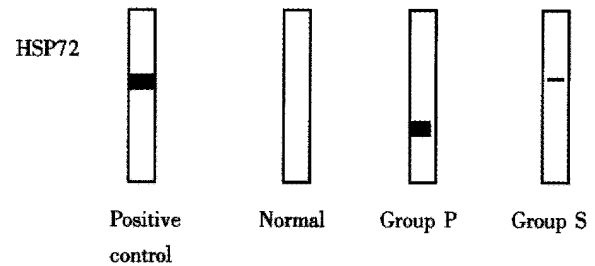

Figure 1 Heat shock protein (HSP) production in the rat liver after 48 hours of reperfusion in each group compared with that in normal liver (5 μg protein per lane). HSC70, which contains inducible HSP72 in approximately 10 per cent, was used as a positive control (0.2 μg protein per lane).

Table 1 Changes in AST, ALT and LDH (IU/L) in serum after reperfusion ($n = 6$)

Parameter	1 h	3 h	48 h
Group *P*			
AST	4832±1589	4896±3270	199±93
ALT	4860±2015	5218±3230	266±155
LDH	45235±21748	44619±29975	588±265
Group S			
AST	4147±1069	2731±1282	204±72
ALT	4230±1460	2689±1459	181±103
LDH	35752±15954	26327±21973	476±248

Results are expressed as means ± SD.
There was no statistically significant difference in AST, ALT and LDH at any time point examined between two groups.

There were no significant differences between the two groups at any time point examined (*P*>0.05, Table 2).

Table 2 Change in ATP levels (mmol/g wet wt tissue) and EC in liver tissue ($n = 6$)

Parameter	1 h	3 h	48 h
Group *P*			
ATP	1.49±0.69	1.91±0.42	3.08±0.42
EC	0.67±0.11	0.62±0.07	0.83±0.03
Group S			
ATP	1.60±0.55	2.22±0.81	3.00±0.22
EC	0.64±0.14	0.68±0.12	0.80±0.05

EC, energy charge. Results are expressed as means ± SD.
There were no significant differences in ATP concentration and EC level at 1 h, 3 h, and 48 h between two groups (*P*>0.05).

DISCUSSION

Heat shock proteins are intracellular proteins that have been well conserved during evolution. They are present in small amounts in normal cells and larger amounts in cells subjected to a variety of stressful stimuli. Heat shock stress, which induces the synthesis of HSPs, is closely related to the physiologic body temperature of the organism[16,27]. This is the reason by which special attention was paid to control the body temperature of the animals

during operations in this study to obviate the possible effects of body temperature changes. The rectal temperature of the animals was strictly controlled in the range of 36℃ ± 1℃ using an electric heating pad.

Schoeniger *et al*[14] reported that prolonged ischemia induced by Pringle's maneuver did not activate the HSP72 gene in the porcine liver until restoration of blood flow. They concluded that transcription of HSP72 mRNA was highly dependent on the generation of superoxide anions. This participation of superoxide anions in the activation of HSP72 gene partly suggests a contribution by the reinflow of portal blood, but the role of portal blood pooling was not a focus of their report. We observed that the reinflow of congested portal blood into ischemic liver after a shortterm Pringle's maneuver contributed to the production of HSP72 in the liver tissue. However, the liver subjected to the same period of ischemia produced less HSP72 when reperfusion was performed with non-congested portal blood. This observation suggests an adverse impact of congested portal blood on liver function after ischemia and reperfusion. In contrast, Tacchini *et al*[28] reported that inflow occlusion to the partial liver for a longer period (up to 60 minutes) also induced expression of HSP72 mRNA in the ischemic lobe in rat liver. In their experiment, portal congestion did not occur because of hemi-occlusion of the hepatic inflow. The discrepancy in the influence of congested portal blood in the production of HSP72 between their results and ours may be due to the difference in ischemic periods. When the ischemic stress is sufficient due to a longer insult, even non-congested blood may cause oxidative stress sufficient to provoke the activation of heat shock genes. However, when the ischemic derangement of hepatic parenchyma is not so severe, the nature of the blood used for reperfusion may have a marked impact on the results. These findings imply that portal pooling would act appreciably as a key stress factor to increase the production of HSP72, but it would not be an essential factor when the degree of pre-existing ischemia is already high.

Although the mechanism by which the reflow of congested portal blood induces HSP72 production is still unclear, the process seems to be complex. The mechanism most likely involves factors that operate in the ischemic reperfused liver. Active oxygen species and free radicals are known to activate heat shock genes[29,30]. It is likely that free radicals generated in the small bowel during portal congestion play a role in the development of liver tissue injury during reperfusion[31-38]. Kawamoto *et al*[7] reported that the temporary occlusion of the portal vein and hepatic artery both increased xanthine oxidase activity in portal plasma and increased the permeability of the intestine during reperfusion. Both of these were prevented when a

portosystemic shunt was performed and splanchnic vascular congestion was prevented. Other potential causative factors in the production of HSP72 include endotoxin and various cytokines translocated from the intestines during portal congestion. Recently, participation of both endotoxin and bacteria in the production of HSP72 has been reported[17,27,39-41]. Pringle's maneuver for 15 minutes was observed to have significantly increased plasma endotoxin levels in the portal blood and enhanced bacterial translocation into the portal blood from the gut[9].

Our results also suggest that HSP72 might prove useful in evaluating the degree of hepatic ischemia-reperfusion injury. Levels of enzymes in serum (including AST, ALT, and LDH), ATP and EC in liver tissue have been commonly used to assess hepatocellular injury. In the present study, no significant difference was observed between the congested and non-congested groups at any time point examined. Thus, even the ATP concentration and EC cannot differentiate these two groups. Animals could fully recover from liver injury as long as the Pringle's maneuver lasted only 15 minutes, and no animals died as a result of it. However, the fact that the stress brought on by the reperfusion of congested portal blood was clearly greater than that produced by the reperfusion of non-congested blood, as demonstrated by the production of HSP72, implies that the accumulation of stress under a repeated or prolonged Pringle's maneuver would likely result in irreversible liver damage. This concept is also consistent with the report of Nitta *et al*[12]. They reported that conventional parameters or arterial ketone body ratio could be used to detect the ill-effect of congested portal blood during reperfusion when Pringle's maneuver was repeated four times, although they failed to detect it after a single 30-minute Pringle's maneuver in dogs. In this sense, the production of HSP72 seems to be more sensitive than conventional parameters to detect the given stress during ischemiareperfusion injury.

In conclusion, HSP72 induced in the liver tissue by a shortterm Pringle's maneuver was associated with the reinflow of congested portal blood into ischemic tissue. The influence of this congested portal blood upon reperfusion of ischemic tissue was detected by HSP72, but not by conventional biochemical parameters. HSP72 is suggested to be useful for determination of the degree of hepatic ischemia-reperfusion injury.

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