Effect of verapamil on hepatic reperfusion injury after prolonged ischemia in pigs

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This study investigated the effect of verapamil on prolonged and severe ischemic injury and elucidated the association of the calcium blocking action with cellular injury, assessing changes in hepatic calcium concentrations during ischemia and reperfusion in pigs. Hepatic ischemia was produced for 180 min by clamping both the hepatic artery and portal vein during temporary portacaval shunt performed before the induction of ischemia. Pigs were divided into two groups: the animals in the verapamil group (Group V, n=6) received continuous administration of 0.025 mg/kg per min of verapamil intraportally for 20 min before ischemia. The control group (Group C) received nothing. A better survival rate was observed in Group V than in Group C (p<0.01), but serum aspartate aminotransferase was higher in Group V after reperfusion (p<0.05). There were no significant changes in hepatic calcium concentrations during ischemia in either group, but it increased immediately after reperfusion in both groups. However, no significant difference was found between the two groups. Recovery of the pyruvate/lactate ratio in Group V tended to be better after reperfusion compared to Group C (p=0.08). These data suggest that the pre-ischemic administration of verapamil produced better survival in animals after prolonged normothermic ischemia. However, the reperfused liver suffered more severe damage in the first 6 h after reperfusion in the verapamil-treated animals. Moreover, there seemed to be very little blocking action of calcium influx. A reduced oxygen requirement may be involved in the protective action of verapamil on animal survival. © Journal of Hepatology.

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Ischemia-reperfusion injury is a common phenomenon in human disease processes (1,2). For example, injury to the allograft by ischemia-reperfusion cannot be avoided during liver transplantation. To improve the result of liver transplantation, several drugs to prevent hepatic ischemia-reperfusion injury have been investigated, such as superoxide dismutase (3), catalase (3), allopurinol (3,4), ATP-MgCl2 (5), chlorpromazine (6), coenzyme Q (7), alpha-tocopherol (8), cyclosporine A (9) and FK 506 (10). It has been demonstrated that calcium channel entry blockers have protective effects in hepatic ischemia reperfusion injury (11-14) and in liver transplantation (15-17). Recently, it has been hypothesized that an excessive accumulation of intracellular calcium is associated with cellular injury (18-20), and the mechanism of the protective effect of calcium blockers has been proposed to occur by blocking calcium influx into cells. On the other hand, other protective actions have been postulated for these drugs: preservation of mitochondrial function (13); an increase of organ perfusion (17,21,22); prevention of lysosomal disruption (23); restoration of depressed immune competence (24,25); and the effect of anti-peroxidant (11,26). Therefore, calcium blocking action as the main mechanism for tissue protection by calcium channel entry blockers is still controversial. Moreover, there are few reports to confirm the beneficial effect of calcium channel

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entry blockers in the model of prolonged, severe hepatic ischemia. Indeed, Nayler (27) concluded that clinical trials relating to the effectiveness of calcium antagonists in the management of patients with myocardial infarction are disappointing, and suggested that the severity of the ischemic episode determines whether calcium antagonists will be beneficial. An investigation of this question is indispensable for the safe clinical use of these drugs in liver transplantation.

The aim of the present study was to investigate the effect of verapamil, a well-known calcium channel entry blocker, in 180-min normothermic ischemia-reperfusion of the liver in pigs, which is fatal without treatment (28).

Material and Methods

Anesthesia and operation

All experiments were conducted in accordance with the Institute of Experimental Animals of Shimane Medical University guidelines for the care and use of laboratory animals. Hybrid pigs (Land Race × Yorkshire) of both sexes weighing 17–23 kg were fasted overnight. The animals were injected with ketamine hydrochloride (25 mg/kg) and atropine sulfate (0.025 mg/kg) intramuscularly before intratracheal intubation. The animals were anesthetized with a bolus intravenous injection of sodium pentobarbital (25 mg/kg) and maintained by adding 5 mg/kg every 2 h with controlled respiration with room air. Cefazolin sodium (Cefazolin®, Fuzisawa Pharmaceutical Co., Ltd., Osaka, 50 mg/kg) was administered intravenously before surgery. The liver hilus was exposed through a midline laparotomy. Hepatic ischemia was produced by clamping both the hepatic artery and the portal vein with a side-to-side portacaval shunt, which was done before the ischemia was induced. Systemic heparinization was not performed throughout the experiment. Reperfusion of the liver was started after 180 min of ischemia by removing the clamp and closing the shunt with a hemostatic clip. Body temperature and blood pressure were monitored until the end of the operation. Normal body temperature was maintained by the infusion of warm saline into the peritoneal cavity. Six hours after reperfusion, when all measurements were completed, the abdomen was closed in two layers. Pigs were returned to the animal room after their spontaneous respiration was confirmed. Pigs were investigated at 12-h intervals after reperfusion for 3 days.

Drug treatment regimens

Pigs were divided into two groups of six each: the verapamil group (Group V) was administered verapamil (Vasolan®, Eisai Co., Ltd., Tokyo) intraportally via a polyethylene catheter at a rate of 0.025 mg/kg per min with a microinfusion pump for 20 min just before ischemia. The control group (Group C) was not given anything before ischemia. The sex distribution was 2:4 (M:F) in Group V and 3:3 in Group C. The mean body weight was 19.5±0.9 kg in Group V and 20.8±0.3 in Group C, respectively.

Sample collections

Hepatic tissues were obtained serially from the margin of the liver for measurement of calcium concentration of the liver. Approximately 2 g of the liver tissue was taken on 16 occasions: before ischemia, 90 min after ischemia, immediately before reperfusion and 5, 10, 15, 20, 25, 30 and 45 min and 1, 2, 3, 4, 5 and 6 h after reperfusion. The left hepatic vein was cannulated, through the right internal jugular vein using Seldinger’s method to assay serum aspartate aminotransferase.

Assay of hepatic calcium concentrations

Calcium concentrations of the liver samples were measured according to a modification of the method described by El-Mofty et al. (29). In brief, the liver specimen liver was cut into small cubes, which were immediately irrigated with a solution of 0.1% ethylenediaminetetraacetic acid disodium salt (EGTA, Nacalai Tesque Inc., Kyoto) for 30 s and rinsed with saline. The samples were then immediately frozen in liquid nitrogen and freeze-dried to a constant weight for 48 h using a lyophilizer (Dura Dry™ Condenser Model, FTS systems, Inc, NY). Approximately 80 mg of the aliquots were dissolved in totally 1 ml solution containing 0.5 ml of concentrated sulfuric acid and 0.5 ml of concentrated nitric acid for 24 h in a 50°C water bath. In tube, the solution was made up to a volume of 5 ml, adding 1% lanthanum chloride (LaCl₃, Sigma Chemical Co., St Louis), which was left at room temperature for 30 min. Calcium concentrations were estimated by atomic absorption analysis using a Polarized Zeeman Atomic Absorption Spectrometer (Hitachi 180-80, Hitachi Co., Ltd., Tokyo).

Assay of oxygen metabolism

Pyruvate and lactate in the hepatic venous blood were measured enzymatically, as described elsewhere (30,31). The assays were carried out using kits (Detarminer PA and LA, Kyowa Medics Co., Ltd., Tokyo). The data were expressed as percentages of pre-ischemic values.

Assay of sAST

Serum AST activities were determined using an automated analyzer (Hitachi 736, Hitachi Co., Ltd., Tokyo).
**Histological examination of liver**
Small pieces of liver tissue were obtained 6 h after reperfusion and fixed with 10% formalin. These samples were stained with hematoxylin and eosin, and then examined microscopically in a blind fashion.

**Statistical analysis**
The data were expressed as mean±SEM. Statistical analyses of the data were performed using one-way analysis of variance (ANOVA) for comparison of means. Actual survival rates were compared using the generalized Wilcoxon test. p<0.05 was considered to be statistically significant.

**Results**
All animals tolerated the experimental procedures well. The blood pressures in both groups were stable during the experimental period.

**Animal survival**
All pigs in Group C died within 20 h after reperfusion, while two of six in Group V (33.3%) lived for 3 days after reperfusion. Four in the latter group died 18, 24, 48, and 49 h after reperfusion. Actual survival curves after reperfusion in both groups are shown in Fig. 1. A statistically better survival rate was observed in Group V than in Group C (p<0.01, generalized Wilcoxon test).

**Liver test**
Fig. 2 represents the changes in sAST activity for the first 6 h after reperfusion. sAST activity increased significantly at all points after reperfusion in both groups as compared with preischemic values (p<0.05 at 30 min after reperfusion in Group V, p<0.01 at the other points in both groups, one-way ANOVA), and it did not decrease within the observation time up to 6 h. Serum AST activity in Group V was significantly higher than that in Group C 3 h after reperfusion (p<0.05, one-way ANOVA).

**Hepatic calcium concentrations**
As shown in Fig. 3, there were no remarkable changes in mean calcium concentrations of the liver in either
group during the ischemic period. After reperfusion, hepatic calcium accumulated significantly compared with preischemic levels ($p<0.01$: at 6 h, $p<0.05$: at 1, 3, 4 h after reperfusion in Group V, and $p<0.01$: at 3 h, $p<0.05$: at 15 min, 1, 4, 5 h after reperfusion in Group C, one-way ANOVA). However, there was no statistical difference in the mean hepatic calcium concentrations between the two groups at any time point after reperfusion.

**Oxygen metabolism**

As illustrated in Fig. 4, serum pyruvate obtained from the hepatic vein increased after reperfusion, but the gains in the two groups were not significant compared with the preischemic value. On the other hand, serum lactate increased significantly after reperfusion in both groups compared with the preischemic value. However, the values of all points in Group V seemed to be lower than those in Group C. Also, the ratio of pyruvate to lactate (Pyr/Lac) decreased significantly after reperfusion in both groups compared with the preischemic value. However, the recovery of Pyr/Lac in Group V was better than that in Group C, although no statistical difference was observed ($p=0.08$: at 1 and 3 h after reperfusion, one-way ANOVA).

![Fig. 4. Changes in pyruvate, lactate and Pyr/Lac (a ratio of pyruvate to lactate) of hepatic venous blood during the first 6 h after reperfusion. The values were expressed as percentages of preischemic values. Closed circles indicate Group V. Open circles indicate Group C. Pyruvate did not increase statistically, but lactate gained significantly after reperfusion as compared with preischemic value in both groups ($\#-- p<0.05$, $\#-- p<0.01$, one-way ANOVA). Also, Pyr/Lac significantly decreased after reperfusion in both groups as compared with the preischemic value. However, the recovery of Pyr/Lac in Group V was better than that in Group C, although no statistical difference was observed ($p=0.08$ but 1 and 3 h after reperfusion, one-way ANOVA).](image)

**Histological findings**

Light microscopic examinations of the liver tissue 6 h after reperfusion are presented in Fig. 5a and b. In the peripheral sinusoids of the hepatic lobules, aggregated red blood cells were observed with moderately centrilobular necrosis in Group C (Fig. 5a). In contrast, the hepatic sinusoids were well preserved in Group V as compared with Group C. However, centrilobular necrosis was more severe with bleeding and infiltration of granulocytes into the hepatic parenchyma in Group V (Fig. 5b).

**Discussion**

Calcium channel entry blockers have been widely used clinically as anti-hypertensive and anti-arrhythmic agents. Also, verapamil has become the drug of first choice for abolishing acute episodes of paroxysmal supraventricular tachycardia (32). Recently, there have been several studies reporting a beneficial effect of verapamil in hepatic ischemia-reperfusion, supporting the hypothesis that the mechanism of protection is through a calcium-blocking effect. However, little is known about actual changes in hepatic calcium levels by using verapamil in vivo. In addition, the safety of the agent has not been established for prolonged and severe hepatic ischemia models. Ischemia-reperfusion injury is reported to be fatal in pigs if the warm ischemic time is longer than 180 min (28); thus a prolonged ischemic time was selected to evaluate the effect of verapamil for safe clinical use in liver transplantation.

In the current study, the preischemic administration of verapamil through the portal vein had a salutary effect on animal survival. However, verapamil had an adverse effect on the liver, at least during the first 6 h after reperfusion. There are several possible explanations of the adverse effect of verapamil on the liver in this study. In light microscopical examination, the sinusoids in the periphery of the hepatic lobules were well preserved in the verapamil-treated liver. This finding was supported by previous studies showing that the calcium channel blocker possesses a vasodilative action and increases the hepatic circulation (17,21,22). Also, verapamil has a suppressive effect on platelet aggregation (33). In addition, our recent study has shown the better hepatic microcirculation with verapamil after reperfusion as compared with controls in a rat hepatic ischemia model using a laser-Doppler technique. The data will be published elsewhere. Therefore, the hepatic reflow at the time of reperfusion may be stronger in verapamil-treated livers than in non-treated livers. Clark & Gewertz (34) have demonstrated that reducing oxygen delivery with a low-flow rate at reperfusion attenuates intestinal reperfusion injury. The verapamil-
treated liver possibly suffered more strongly from toxic substances upon reperfusion. The finding of more severe centrilobular necrosis in the verapamil-treated group is compatible with the above speculation. There is a possibility that a higher sAST was reflected by washout of the liver due to increased liver flow with verapamil. However, the finding of more severe centrilobular necrosis in the group administered verapamil may also suggest that more severe injury to liver cells occurred in this group.

In this study, there was no statistical difference in calcium entry into the liver between the two groups. Instead, the calcium level seemed to be higher in verapamil-treated livers during the late reperfusion period in this drastic model. Using a cultured cell model in vitro, there have been several reports on calcium metabolism after treatment with verapamil during ischemia-reperfusion. Murphy et al. (35) suggested that verapamil was effective in reoxygenation-induced calcium overload using cultured chick embryo heart cell. On the other hand, Franceschi et al. (36) reported that no protection from intracellular calcium overload after exposure to oxygen-derived free radicals was observed with verapamil in cultured endothelial cells from bovine aorta. We assume that these conflicting results, including our study, may be dependent on the degree of destruction of cellular membranes. Generally, the routes of calcium influx are the voltage-dependent calcium channels, which are regulated by calcium channel entry blockers under physiologic conditions. However, nonspecific membrane pores are possibly generated as the result of membrane lipid peroxidation. Also, an unphysiologic influx of calcium may occur through them in accordance with the calcium gradient between intracellular and extracellular spaces (20). Therefore, we suggest that the calcium influx through the nonspecific membrane pores may be largely dominant in this severe ischemic model, resulting in severe destruction of cellular membranes. Some of the voltage-dependent calcium channels might also be broken at this time, thus gaining little benefit from the calcium blocking-action. Further studies are required to investigate calcium metabolism in models with different ischemic times.

There was a problem relating to calcium assay in this study, which was an indirect method for the assessment of intracellular calcium metabolism. Therefore, it was questionable whether this total calcium concentration of the liver really reflected the effect of verapamil on calcium metabolism. In myocardial ischemia-reperfusion, Nayler (37) demonstrated that verapamil suppressed tissue calcium gain in the early reperfusion period after short ischemia, using the same atomic absorption analysis as we used. We therefore believe that this method of analyzing calcium is reliable for assessing the effect of verapamil on calcium metabolism. In addition, our recent study (38) ascertained the different pattern of serial changes in the total hepatic calcium concentration measured by this method in 90- and 180-min ischemia models in pigs. Therefore, we suggest that total tissue calcium concentration is an important parameter for measurement of cellular injury in vivo.

Why did verapamil give better survival of animals in spite of more severe hepatic injury 6 h after reperfusion? To elucidate this conflicting result, we evaluated oxygen metabolism because calcium antagonists may have a potent function in decreasing oxygen requirements. The recovery of Pyr/Lac ratio, which reflects the oxidoreduction state, tended to be better after reperfusion in the group...
administered verapamil. The data indicated that oxygen metabolism was well maintained in the treated group. In addition, verapamil has been known to have a potent function in stabilizing organ perfusion. Unfortunately, we could not estimate such parameters in this study. However, it is quite possible that the stabilized circulation of vital organs due to verapamil might have a protective effect. Indeed, the rat administered verapamil and which is presented in Fig. 5b (severe centrilobular necrosis) survived until sacrifice. Further trials are required to clarify this paradoxical result.

In summary, the pre-ischemic administration of verapamil produced better survival in animals after prolonged normothermic ischemia (180 min). However, the reperfused liver suffered from more severe tissue damage during the first 6 h after reperfusion in the verapamil-treated animals. Moreover, the blocking action of calcium influx seemed to be very slight. A reduced oxygen requirement may be involved in the protective effect of verapamil on animal survival.

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