PROTECTIVE EFFECTS OF VERAPAMIL ON EXPERIMENTAL ISCHEMIA-REPERFUSION-INDUCED LIVER INJURY IN CIRRHOTIC RATS

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SUMMARY

The effect of verapamil on the survival rate, hepatic adenosine 5'-triphosphate, and hepatic malondialdehyde levels of cirrhotic rats exposed to a warm hepatic ischemia-reperfusion episode was investigated. While 90% of saline-treated cirrhotic rats survived after 30 minutes of total hepatic ischemia, the survival rate decreased to 20% when ischemia was prolonged to 40 minutes. A survival rate of 50% was obtained when verapamil was administered to cirrhotic rats exposed to hepatic ischemia of 40 minutes duration, showing a remarkable protective effect of verapamil in this model. The decrease in hepatic adenosine 5'-triphosphate level after 40 minutes of ischemia was significantly less in the verapamil-treated rats and the recovery after 1 hour of reflow was more prominent, compared with saline-treated controls. Nevertheless, control animals were able to restore their hepatic adenosine 5'-triphosphate levels after reperfusion. Hepatic malondialdehyde levels after ischemia and after reflow were significantly lower in the verapamil-treated group than those of controls. The findings suggest that the beneficial effect of verapamil on ischemia-reperfusion-induced liver injury in cirrhotic rats is related to the inhibition of lipid peroxidation. This effect, by inference, might overlap or bring about an enhanced post-reflow mitochondrial energy charge restoration, which might contribute to the protection afforded by verapamil.

Key Words: Verapamil, Hepatic Ischemia, Liver cirrhosis, Experimental

INTRODUCTION

Ischemia is a well-documented cause of hepatic cell injury (1-5). The intolerance of the liver to ischemia has lead to the search for the involved mechanisms and protective agents addressing these mechanisms. For example; steroids(6), ATP(7), alpha-tocopherol(8), aprotinin(9), superoxide dismutase and catalase (10-11), allopurinol(12), and recently calcium channel blockers(13,14) have been implicated as protective agents interfering with potentially damaging factors, such as mitochondrial dysfunction, ATP depletion, and superoxide-induced membrane changes .Among these, calcium has been implicated as the primay propagating agent in ischemic cell injury, and verapamil, a well-known calcium channel blocker, has been shown to prevent ischemia-reperfusion-induced liver injury by inhibiting several proposed pathological processes such as conversion of xanthine dehydrogenase to xanthino oxidase or mitochondrial calcium loading (13-15).

Hepatectomy, as well as hepatic transplantation, is frequently indicated in patients with liver cirrhosis(16,17). On the other hand, patients with cirrhosis often have portal hypertension and coagulation defects and therefore, hepatic resection, even if minor, is difficult (17,18). The cirrhotic liver, with a limited functional reserve, is more prone to the ischemic insult that may result from profound hemorrhage and/or temporary hepatic inflow occlusion(19,20). Protection of the cirrhotic liver from ischemic damage would make it possible to prolong hepatic inflow occlusion and carry out more extensive liver surgery safely.

How long the cirrhotic liver may tolerate ischemia has largely been ignored and experiments designed to study the effects of calcium channel entry blockers on the cirrhotic liver exposed to ischemia are lacking(20). The present study was designed to investigate the effect of administered verapamil on the response of the cirrhotic liver to a warm ischemia-reperfusion episode and to elucidate the possible mechanisms responsible for this hypothesized protection.

METHODS

Experimental design

Forty male Wistar rats, 12-20 weeks old and weighing 200-250 grams(g) were obtained from Gazi University Surgical Research Center. Cirrhosis was induced by

chronic exposure to phenobarbital and carbontetrachloride(CCl_4) according to McLean et al (21). Treatment was continued for six weeks, and liver biopsies were obtained from 5 randomly chosen rats in order to confirm histologically the development of specific CCl_4 cirrhosis. Thirty of the remaining rats were divided into 3 equal groups randomly.

Group 1 (n=10):
Animals were anesthetized with ketamine hydrochloride 50 mg/kg intramuscularly(im). The abdomen was exposed through a midline incision and a polyethylene catheter was inserted into the portal vein after systemic heparinization (1000IU/kg). 1mL of isotonic sodium-chloride(NaCl) was infused through the catheter in 5 min. The hepatic artery, portal vein, common bile duct, and collateral vessels, if any, were isolated and clamped with a nun-crushing instrument. Total hepatic ischemia was induced for 30 min. After the completion of hepatic ischemia, 1 mL of saline was again infused through the portal catheter in 5 min. The adbominal incision was closed with a 4/0 continuous silk suture.

Group 2 (n=10):
The same procedures were followed. Once again, 1 mL of saline was infused through the portal catheter before and after the induction of ischemia. Total hepatic ischemia was induced for 40 min in this group of animals.

Group 3 (n=10):
Following portal vein cannulation, 0.3 mg/kg of verapamil was administered intraportally in 5 min. Hepatic ischemia was induced for 40 min and the same dose of verapamil was given again intraportally in 5 min immediately following reflow. The volumes infused were completed to 1 mL with isotonic saline. Liver samples were obtained from each animal at arbitrary points before the administration of verapamil or control vehicle(before ischemia), immediately after the completion of ischemia, and one hour following reflow. The samples obtained from groups 2 and 3 were immediately crushed with aluminum tongs precooled in liquid nitrogen and freeze-dried. Postoperatively, the rats were allowed to take food and water freely.

Determination of survival rate
All rats that died postoperatively were autopsied. Rats in which complications, such as intraperitoneal bleeding, developed were excluded from the group and replaced by other cirrhotic rats. Rats that survived more than 10 days postoperatively were counted.

Measurement of hepatic adenosine 5'-triphosphate(ATP) levels
Liver samples were homogenized twice with 6% perchloric acid and centrifugated at 17500 rpm, at +4°C for 20 min. The supernatant was neutralized with 5 M K_2CO_3 solution. ATP was measured by the enzymatic method using glucose-6-phosphate dehydrogenase and hexokinase enzymes(22). The results were expressed in µmol/g tissue.

Measurement of lipid peroxidation
Liver samples were homogenized with 1.15% KC1 solution and liver lipid peroxide levels was measured using the method of colorimetric reaction with thiobarbituric acid(23). The results were expressed in nmol/g tissue. The lipid peroxide determined was referred to as malondialdehyde (MDA).

Statistical analysis
All values are given as mean ± SEM. Hepatic ATP and MDA levels were compared between the groups using Mann-Whitney-U test, and survival rates using the chi square method. Differences within each group was evaluated using Wilcoxon test.

RESULTS

Five rats randomly chosen in order to confirm the development of specific CCl_4 cirrhosis, histologically following treatment for 6 weeks with phenobarbitone and CCl_4, all proved to have developed moderate to severe cirrhosis with hepatocyte necrosis, steatosis, sinusoidal congestion, periportal chronic inflammation, and bile duct proliferation.

Survival after ischemia
Hepatic ischemia of 30 minutes duration caused only 1 death in group 1, resulting in a survival rate of 90%. When hepatic ischemic time was prolonged to 40 min (group 2), 8 of 10 rats died; so the rate of survival decreased to 20%. The difference between the groups was statistically significant ($p<0.05$). In the verapamil-treated group (group 3), 5 of 10 rats survived after 40 min of ischemia. The 50% survival rate differed significantly from that of saline-treated group, indicating a marked improvement in the survival rate of rats treated with verapamil ($p<0.05$).

Changes of ATP and MDA levels
Hepatic ATP and MDA levels were determined in groups 2 and 3 before ischemia, immediately after ischemia, and 1 h after reflow. In the saline-treated cirrhotic group, the mean ATP level before ischemia was 8.87±3.01 µmol/g tissue, and decreased significantly to 1.11±2.06 µmol/g tissue following 40 min of ischemia ($p<0.05$). 1 hr after reperfusion, the ATP level was restored to 5.80 ±3.55 µmol/g tissue ($p<0.05$ with respect to ATP-after ischemia). In the verapamil-treated group, the initial ATP level of 7.64 ± 3.88 µmol/g tissue decreased to 5.67 ± 1.65 µmol/g tissue at the end of 40 min of ischemia, and this difference was not significant (Fig. 1). 1 h after reflow, the hepatic ATP was restored to a level very close to that of the preischemic period (7.18 ± 2.53 µmol/g tissue). Comparing groups 2 and 3, the initial preischemic ATP levels were similar. Hepatic ATP level after 40 min of ischemia was significantly lower in the saline-treated control group than the verapamil-treated group ($p<0.05$). Interestingly, although the

recovery of ATP after 1 hr of reflow was more prominent in the verapamil-treated group, a significant difference did not exist between the two group with respect to post-reflow ATP levels.

In the saline-treated control group, the MDA level after 40 min of ischemia did not differ significantly from the preischemic level (17.62 ± 4.09 and 17.01 ± 3.12 nmol/g tissue, respectively). Following 1 h of reperfusion, it rose up to 33.20 ± 11.2 nmol/g tissue ($p<0.05$). In the verapamil-treated group, the mean MDA level increased insignificantly from the baseline value of 18.51 ± 3.12 nmol/g tissue to 21.72 ± 5.63 nmol/g tissue after 40 min of ischemia. The rise in MDA level caught statistical significance after 1 h of reflow (23.88 ± 5.6 nmol/g tissue) ($p<0.05$ with respect to preischemic MDA) (Fig. 2). Comparing the two groups, MDA levels after 1 h of reflow were significantly lower in the verapamil-treated group than those of controls ($p<0.05$).

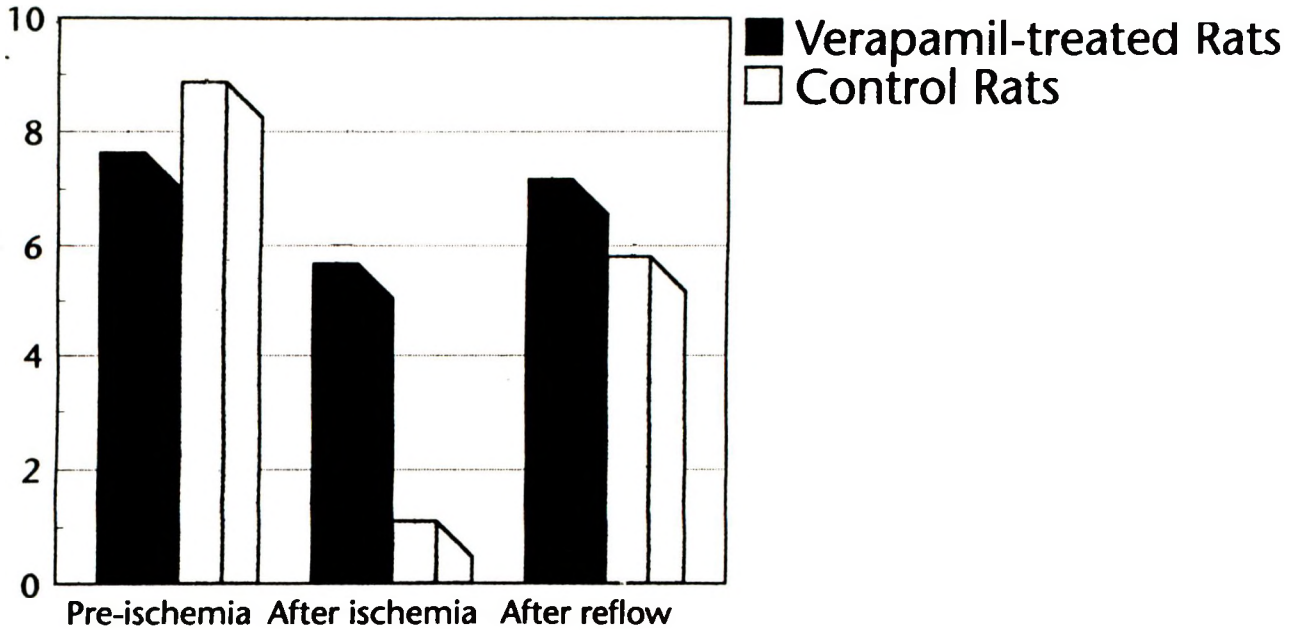

Fig. 1. Hepatic ATP levels of verapamil-treated and control cirrhotic rats before total hepatic ischemia, after 40 minutes of ischemia, and 1 hour after reflow. *significantly different from control group ($p<0.05$)

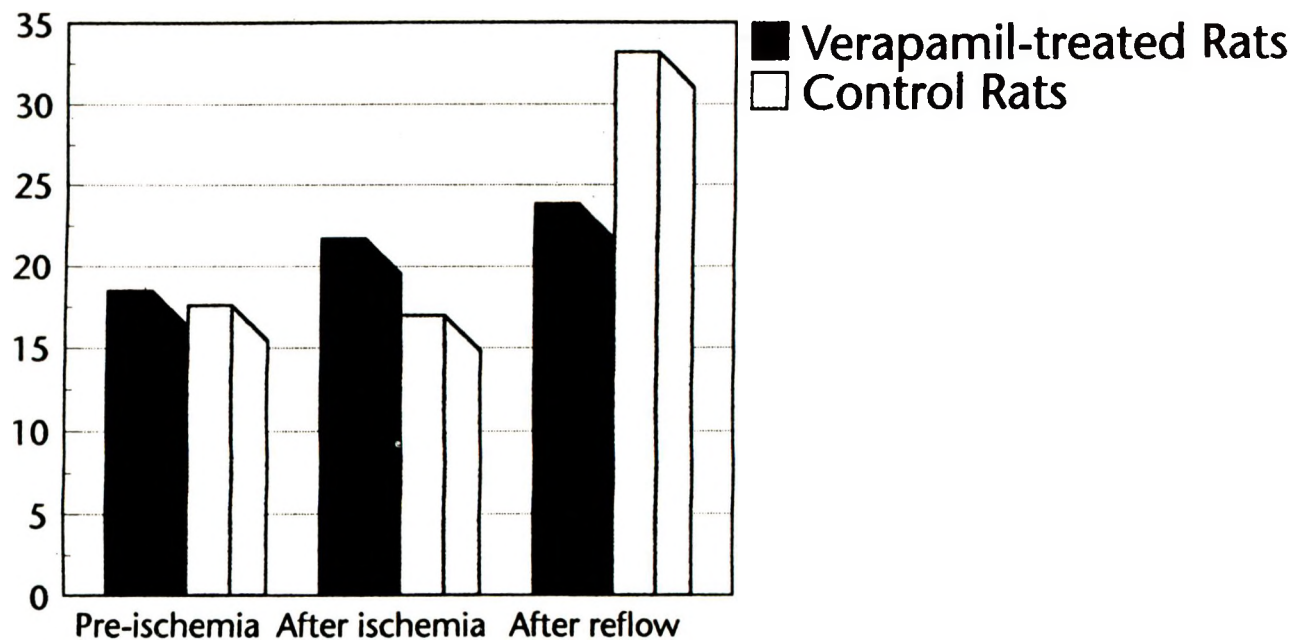

Fig. 2. Hepatic MDA levels of verapamil-treated and control cirrhotic rats before total hepatic ischemia, after 40 minutes of ischemia, and 1 hour after reflow. *significantly different from control group ($p<0.05$)

DISCUSSION

Liver ischemia is a well-known phenomenon in patients operated on for trauma or cancer (1,24). Hypoperfusion of liver cells and consequent hepatocyte dysfunction may also occur following a period of hemodynamic or cardiogenic shock (1,5,25). Temporary occlusion of hepatic inflow, namely the Pringle maneuver, has been extensively used by those who participate in liver surgery in order to prevent profound hemorrhage from the raw surface of the liver encountered during liver resection(26). Total hepatic inflow occlusion also provided a reliable model to investigate liver ischemia, which was first challenged by Huguet and associates who found human livers to tolerate ischemia for up to 65 minutes under normothermia(2,3). Ischemic liver damage has been, therefore, a subject of a great deal of investigation. Despite this, the mechanisms by which (post)ischemic liver damage occurs are not well-defined, and the exact nature and sequence of events leading to irreversible injury of ischemic cells are uncertain. Several theoretically protective agents addressing potentially damaging factors have been tried in order to atenuate ischemic liver injury (6,7,9-15). Little is known about the mechanisms of protection of the cirrhotic liver from the ischemic insult. However, hepatocellular carcinomas are more frequently met in patients with liver cirrhosis, and therefore, hepatectomy is more frequently needed (16,17). On the other hand, major hepatic resection is usually contra-indicated and even minor resection is difficult because of coagulation defects and limited functional reserve in cirrhotic patients (17,18). The risk of hepatic failure unassociated with surgery and following hemodynamic or cardiogenic shock also exists in this group of patients, maybe to a higher extent. Total hepatic inflow occlusion, which is actually applied to prevent the ischemic injury due to hemorrhage, is itself an ischemic insult and its upper time limit must be well-defined. Nagasue and associates successfully performed nonanatomical resections of cirrhotic livers with temporary occlusion of hepatic inflow (and outflow) for up to 30 minutes (20). A survival rate of 63% was reported in cirrhotic rats following 30 minutes of hepatic ischemia (27). Studies designed to improve the tolerance of the cirrhotic liver to ischemia are lacking.

The idea that calcium channel blockers may attenuate the ischemic damage of tissue has been supported by studies of the heart, brain, and kidney (28-30).

Using a rat total hepatic ischemic model, Shinohara et al. obtained a marked improvement in the survival rate by verapamil administration. In this study, the recovery of hepatic ATP level following ischemia was significant in the verapamil-treated group, showing well-preserved mitochondrial function(13). Maruyabashi et al. reported that the ability of the liver to regenerate its ATP and to maintain an adequate energy charge during restoration of hepatic inflow determined the survival rate (31). The recent proposed mechanisms of protection of the ischemic liver afforded by verapamil have been the inhibition of xanthine dehydrogenase-xanthine oxidase conversion, and the inhibition of potentiation of the damaging effect of free radicals on the mitochondrial electron transport chain due to impairment of NADH-coenzyme Q-reductase activity by mitochondrial calcium loading (14,28,32). Improvement of the hepatic microvascular circulation, and prevention of the lysosomal destruction of ischemically injured hepatic cells were other proposed mechanisms of protection afforded by calcium channel blockers (33,34).

In the present study, 90% of the cirrhotic rats tolerated hepatic ischemia for 30 minutes. The survival rate decreased to 20% when hepatic ischemia was prolonged to 40 minutes. In the verapamil-treated group, however, 5 of 10 rats survived after 40 minutes of ischemia, showing a remarkable protective effect of verapamil against ischemic liver damage in cirrhotic rats.

In order to clarify this protective effect of verapamil, hepatic ATP levels and the degree of lipid peroxidation were investigated. Within the time limits of this study, the decrease in hepatic ATP level after ischemia was significantly less in the verapamil-treated rats and the recovery of ATP after 1 of reflow was more prominent, compared with controls. Nevertheless, the saline-treated controls were also able to restore their hepatic ATP levels after 1 of reflow. Therefore, it seems likely that the effect of verapamil on mitochondrial ATP regeneration constitutes a component of the protection it affords.

Another proposed mechanism of the protection afforded by verapamil relates to the conversion of xanthine dehydrogenase to xanthine oxidase. The elevated cytosolic calcium concentration during ischemia, it is believed, activates a protease capable of this conversion, resulting in enhanced free radical formation and consequent lipid peroxidation (10,15). In our study, hepatic MDA levels were investigated in order to determine the degree of lipid peroxidation after ischemia and reperfusion in verapamil-treated and control cirrhotic rats. Comparing the two groups, MDA levels after 1 hour of reflow were significantly lower in the verapamil - treated group than those of controls. This finding suggests that the inhibition of lipid peroxidation during hepatic ischemia-reperfusion is a major mechanism by which verapamil exerts its beneficial effects in cirrhotic rats. We cannot contradict that this effect overlaps with an improved mitochondrial respiratory function, which would otherwise be impaired due to the high levels of oxygen - derived free radicals potentiated by calcium and/or due to diminished NADH-coenzyme Q-reductase activity. Other proposed effects of verapamil on hepatic microvascular spasm or on leaking lysosomal enzymes were not investigated in this study, and await future experiments with cirrhotic models.

Our results demonstrate the beneficial effects of verapamil for the protection of the cirrhotic liver from ischemic injury. Further detailed studies are required to elucidate the mechanisms by which the cirrhotic liver suffers from the ischemic insult and to investigate other theoretically protective agents.

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