

Protective mechanism of L-arginine against liver ischemic-reperfusion injury in rats

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OBJECTIVE: To investigate the protective effect of L-arginine (L-arg) against liver ischemic-reperfusion (I/R) injury in rat model and its possible mechanism.

METHODS: Male Sprague-Dawley rats were randomized into sham group; hepatoduodenal ligament mobilized, but not clamped; control group; hepatic ischemia only; Arg group: 5 minutes before hepatic ischemia, L-arg (200 mg/kg) injected via the dorsal penis vein; Arg+L group: 10 and 5 minutes before hepatic ischemia, L-NAME (30 mg/kg) and L-arg (200 mg/kg) injected via the dorsal penis vein, respectively; and Fmk group: 5 minutes before hepatic ischemia, ZVAD-fmk (15 mg/kg) injected via the dorsal penis vein. The liver was subjected to ischemia for 40 minutes by Pringle's maneuver, and reperfusion was initiated by removing clamp. The 7-day survival rate, alanine transaminase (ALT) level, caspase-3 activity and apoptotic hepatocyte count were compared among these groups.

RESULTS: After 40 minutes of ischemia and 6 hours of reperfusion, the 7-day survival rate of the Arg group was significantly higher than that of the control and Arg+L groups ($P < 0.05$). The ALT level, caspase-3 activity and apoptotic hepatocyte count in the Arg group were decreased significantly than those in the control and Arg+L groups ($P < 0.01$). The caspase-3 activity and apoptotic hepatocyte count in the Arg group were slightly higher than those in the sham and Fmk groups, but there was no statistical significance.

CONCLUSIONS: L-arg could ameliorate liver I/R injury and the possible protective mechanism is inhibition of hepatocyte apoptosis via inhibition of caspase-3 activity by nitric oxide synthesis.

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Key words: liver; ischemia; nitric oxide; caspase-3; rat

Introduction

Liver ischemic-reperfusion (I/R) injury is one of the potential risk factors contributing to postoperative liver dysfunction. Nitric oxide (NO) has been reported to protect liver from I/R injury in rat models,^[1,8] but its underlying protective mechanism is still under investigation. Our previous

studies have shown that hepatocytes apoptosis mediated by caspase-3 is a pivotal pathologic feature in cirrhotic liver I/R injury.^[2,3] In this study, we used L-arginine as a source of NO to investigate whether L-arginine can protect hepatocytes from undergoing apoptosis by inhibiting caspase-3 in a rat liver I/R model.

Methods

Animals and grouping

Male SD rats weighing 250–300 g were obtained from the Animal Center of Sun Yat-Sen University, Guangzhou, China. The animals were randomized into 5 groups. Sham group: under ester anesthesia, the abdomen of rats was opened by a midline incision. The hepatoduodenal ligament was mobi-

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lized instead of clamping. Control group: hepatic inflow was occluded in the period of study. Arg group: prior to ischemia, L-arg (200 mg/kg) was injected through the dorsal vein of the penis. Arg+L group: 10 and 5 minutes before ischemia, NO synthetase inhibitor, L-NAME (30 mg/kg, Sigma Co., USA) and L-arg (200 mg/kg) were injected via the dorsal vein of the penis subsequently. Fmk group: prior to ischemia, caspase-3 inhibitor, and ZVAD-fmk (15 mg/kg, Sigma Co., USA) were injected through the dorsal vein of the penis.

Liver I/R model

Pringle's maneuver^[4] was used to induce liver ischemia in rats anesthetized with ether. Their abdomen was opened through a midline incision. After isolation of the hepatoduodenal ligament, the structures in the portal triad (the hepatic artery, portal vein and common bile duct) were clamped by a Bull-dog clamp for 40 minutes. Reperfusion was initiated by removal of the clamp. The abdomen was closed by double suturing, and the rats were allowed to recover for liver sampling and survival observation.

Alanine transaminase (ALT) determination

0.5 ml blood was obtained from the tails of the rats at the time points of before liver ischemia and 6, 24, 72 hours after reperfusion, respectively. The serum was collected after centrifugation and stored at -80°C for further examination. Serum ALT was measured by a chemical analyzer (Hitachi 7170A, Japan).

Measurement of caspase-3 activity

Caspase-3 activity was quantified by proteolytic cleavage of the fluorogenic substrate 7-amino-4-trifluoro-methylcoumarin-conjugated Asp-Glu-Val-Asp tetrapeptide (AMC-DEVD).^[5] Liver tissues were obtained by wedge resection of the middle segment of the liver before I/R and at 6 hours after reperfusion under ether anesthesia. Fresh liver tissue (20 mg) was homogenized in a lysis buffer at 4°C . After freezing and thawing 4 times, the lysates were centrifuged at 15 000 rpm for 10 minutes, and the supernatant was collected. Then the

caspase-3 activity was measured at a fluorescence spectrophotometer (Hitachi F-3010, Japan) using a caspase-3 fluorescent kit (Sigma Co., USA) according to the manufacturer's instructions and our previous study.^[2] Caspase-3 activity unit was expressed as nmol AMC release per hour per mg liver tissue.

TUNEL assay

Apoptosis was detected by terminal deoxynucleotidyl mediated nick end labelling (TUNEL) assay.^[6] Paraffin-embedded liver tissue was cut into $5\ \mu\text{m}$ sections. After de-paraffining in a series of alcohol solutions, a TUNEL kit (Boehringer Mannheim Co., Germany) was used according to the manufacturer's protocol. Positive and negative controls were done using sections pretreated with DNase I (Sigma Co., USA) and stained without deoxynucleotide substrate, respectively. Five high power fields ($\times 400$) were selected randomly in each specimen, in which apoptotic hepatocytes were calculated.

Statistical analysis

The data were expressed as mean \pm standard deviation. The means were compared by analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant.

Results

7-day survival

The 7-day survivals were 100% in the sham, Arg, Fmk groups respectively, but 73.3% (11/15) and 66.7% (10/15) in the control and Arg+L groups. The survival rates in the Arg and Fmk groups were significantly higher than those in the control and Arg+L groups ($P < 0.05$).

Changes of ALT during reperfusion

Except the sham group, the ALT levels of the other 4 groups were markedly elevated at 6 hours after reperfusion, and then decreased gradually (Table 1). At 6, 24 and 72 hours, the ALT level in the Arg group was significantly lower than that of the control and Arg+L groups respectively, but

Table 1. Changes of ALT levels of these groups ($n = 10$) at different reperfusion time points (u/L)

Groups	Before ischemia	R 6 h	R 24 h	R 72 h
Sham	38.2±12.1	55.6±17.2**	41.4±13.7	34.6±10.1
Control	37.5±12.4	879.5±191.8	537.7±191.4	230.8±105.3
Arg	41.3±13.4	493.1±126.2*	246.0±103.8 Δ	127.5±57.9 $\Delta\Delta$
Arg+L	37.8±14.0	961.8±192.2	609.1±186.6	227.2±102.4
Fmk	32.0±10.1	162.2±74.0	109.0±56.6	64.9±25.5

Compared with other groups, * $P < 0.01$; compared with the control group, Arg group and Arg+L group, ** $P < 0.01$; compared with the Fmk group, ** $P = 0.119$; compared with the other groups, Δ $P < 0.05$; compared with the control group, Arg group and Arg+L group, $\Delta\Delta$ $P < 0.05$; compared with the Fmk group, $\Delta\Delta$ $P = 0.06$. R: reperfusion.

Table 2. Comparison of hepatic caspase-3 activity and apoptotic hepatocytes among the groups ($n = 10$) at 6 hours of reperfusion

Groups	Caspase-3 activity (nmol AMC·h ⁻¹ ·mg ⁻¹ tissue)	Apoptotic hepatocytes (%)
Sham	4.3±1.4	1.6±0.9
Control	11.7±3.2	11.2±3.1
Arg	6.1±1.7*	4.4±2.7**
Arg+L	12.2±4.0	12.8±3.1
Fmk	3.2±1.4	2.8±1.5

Compared with the control group and Arg+L group, * $P < 0.01$; compared with the sham group, * $P = 0.277$; compared with the Fmk group, * $P = 0.089$; compared with the control group and Arg+L group, ** $P < 0.01$; compared with the sham group, ** $P = 0.086$; compared with the Fmk group, ** $P = 0.314$.

it was still higher than that of the sham and Fmk groups. At 72 hours after reperfusion, the ALT level of the Arg group was comparable with that of the Fmk group ($P > 0.05$).

Caspase-3 activity among the groups

At 6 hours of reperfusion, the hepatic caspase-3 activity of the control group was significantly higher than that of the sham group ($P < 0.05$). The caspase-3 activity of the Arg group was significantly lower than that of the control and Arg+L groups respectively, but there was no statistical difference compared with the sham and Fmk groups ($P > 0.05$, Table 2).

Hepatocyte apoptosis

At 6 hours after reperfusion, apoptotic hepatocytes were remarkable, but no typical necrosis was shown by routine HE staining. The count of apoptotic hepatocytes was less than that of the control and Arg+L groups, respectively ($P < 0.05$), but no difference was seen compared with the sham and Fmk groups ($P > 0.05$, Table 2).

Discussion

Our results showed that L-arg has a potential protective effect on liver I/R injury in rats. It is shown by a remarkable reduction of ALT level at different time points of reperfusion when compared with the control group. However, before administration of extrinsic L-arg, a NO synthetase inhibitor, L-NAME, was used to inhibit intrinsic NO synthesis. The ALT levels were significantly increased under the same I/R condition. These indicated that the protective effect of L-arg against I/R injury was produced by NO synthesis instead of L-arg itself.

Hepatocyte apoptosis mediated by caspase-3 is a critical pathologic feature in liver I/R injury.^[2,3,7,9] After 40 minutes of ischemia and 6 hours of reperfusion in this study, the caspase-3 activity of the control group was significantly higher than that of the sham group, indicating that caspase-3 is highly activated during I/R. TUNEL assay showed that typical hepatocyte apoptosis during the process of liver I/R injury, however there was no evidence of necrosis shown by HE staining. When ZVAD-fmk was used to inhibit caspase-3 prior to ischemia, the caspase-3 activity was significantly reduced, and subsequently the count of apoptotic hepatocytes was markedly decreased. These findings indicated that hepatocyte apoptosis is a key pathologic feature in the rat liver subjected to 40 minutes of ischemia, as we have reported previously.^[2,3]

In this study, both of the caspase-3 activity and the count of apoptotic hepatocytes of the Arg group were significantly lower than those of the control and Arg+L groups, respectively. It is indicated that NO could inhibit caspase-3, and as a result hepatocyte apoptosis is inhibited. L-arg itself could not inhibit caspase-3, but caspase-3 is in-

hibited by NO after L-arg is transformed to NO under the action of NO synthetase.

In conclusion, L-arg could potentially ameliorate liver injury after 40 minutes of ischemia and reperfusion. Its protective mechanism might be inhibition of hepatocyte apoptosis via inhibiting caspase-3 activity by NO synthesis.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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