Glycyrrhizinate reduces portal hypertension in isolated perfused rat livers with chronic hepatitis

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Abstract

AIM: To investigate the effects of diammonium glycyrrhizinate (Gly) on portal hypertension (PHT) in isolated portal perfused rat liver (IPPRL) with carbon tetrachloride (CCL₄)-induced chronic hepatitis.

METHODS: PHT model was replicated with CCL₄ in rats for 84 d. Model was identified by measuring the ascetic amounts, hepatic function, portal pressure in vivo, splenic index, and pathological alterations. Inducible nitric oxide synthase (iNOS) in liver was assessed by immunohistochemistry. IPPRLs were performed at d₀, d₂₈, d₅₆, and d₈₄. After phenylephrine-induced constriction, Gly was geometrically used to reduce PHT. Gly action was expressed as median effective concentration (EC₅₀) and area under the curve (AUC). Underlying mechanism was exploited by linear correlation between AUC values of Gly and existed iNOS in portal triads.

RESULTS: PHT model was confirmed with ascites, splenomegaly, serum biomarkers of hepatic injury, and elevated portal pressure. Pathological findings had shown normal hepatic structure at d₀, degenerations at d₂₈, fibrosis at d₅₆, cirrhosis at d₈₄ in PHT rats. Pseudo lobule ratios decreased and collagen ratios increased progressively along with PHT development. Gly does dose-dependently reduce PHT in IPPRLs with CCL₄-induced chronic hepatitis. Gly potencies were increased gradually along with PHT development, characterized with its EC₅₀ at 2.80 × 10⁻¹⁰, 3.03 × 10⁻¹¹, 3.77 × 10⁻¹¹ and 4.65×10⁻¹¹ mol/L at d₀, d₂₈, d₅₆ and d₈₄, respectively. Existed iNOS was located at hepatocyte at d₀, stellate cells at d₂₈, stellate cells and macrophages at d₅₆, and macrophages in portal triads at d₈₄. Macrophages infiltrated more into portal triads and expressed more iNOS along with PHT development. AUC values of Gly were positively correlated with existed iNOS levels in portal triads.

CONCLUSION: Gly reduces indirectly PHT in IPPRL with CCL₄-induced chronic hepatitis. The underlying mechanisms may relate to rescue NO bioavailability from macrophage-derived peroxynitrite in portal triads.

Key words: Chronic hepatitis; Portal hypertension; Isolated portal perfused rat liver; Diammonium glycyrrhizinate; Inducible nitric oxide synthase
INTRODUCTION

Portal hypertension (PHT) is a fatal complication in patients with advanced chronic hepatitis. Some changes are reversible in PHT pathogenesis, such as an increase of hepatic vascular resistance and an elevation of portal hyperemia. Therefore, it is possible to develop drugs for PHT therapy.

Insufficient intrahepatic nitric oxide (NO) bioavailability is involved in PHT development. Macrophages in portal triads generate peroxynitrite via inducible nitric oxide synthase (iNOS) and superoxide via NADPH oxidases. Under chronic hepatitis, macrophages infiltrated more into portal triads and expressed more iNOS. Peroxynitrite was generated from NO and superoxide, to reduce NO bioavailability. The decreased NO availability leads to an increase in intrahepatic portal resistance, resulting in PHT.

Diammonium glycyrrhizinate (Gly), a molecule derived from a medical plant of Radix glycyrrhizae, is effective in treatment of PHT patients and animals, but the underlying mechanisms are still unclear. In our previous studies, we demonstrated that Gly lower PHT in isolated portal perfused rat livers (IPPRLS) at physiological status. Suitable perfuse velocities were designated as anatomic preloads in IPPRLs with CCl4-induced chronic hepatitis at four stages. A pharmacodynamic model of PHT had been developed in IPPRLs with chronic hepatitis, as median effective concentration (EC50) values of phenylephrine and acetylcholine. This model makes it possible to evaluate candidates for PHT therapy. Furthermore, several studies have shown that Gly enhances NO generation from inflammatory macrophages, limits superoxide release and increases NO bioavailability.

In this study, we investigated the effects of Gly on PHT in the IPPRLs with CCl4-induced chronic hepatitis, and on NO bioavailability from the macrophages in portal triads.

MATERIALS AND METHODS

Animals and materials

Male Wistar rats (200 ± 13 g) were obtained from Animal Centre of Chinese Academy of Medical Sciences. Rats were maintained in a Special Pathogen Free laboratory, with a 25.0 ± 0.2 °C, a 12-h/12-h light/dark photoperiod and 45% ± 2% humidity. All rats were fed standard rodent pellets and allowed free access to filtered water. All experiment procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China.

Carbon tetrachloride (CCl4, CAS 56-23-5), olive oil (CAS 8001-25-0) and heparin sodium (MW 12000, CAS 9041-08-1) were obtained from Sinopharm Chemical Reagent Company; Acetylcholine chloride (CAS 60-31-1) and phenylephrine hydrochloride (CAS 61-76-7) were from Sigma (United States); Diammonium Gly (CAS 79165-06-3) were from Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

Portal hypertensive model

As described previously, PHT model was replicated by subcutaneous injection with 3 mL/kg mixture of 40% (v/v) CCl4 in olive oil, twice weekly for 0, 4, 8 and 12 wk; age-matched animals did with olive oil along as vehicle control (Figure 1). Forty-eight hours after last injection, rat was weighted (Wb) and anesthetized with pentobarbital sodium (50 mg/kg) subcutaneously, a midline incision was made to open abdominal cavity, soak up ascitic samples, expose liver vessels, measure portal pressure, collect blood sample, and canelize hepatic artery, portal vein, hepatic vein. The remained blood in the isolated livers was eliminated with perfusate containing 20.0 μg/mL of heparin sodium through hepatic artery.

Isolated perfused liver system

As described previously, the velocity in each IPPRL was finely controlled by a quantified roller pump. The perfuse pressure was continuously recorded with the Powerlab linked to a computer with a pressure transducer immediately ahead of the portal inlet cannula. The global viability of portal perfused livers was assessed with gross appearance, a stable perfusate pH (7.40 ± 0.10), a stable perfuse pressure, and active response to acetylcholine.

Ascitic quantification

As described previously, exudative liquid was soaked up by dried soft paper in a tube (W1). Before (W1) and after (W2) the wet paper with exudation was dried enough, the paper with tube was weighed exactly. Basing on the body weight (Wb), exuded water (EWR) and dried mass ratios (EDMR) were calculated as EWR = (W1 - W2)/Wb × 1000 and EDMR = (W2 - W1)/Wb × 1000, expressed as exudative weight (g) per kilogram body weight (g/kg).

Hepatic function

Sera were separated by centrifugation at 300 g for 5 min and were stored at -80 °C. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP) and albumin (Alb) were determined by an autoanalyzer (Hitachi 7060; Hitachi, Japan) with commercial kits (Biosino Biotechnology and Science, China) according to the manufacturer’s instructions.

Pathological changes

Organ indexes: Liver, spleen and kidneys were weighed (W) and the organ indexes (OI) were calculated for each
**RESULTS**

**General feature**

**Ascites**: As one of PHT consequences, exuded watery and dried mass ratios increased progressively along with PHT development ($P < 0.01$, Table 1).

**Table 1  Exudations and organ indexes (mean $\pm$ SE, $n = 8$)**

| Groups | Water (g/100g) | Dried mass (g/100g) | Liver (g/100g) | Spleen (g/100g) | Kidneys (g/100g) |
|--------|----------------|---------------------|----------------|----------------|-----------------|
| d0     | 0.223 $\pm$ 0.091 | 0.490 $\pm$ 0.260 | 2.801 $\pm$ 0.347 | 0.163 $\pm$ 0.040 | 0.731 $\pm$ 0.096 |
| d8     | 0.372 $\pm$ 0.127 | 1.250 $\pm$ 0.210 | 5.936 $\pm$ 1.081 | 0.236 $\pm$ 0.037 | 0.854 $\pm$ 0.095 |
| d16    | 0.791 $\pm$ 0.134 | 1.540 $\pm$ 0.150 | 4.472 $\pm$ 0.909 | 0.292 $\pm$ 0.103 | 0.861 $\pm$ 0.117 |
| d48    | 2.267 $\pm$ 0.732 | 3.590 $\pm$ 1.610 | 3.057 $\pm$ 0.349 | 0.409 $\pm$ 0.095 | 1.071 $\pm$ 0.117 |

$^{a}P < 0.05$, $^{b}P < 0.01$ vs d0; $^{c}P < 0.01$ vs d8; $^{d}P < 0.05$, $^{e}P < 0.01$ vs d8.

Mean optical density (OD), positive staining area (Av) and total observed area (At) were measured using Image Pro Plus Analysis System. Levels of existed iNOS were represented as dianamobenzidine-OD average per volume ($OD \times (Ar/At)^{1/2}$); the average of values from ten random fields generated a datum.

**Pharmacodynamic actions**

As described previously[^13][^14], pharmacodynamical model in IPPRLs with chronic hepatitis was modified delicately with structural and functional preloads at d0, d8, d16 or d48 in PHT development. Perfused velocity as structural preload was defined as 3935.50, 4720.63, 4753.35, or 5164.16 μL/min[^13]. Concentration of phenylephrine as functional preload was designated as $1.69 \times 10^{-10}$, $2.64 \times 10^{-10}$, $5.82 \times 10^{-10}$ or $8.24 \times 10^{-10}$ mol/L[^14].

Port pressure in each IPPRL was initially maintained at defined velocity for 30 min[^13]. Maintained pressure was near to portal pressure in vivo. Elevated pressure was stabilized for 10 min after phenylephrine-induced constriction at designated concentration[^14]. Elevated pressure was considered as the baseline for analyzing Gly to relax hepatic portal venules. Cumulative geometric concentrations of Gly ($10^{-10}$ mol/L, $k = 0.10$) were finally used in recirculating perfusate to reduce elevated portal pressure. Gly concentration-response curve was regressed from the cumulative concentrations and the changed percentage of perfused pressure from the baseline of phenylephrine constriction.

**Statistical analysis**

Data are expressed as mean ± SD in each stage. Unpaired t test was used, $P < 0.05$ was considered significance. Equation, $EC_{50}$ with its 95%CI and area under the curve (AUC) of Gly were calculated by regression analysis using Graph-Pad Prism 4 (Graph-Pad Software) in nonlinear fit and various slopes. $EC_{50}$ values of Gly were regressed linearly with the duration (0, 4, 8 and 12 wk) in chronic hepatitis; AUC values of Gly did with the levels of existed inNOS in portal triads.

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[^14]: As one of PHT consequences, exuded watery and dried mass ratios increased progressively along with PHT development ($P < 0.01$, Table 1).

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**Histological morphometry**: After perfusion as described previously[^13][^14], a portion of left lobe from each liver was fixed in 10% buffered formaldehyde, embedded in paraffin, cut as 6 µm sections, and stained with haematoxylin-eosin (HE) and Masson’s trichrome stain. Images were acquired with a Digital Pathology system (Hamamatsu, Japan). Ten fields from each liver were randomly selected, the percentage of lobules (ratio of lobule area per total analyzed field area $\times 100$, under $\times 10$, in HE) and one-ten thousandth of collagen (ratio of collagen area per total analyzed field area $\times 10000$, under $\times 40$, in Masson) were measured using Image Pro Plus analysis system 7.0.1 (No41N70000-60555, Media cybernetics, United States); the average of values from ten random fields generated a datum.

**Immunohistochemical morphometry**: Formalin-fixed, paraffin-embedded, 6 µm sections were used. Rat iNOS was stained with a rabbit polyclonal antibody (1:200, Santa Cruz Biotechnology) and an avidin-biotin peroxidase immunostaining kit with diaminobenzidine (Boster, Wuhan, China). One set of sections was counterstained with hematoxylin for observing cellular location; the other set did not for quantifying existed levels. Microscopic images under $\times 40$ were acquired with Digital Pathology System.

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**Figure 1  Experimental design of glycyrrhizinate on portal hypertension.** Rat model of portal hypertension was replicated with CCl4 in olive oil (full line), age-matched rats did with olive oil (dotted line). PP: Portal pressure in vivo; IPPRL: Isolated portal perfused rat liver; EVs: Median effective velocity; PE: Phenylephrine; HE: Haematoxylin-eosin stain; Masson: Masson’s trichrome stain; IHC: Immunohistochemistry; Gly: Diammonium glycyrhizinate; EC50: Median effective concentration of Gly relaxation.

**Formula**: Rat (OI = W/L/W50$\times 100$).

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|        | Water               | Dried mass             | Liver            | Spleen         | Kidneys        |
| d0     | 0.223 $\pm$ 0.091   | 0.490 $\pm$ 0.260      | 2.801 $\pm$ 0.347 | 0.163 $\pm$ 0.040 | 0.731 $\pm$ 0.096 |
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$^{a}P < 0.05$, $^{b}P < 0.01$ vs d0; $^{c}P < 0.01$ vs d8; $^{d}P < 0.05$, $^{e}P < 0.01$ vs d8.
Table 2 Changed levels of serum biomarkers (mean ± SE, n = 8)

| Groups | ALT (IU/L) | AST (IU/L) | ALP (IU/L) | TP (g/L) | Alb (g/L) |
|--------|------------|------------|------------|----------|----------|
| d0     | 49.63 ± 14.03 | 124.13 ± 20.20 | 72.88 ± 14.58 | 63.63 ± 4.26 | 33.12 ± 1.97 |
| d28    | 237.13 ± 107.91 | 500.25 ± 235.12 | 318.75 ± 147.81 | 56.84 ± 5.43 | 25.93 ± 3.16 |
| d56    | 160.25 ± 42.39 | 411.63 ± 143.51 | 363.50 ± 170.36 | 54.91 ± 5.27 | 24.13 ± 4.25 |
| d84    | 230.00 ± 58.58 | 475.50 ± 201.02 | 303.00 ± 225.94 | 58.36 ± 7.92 | 26.98 ± 6.86 |

\*P < 0.05, \*P < 0.01 vs d0; \*P < 0.05 vs d36; \*P < 0.01 vs d50; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; TP: Total protein; Alb: Albumin.

Table 3 Pathological changes and inducible nitric oxide synthase levels (mean ± SE, n = 8)

| Groups | PP (mmHg) | Lobule | Collagen | iNOS-OD/V |
|--------|-----------|--------|----------|-----------|
| d0     | 6.648 ± 2.235 | 0.564% ± 0.022% | 0.088% ± 0.013% | 0.165 ± 0.011 |
| d28    | 9.225 ± 2.114 | 0.511% ± 0.031% | 0.248% ± 0.120% | 0.197 ± 0.005 |
| d56    | 24.724 ± 3.368 | 0.230% ± 0.024% | 1.974% ± 0.637% | 0.132 ± 0.010 |
| d84    | 26.752 ± 3.263 | 0.134% ± 0.009% | 5.925% ± 1.761% | 0.236 ± 0.040 |

\*P < 0.01 vs d0; \*P < 0.05 vs d0; PP: Portal pressure; iNOS: Inducible nitric oxide synthase.

Organ indexes: Hepatic indexes were the lowest at d0, the highest at d28, higher at d36 and lower at d84. Splenic or renal indexes increased gradually from d0 to d84 (P < 0.01, Table 1).

Hepatic function: ALT and AST activities in sera at d50 increased progressively from that at d0, then decreased to d84. ALP activities at d7 and d84 increased from that at d0, then decreased to d84. Collagen ratio of d7 and d84 decreased from that at d0, then decreased at d84 (P < 0.05, Table 2).

Portal hypertensive feature

PHT: The portal pressures in vivo increased progressively from d0 to d84 in PHT development. Elevated portal pressures (> 20 mmHg) at d84 and d50 reached to the diagnostic criteria of clinic PHT (P < 0.01, Table 3).

Pathologic changes: At d0, histological observation showed normal hepatic structure; some droplet steatosis appeared in hepatocytes, collagen located only at portal triads, at d84, deposit collagen, relieved enlarged hepatic cords, and increased collagen ratios increased progressively from d0 to d84 in PHT development (P < 0.01, Table 3).

Collagen ratio: Collagen ratios increased progressively from d0 to d84 in PHT development (P < 0.01, Table 3).

iNOS distribution

Localization: At d0, iNOS was mainly located in hepatocytes and scattered stellate cells of lobules (Figure 2E). At d28, positive granules were thinner in scattered hepatocytes, thicker in the stellate cells in lobules (Figure 2F). At d50, granules in hepatocytes were completely quenched; positive granules limited within macrophages in portal triads and stellate cells in lobules (Figure 2G). At d84, thick positive granules located in macrophages in portal triads and stellate cells in pseudo lobules (Figure 2H).

Quantification: Levels of existing iNOS per volume in portal triads at d0, d36 and d50 were significantly decreased by 19.39%,-20.00%, and 43.03% from that at d0 (P < 0.01, Table 3). These at d36 and d50 were decreased by 32.99% and increased by 19.80% from that at d0, respectively (P < 0.01). So did it increased by 78.79% at d84 from that at d0 (P < 0.01).

Gly reducing PHT

Dose-effective relation: Gly had the same shape of dose-effective curves at d0 (Figure 2I), d28 (Figure 2J), d50 (Figure 2K), d84 (Figure 2L), respectively, in PHT development. The equations of effective potency to reduce PHT were as follows:

\[ y = -0.3691 + 0.29847/\left(1 + 10^{0.5121 + 0.9844}x \right) \] (R = 0.9975, P < 0.01),
\[ y = -0.1162 + 0.09902/\left(1 + 10^{0.9634 + 0.8646}x \right) \] (R = 0.9942, P < 0.01),
\[ y = -0.03321 + 0.02189/\left(1 + 10^{3.547 + 0.3404}x \right) \] (R = 0.9994, P < 0.01), and
\[ y = 0.10095 + 0.00674/[1 + 10^{4.7521 + 0.53363}x] \] (R = 0.9981, P < 0.01);

EC50 values with their 95%CIs were 2.80 × 10^{-10} (8.20 × 10^{-11} - 9.59 × 10^{-10}), 3.03 × 10^{-11} (9.14 × 10^{-12} - 1.00 × 10^{-11}), 3.77 × 10^{-11} (1.51 × 10^{-11} - 9.38 × 10^{-11}), and 4.65 × 10^{-11} (1.73 × 10^{-11} - 1.25 × 10^{-10}) mol/L, respectively.

Time-effective relation: Pathological development af-
Mechanic connection: Existed iNOS was involved in Gly potency to reduce PHT. Therefore, AUC values of Gly regressed linearly with existed iNOS levels in portal triads at d28, d56, d84 in PHT development (y = 0.2669x + 0.0931, R = 0.9517, P < 0.05).

DISCUSSION

PHT is a common complication in patients with advanced chronic hepatitis[1]. It is possible to develop therapeutic candidates against reversible mechanisms in PHT pathogenesis[1,2]. In this study, we use PHT model in IPPRL with CCl4-induced chronic hepatitis to investigate the potential effects of Gly on PHT, and to explore further the possible underlying mechanisms. NO does directly decrease PHT[2,18]. However, eNOS-derived NO
Portal hypertension (PHT) is a common complication of chronic hepatitis, with levels of existed iNOS in portal triads.

Gly had been shown to cause renal and central systemic hypertension via inhibiting type 2 11β-hydroxysteroid dehydrogenase. Possible mechanisms of Gly to relax directly portal vein included inhibiting gap junction intercellular communications and activating peroxisome proliferator-activated receptor. As hepatic artery being ligated in this study, IPPRL was made to evaluate the effects of Gly on portal venules alone. Under this condition, portal resistance is mainly originated from both of smooth muscle cells in terminal portal venules and sphincter-like endothelia at hepatic sinusoid inlets. Different from extrahepatic vasodilator, Gly relaxes indirectly portal vein via improving intrahepatic NO bioavailability. In this study, AUC values of Gly depend on macrophage-derived iNOS in portal triads. Macrophages infiltrated in portal triads expressed more iNOS and oxidative enzymes, consequently generated more peroxynitrite to decrease NO bioavailability. Gly reduces superoxide, decreases peroxynitrite, increases NO bioavailability, and relieves PHT (Figure 3).

Our results suggested that Gly reduces PHT, which might explain therapeutic effectiveness of Gly-contained prescriptions for patients with PHT ascites. It is also a clue to find more effective candidates related to macrophage-generated iNOS or NADPH oxidase, which partially contributes to the effects of Gly to reduce PHT. Gly or its more effective derivates should be exploited as candidates to maintain NO bioavailability in terminal portal venules, especially against free radical injury.

**COMMENTS**

**Background**

Portal hypertension (PHT) is a common complication of chronic hepatitis, with...
significant morbidity and mortality in clinic. It is important to develop new drugs for this disease. A sensitive pharmacological model has been established for PHT in the isolated perfused rat livers, which supplies suitable methods for evaluating candidates for PHT.

Research frontiers

Diammonium glycyrrhizinate (Gly) is one of the representative candidates for PHT in experiment and clinic. It relaxes portal veins in isolated portal perfused rat livers at physiological status. It is also believed that the inducible nitric oxide synthase (iNOS) expressed from infiltrated macrophages in portal triads is important to increase portal resistance. The sensitive pharmacological model was used here to investigate the effect of Gly on PHT, and to exploit further the possible mechanisms of its actions.

Innovations and breakthroughs

The intrahepatic portal resistance of PHT originates mainly from the smooth muscle cells in terminal portal venules and the sphincter-like endothelium at hepatic sinusoid inlets. Gly is effective for reducing PHT in isolated portal perfused rat livers with chronic hepatitis, with the similar S shape and different potency from its dose-effective curves. As PHT advanced, it was found that more macrophages infiltrated in portal triads and expressed more iNOS. Therefore, macrophage iNOS in portal triads involves in the pathogenesis, on which with Gly acted. The mechanisms of Gly for PHT in isolated portal perfused rat liver with chronic hepatitis are related to increase of nitric oxide (NO) bioavailability.

Applications

Gly reducing PHT might explain the actions of medical plants in Chinese prescriptions. More effective derivate might be generated from Gly molecule structure. NO bioavailability is a candidate target for PHT.

Terminology

Peroxynitrite is the anion with the formula ONOO−, an unstable structural isomer of nitrate. It derives from both of NO and O2•− in activated macrophages at portal triads with chronic hepatitis. It damages all biomolecules in cells, such as DNA and proteins. Peroxynitrite could let NO availability decrease severely.

Peer review

The authors investigated the effect of Gly on PHT. They found that Gly reduce PHT in isolated portal perfuse rat livers with chronic hepatitis. Such action may explain Chinese medical herbs for PHT. It also suggests that NO availability involve in the pathogenesis of PHT.

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