Protective effects of polydatin against CCl₄-induced injury to primarily cultured rat hepatocytes

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Subject headings: polydatin; injury, hepatocyte; CCl₄.

Abstract

AIM To investigate the protective effects of polydatin (PD) against injury to primarily cultured rat hepatocytes induced by CCl₄.

METHODS Rat hepatocytes were separated by methods of liver infusion in vivo and cultured medium (7.5×10⁴ cells/mL). Two mL or 0.2 mL was added into 24-well or 96-well plates respectively. Twenty-four hours after cell preculture, PD at concentrations of 10⁻⁷ mol/L-10⁻⁴ mol/L was added into each plate. At the same time injury to hepatocytes was induced by adding 10mmol/L-CCl₄. Then, 0.1 mL or 1 mL culture solution was removed from the 96-well or 24-well plates at 6 h, 12 h, 24 h and 48 h after CCl₄ intoxication respectively for the determination of GPT, GSH and MDA. At 48 h, the survivability of rat hepatocytes was assayed by the MTT colorimetric method.

RESULTS After CCl₄ challenge, the release of GPT and the formation of MDA in rat hepatocytes markedly increased and maintained at a high level in 48 h, whereas PD with different concentrations could markedly inhibit this elevation with 10⁻⁴ mol/L PD having the strongest effects and inhibiting rate was over 50%. PD could also improve the decreased content of GSH caused by CCl₄ in accordance with the doses used. CCl₄ evidently decreased the hepatocyte survivability from 91.0±3.8% to 35.4±3.8%. On the other hand, PD at 10⁻⁷ mol/L-10⁻⁴ mol/L could reverse this change and improve the cell survival rates to 56.1±5.2%, 65.8±5.0%, 88.7±6.8% and 75.2±7.3%, respectively.

CONCLUSION PD at 10⁻⁷ mol/L-10⁻⁴ mol/L could protect primarily cultured rat hepatocytes against CCl₄ induced injury.

INTRODUCTION

Polygonum cuspidatum Sieb. et Zucc. (Polygonaceae) is a traditional Chinese herbal drug, with bitter taste and cold nature. It mainly acts upon the liver, gallbladder and lung meridians. It is well known that P. cuspidatum has various activities such as promoting blood circulation, relieving swelling and pain, eliminating phlegm, alleviating cough, clearing away heat, and removing dampness and toxin. The drug has been widely used for cardiovascular and liver diseases. Its active compounds mainly consist of free anthraquinones which include emodin, physcion and chrysophanol. Another important compound is resveratrol.[1]

Polydatin (PD), 3, 4’, 5-trihydroxystibene-3-β-mono-D-gluc oxide, also named piceid, is the glycoside of resveratrol[1]. Some previous studies demonstrated that PD could lower the level of blood lipid, inhibit the platelet aggregation, dilate blood vessels, protect cardiocytes, reduce cerebral ischemic damage and inhibit lipid peroxidation[2-6]. However, the effects of PD on hepatocytes and its mechanisms have not been reported up to date. In this paper we report the details of protective effects of polydatin against injury to primarily cultured rat hepatocytes induced by CCl₄.

MATERIALS AND METHODS

Materials

Collagenase (type IV), 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), dexamethasone, N-2-hydroxyethyl-piperazine-N’-2’-ethane sulfonic acid (HEPES), insulin, penicillin and streptomycin were purchased from Sigma Chemical Corp. (St. Louis, USA). RPMI 1640 was

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a product of Gibco Life Technologies INC (Grand Island, NY). Fetal calf serum was obtained from Institute of Hemopathy, Chinese Academy of Medical Sciences (Tianjin). PD (Purity>90%), which was isolated from the root and rhizome of P. cuspidatum[7], provided by the Department of Chemistry, the First Military Medical University.

Animals
Wistar rats, male, 6 weeks old, weighing 160-180 g, were used for hepatocyte isolation. They were provided by Laboratory Animal Center, Guangzhou University of TCM.

Isolation and culture of rat hepatocytes
Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Then the liver parenchymal cells of rat were isolated by the collagenase perfusion method following the procedure of Seglen and Kojji[8,9]. Simply, the portal vein of rat liver was exposed and cannulated with a teflon catheter. The liver was perfused with Ca²⁺ free solution containing NaCl 142, KCl 6.7, HEPES 10, NaOH 5.5 (mmol/L), pH 7.4, at 37°C, with a flow rate of 40 mL/min. Twelve minutes later, recirculation started with collagenase solution composed of NaCl 67, KCl 6.7, CaCl₂·2H₂O 5, HEPES 100, NaOH 66, collagenase 0.2 g/L, pH 7.6. Isolated cells were cultured in RPMI 1640 containing 10% fetal calf serum, 10 mmol/L- penicillin and streptomycin, 10 mmol/L- dexamethasone. The content of hepatocytes was adjusted to 7.5 × 10⁸ cells/L with the above medium. Cultured medium 2mL and 0.2mL were added into 24-well and 96-well plates respectively. The cells were incubated for 4h at 37°C under 50mL/L CO₂ in air. Non-adherent hepatocytes were eliminated by replacing the medium, and adherent hepatocytes continued to be incubated, and the medium was changed every 24 h.

CCl₄-induced hepatocytes injury
After pre-culture for 24 h, the hepatocytes were exposed to fresh medium containing 10mmol/L-CCl₄ and various concentrations of PD. At 6, 12, 24 and 48 h after CCl₄ intoxication, 0.1 mL and 1 mL culture solution were removed from 96-well and 24-well plates respectively for determination.

Measurement of glutamic pyruvic transaminase (GPT)
The kits of GPT analysis, provided by the Shanghai Institute of Biological Products of Ministry of Health, were used to measure the activity of GPT in 0.1 mL-culture medium.

**RESULTS**

**Effects of PD on GPT activity in culture medium**
The concentration of GPT in culture medium significantly increased after CCl₄ challenge, and maintained at a high level in 8 h (Table 1). Furthermore, a progressively elevated trend existed with time-dependence. PD could significantly inhibit the level of GPT in accordance with the doses used. Especially, PD 10 µmol/L had the strongest effects and the inhibiting rate was over 50%.

**Table 1** Effect of PD on GPT activity in culture medium

| Group          | 6 h (U/L) | 12 h (U/L) | 24 h (U/L) | 48 h (U/L) |
|----------------|-----------|------------|------------|------------|
| Normal         | 13.5±2.5  | 13.8±3.1   | 13.7±5.6   | 14.1±3.3   |
| Control        | 72.3±14.1 | 79.7±10.3  | 85.4±9.2   | 88.3±19.6  |
| PD 10⁻⁷        | 60.3±17.1 | 62.0±15.6  | 68.8±17.5  | 71.4±20.5  |
| PD 10⁻⁶        | 55.0±10.3 | 58.3±16.7  | 64.1±13.6  | 69.1±19.2  |
| PD 10⁻⁵        | 50.6±10.6 | 58.3±5.5   | 42.5±7.0   | 45.0±7.6   |
| PD 10⁻⁴        | 42.1±7.8  | 47.5±9.8   | 56.8±11.3  | 59.2±10.7  |

*P<0.05, **P<0.01, vs CCl₄-treated control group.

**Effects of PD on GSH content in culture medium (Table 2).**
The content of GSH in culture medium decreased obviously as compared with that in normal hepatocytes after 6 h incubation with CCl₄ (Table 2). On the other hand, PD of various concentrations could
improve GSH in a dose-dependence manner, and 10 μmol/L PD showed a most significant activity.

Table 2 Effects of PD on GSH content in culture supernatant (mean ± SD, n = 8)

| Group | GSH (ng/L) after CCl4 challenge |
|-------|---------------------------------|
|       | 6 h | 12 h | 24 h | 48 h |
| Normal | 4.0±0.4<sup>a</sup> | 4.5±0.6<sup>a</sup> | 4.8±0.4<sup>a</sup> | 4.6±0.7<sup>a</sup> |
| Control | 15.5±1.8 | 16.0±2.7 | 17.5±2.1 | 19.0±2.4 |
| PD 10<sup>-7</sup> | 13.5±2.0 | 13.8±3.3 | 13.0±4.3<sup>b</sup> | 14.5±1.8<sup>b</sup> |
| PD 10<sup>-6</sup> | 11.4±1.7<sup>a</sup> | 12.0±1.8<sup>a</sup> | 12.1±3.1<sup>a</sup> | 12.5±2.0<sup>a</sup> |
| PD 10<sup>-5</sup> | 6.5±1.2<sup>b</sup> | 6.7±1.2<sup>b</sup> | 7.5±2.3<sup>b</sup> | 8.2±2.7<sup>b</sup> |
| PD 10<sup>-4</sup> | 8.7±3.5<sup>b</sup> | 8.9±2.8<sup>b</sup> | 9.8±2.6<sup>b</sup> | 10.3±3.0<sup>b</sup> |

<sup>a</sup>P<0.05, <sup>b</sup>P<0.01, vs CCl4 treated control group.

Effects of PD on cell survivability in primary culture rat hepatocytes

The results of MTT assay showed that normal hepatocytes had high level of cell viability (91.0%±7.9%) and CCl4 induced marked decrease of hepatocytes survivability (35.4%±3.8%, P<0.01 vs normal group), whereas the level of cell survivability could be significantly enhanced by PD at the concentrations of 10<sup>-7</sup> mol/L - 10<sup>-4</sup> mol/L to 56.1%±5.2% (P<0.05, vs CCl4-treated control group), 65.8%±5.0% (P<0.05), 88.7%±6.8% (P<0.001) and 75.2%±7.3% (P<0.01) respectively. It reached a maximum value at 10<sup>-5</sup> mol/L and slightly declined when the concentration of PD was up to 10<sup>-4</sup> mol/L (Figure 1).

Figure 1 Effects of PD on cell survivability in primary culture rat hepatocytes.

1. CCl4-treated control group; 2. normal hepatocytes; 3. PD 10<sup>-7</sup> mol/L; 4. PD 10<sup>-6</sup> mol/L; 5. PD 10<sup>-5</sup> mol/L; 6. PD 10<sup>-4</sup> mol/L.

Discussion

*P. cuspidatum* has been used to treat some chronic liver diseases such as hepatitis and hepatocirrhosis. We have been trying to search for hepatoprotective compounds of *P. cuspidatum*. Our previous in vitro studies showed that emodin, another active compound, had a hepatoprotective effect<sup>[11]</sup>. The present in vitro study also indicated that PD had a protective effect against CCl4 induced injury to primarily cultured rat hepatocytes. Since the extraction and isolation of PD are relatively simple and have a high content of 1.23% in the root of *P. cuspidatum*<sup>[7]</sup>, we may take these advantages to further study its mechanisms of hepatoprotective effect and develop a new drug from it.

CCl4 is a well-known example of a chemical that produces free radical-mediated liver injury. It generates CCl4 by the activation of liver cytochrome P-450, initiating lipid peroxidation of bio-membranes<sup>[12]</sup>. In the present experiment, it was found that CCl4 induced both the increase of GPT in supernatant and the elevation of MDA in rat hepatocytes. However, administration of 10<sup>-7</sup> mol/L - 10<sup>-4</sup> mol/L PD could partly reduce GPT and MDA. Therefore, there may be two possible mechanisms contributing to the hepatoprotective actions of PD. One is that PD inhibits further production of lipid peroxidation in rat hepatocytes, and the other is that it inhibits the destructive action of lipid peroxidation on liver cells.

GSH is an important endogenous anti-oxidant substance. The decrease of GSH content may be due to increased GSH consumption as it participates in the detoxification system for the metabolism of CCl4, and results in an enhanced susceptibility of hepatocytes to CCl4 toxicity<sup>[13]</sup>. Our results showed that CCl4 obviously decreased GSH content in the...
hepatocytes, but PD could partly reverse it. This suggested that the nature of PD protecting-SH compounds (such as GSH) from CCl4 injury may be the third mechanism of its hepatoprotection. It is interesting that PD of 10^{-5} mol/L was more effective than that of 10^{-4} mol/L, at the same time, the hepatoprotective action of PD was in dose dependence at concentrations of 10^{-7} mol/L - 10^{-5} mol/L. Its mechanisms of action need to be further studied.

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