Regular Article

Plasma Retinol Binding Protein for Monitoring the Acetaminophen-induced Hepatotoxicity

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Summary: Retinol-binding protein (RBP) is a specific transport protein which carries retinol in the circulation. RBP concentration in plasma and liver of rats following a large dose of acetaminophen (APAP) intraperitoneally was examined. The RBP concentration in plasma decreased significantly at 12 hr after the APAP administration, while the plasma albumin concentration was affected a little. Western blot and northern blot analyses showed marked changes in RBP but not in albumin. Thus, RBP was suggested to be more sensitive for the acute drug-induced hepatotoxicity than albumin. The decrease of RBP concentration in plasma was suggested to be caused by the dysfunction of RBP synthesis in the liver.

Key words: retinol binding protein; hepatotoxicity; acetaminophen; albumin

Introduction

Retinol-binding protein (RBP), a specific transport protein for retinol in the circulation, is mainly synthesized in the liver and catabolized in the kidney and forms a complex with retinol as 1:1 molecular ratio in the hepatocytes. After the secretion from the hepatocytes, RBP-retinol complex associates with transthyretin (TTR) in the liver, forming retinol-RBP-TTR complex. This complex formation prevents the renal glomerular filtration of RBP. Retinol is delivered to the target cells through the specific RBP receptor on the cell membrane by dissociating from the RBP-TTR complex. It is known that RBP concentration in plasma is sensitively variable in some diseases such as hepatitis and cirrhosis. RBP in plasma of patients with liver disease decreases due to the reduced synthesis of RBP in liver parenchymal cells. It increases in patients with kidney disease and diabetes due to the reduced glomerular filtration of RBP. Therefore, RBP should be potentially a good indicator of clinical and nutritional status, compared with other plasma proteins like albumin, because the half-life time of RBP in plasma is short and the concentration in plasma is low.

The drug-induced hepatotoxicity is generally evaluated by the release of cytosol enzymes, e.g. lactate dehydrogenase (LDH) and alanine aminotransferase (ALT), from hepatocytes, suggesting the injury of membranes. In addition to such releasing enzymes, the examination of physiological function of liver such as protein synthesis would be useful to evaluate the drug-induced hepatotoxicity more exactly. A concentration change of plasma RBP would be largely related to the change of RBP protein synthesis and/or that of RBP intracellular transport in the hepatocytes. Thus, RBP could be a potent marker to evaluate the drug-induced hepatotoxicity from the viewpoint of the change in the physiological function of liver.

Acetaminophen (APAP) is widely used as an analgesic-antipyretic drug, but a large dose of APAP causes the severe hepatotoxicity in human and animal models. The APAP-induced hepatotoxicity, a typical example of the drug-induced hepatotoxicity, has been widely studied. In the present paper, RBP in plasma of rats treated with a large dose of APAP was studied in order to examine the usefulness of RBP as a marker of the drug-induced hepatotoxicity.

Materials and Methods

Chemicals: DEAE-cellulose (DE-52) was purchased from Whatman International Co., Ltd. (Kent, UK). Sephadex G-100 Superfine and ECL Western Blotting Detection Reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Albumin, LDH-UV and GPT-UV Test Wako were from Wako Pure Chemicals Co., Ltd. (Tokyo, Japan). Freund's complete adjuvant was purchased from Nakalai Tesque Co., Ltd. (Kyoto, Japan). Anti-albumin antiserum was...
purchased from Inter-cell Technologies, INC (New Jersey, USA). APAP, all-trans retinol and bovine serum albumin were purchased from Sigma Chemical Co. (St.Louis, USA). All other chemicals were of analytical grade.

**Treatment of animals:** Male Wistar rats (8~9 weeks old), obtained from Takasugi Experimental Animals (Saitama, Japan), were treated intraperitoneally with β-naphthoflavone (80 mg/kg body weight) for 3 days. Then APAP dissolved in corn oil (500 mg/kg body weight) was intraperitoneally administered to the rats. Corn oil alone was administered to the rats as the control. Blood samples were withdrawn by heart puncture at 0, 12 and 24 hr after the APAP administration. They were housed under 12 hr light/dark cycle at an ambient temperature of 25°C.

**Purification of rat RBP:** Rat RBP was purified from rat plasma using DE-52 and Sephadex G-100 according to a slightly modified method of Blaner & Goodman.12

**Preparation of anti-RBP antiserum:** Purified rat RBP, after dialyzed, was diluted to 1 mg protein/mL with 0.9% NaCl and emulsified in an equal volume of Freund’s complete adjuvant. A rabbit was immunized with the emulsion containing 1 mg RBP by intradermally injecting it at 10 sites of its back. The rabbit was further immunized by injecting the emulsion containing 0.25 mg RBP on its back at 1 month after the first injection. Anti-RBP antiserum was taken from the vein of ear at 2 weeks after the second injection. The rabbit anti-RBP antiserum was prepared by centrifuging it at 2000 × g for 10 min.13

**Determination of RBP:** 125I-RBP was prepared using lactoperoxidase according to the method of Marcalonis.16 Plasma RBP concentration was determined using the antiserum according to the radioimmunoassay method of Blaner & Goodman13 with a slight modification.

**Determination of all-trans retinol:** Plasma retinol concentration was determined by HPLC. TSK-gel (18 cm × 4.6 mm; TOSOH, Tokyo, Japan) was used for the chromatograph column. All-trans retinol and retinyl acetate as an internal standard were monitored by the absorbance at 325 nm. The mobile phase was acetonitrile-tetrahydrofuran-1% ammonium acetate-methanol (65:25:7:3) at a flow rate of 1.0 mL/min.15

**Determination of LDH, ALT and albumin in plasma:** The activities of LDH and ALT were measured using LDH-UV and GPT-UV Test Wako. Albumin concentration in plasma was measured by the enzyme-linked immunosorbbent assay.16

**Western blot analysis:** Liver homogenates were subjected to 10% SDS-polyacrylamide gel electrophoresis. The fractionated proteins were electrically transferred onto a PVDF membrane filter using 25 mM Tris-HCl buffer (pH 8.4) containing 192 mM glycine and 20% methanol for 1 hr at 15 V. The membrane was blocked overnight at 4°C with TBS containing 3% BSA and 0.05% Tween 20. Then it was incubated for 2 hr at room temperature with TBS containing 0.1% BSA, 0.05% Tween 20 and rat RBP or albumin antibodies at final dilution of 1:500, then washed three times with TBS containing 0.05% Tween 20, and incubated for 1 hr at room temperature with TBS containing 0.1% BSA, 0.05% Tween 20 and anti-goat antibody at final dilution of 1:2000. Then it was washed three times with TBS containing 0.05% Tween 20, and detected by ECL method.

**RNA isolation:** Male Wistar rats (7 weeks old) were killed to excise the liver at 0 and 12 hr after the APAP administration (500 mg/kg body weight). Total RNA was prepared from 1 g of liver tissue by the phenol-chloroform procedure. mRNA was prepared from total RNA (500 μg) by oligotex-dT30. mRNA concentration was determined by the UV absorption at 260 nm.

**Northern hybridization:** Northern hybridization was performed according to the method described previously. Poly (A)+ RNA (10 mg) was separated on 1% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane (Biodyne A, Pall Gelman Laboratory, Ann Arbor, MI, USA), prior to fixation by baking for 2 hr at 80°C. Blots were prehybridized in hybridization buffer containing 4 × SSC, 5 × Denhardt’s solution, 0.2% SDS, 0.1 mg/mL sonicated salmon sperm DNA and 50% formamide at 42°C for 2 hr. Specific cDNA probes for rat RBP, albumin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were prepared from the sequence between bases 1 and 519 (519 bp) of rat RBP partial cDNA sequence (genbank accession M10934), 195 and 630 (436 bp) of rat albumin (genbank accession NM_134326) and 523 and 1003 (481 bp) of rat G3PDH (genbank accession M17701). Hybridization was performed overnight in the same buffer containing 106 cpm/mL of [32P] labeled cDNA probes prepared by a random primed labeling method (Rediprime, Amersham International Ltd, Little Chalfont, UK). The hybridized membrane was washed in 2 × SSC and 0.1% SDS at 55°C for 10 min, followed by washing in 0.1 × SSC and 0.1% SDS at 55°C for 10 min. Filters were exposed to Fuji imaging plates (Fuji Photo Film Co., Ltd., Kanagawa, Japan) for 24 hr and analyzed by a BAS imaging analyzer (Fuji Photo Film Co., Ltd., Kanagawa, Japan).

**Statistical analysis:** Data were expressed as mean ± SD. Differences between experimental groups were assessed by analysis of variance (ANOVA). A significant difference was accepted when p < 0.05.

**Results**

LDH, ALT, retinol and RBP in plasma of rats treated with APAP: Blood was withdrawn from the heart of
rats at 0, 12 and 24 hr after the APAP administration. Clinical test parameters of hepatotoxicity in plasma were measured. LDH and ALT activities in plasma of the APAP administered rats increased significantly, compared with those of rats at 0 hr after the APAP administration, respectively (Figs. 1 and 2). Retinol concentration in plasma decreased by the APAP administration, although not significant (Fig. 3). RBP concentration in plasma significantly decreased at 12 hr after the APAP administration, compared with that of rats at 0 hr after the APAP administration (Fig. 4).

**Comparison between RBP and albumin concentrations in plasma of rats treated with APAP:** RBP concentration in plasma at 12 hr after the APAP administration to rats decreased significantly, compared with that at 0 hr, while albumin concentration in plasma was not affected so much as RBP (Fig. 5).

**Protein expression of RBP and albumin in liver of rats treated with APAP:** RBP and albumin in liver at 12 hr after the APAP administration to rats were examined by Western blot analysis (Fig. 6). The protein expression of RBP was markedly reduced, compared with the control, but that of albumin was scarcely affected.

**mRNA expression of RBP and albumin in liver of rats treated with APAP:** mRNA levels for RBP and albumin in liver at 0, 6, 12 and 24 hr after the APAP administration to rats were examined by Northern blot analysis (Fig. 7). The mRNA level for RBP in liver of rats treated with APAP markedly decreased at 12 hr after the APAP treatment and then was recovered to the initial level at 24 hr. On the other hand, the mRNA level
for albumin in liver of rats treated with APAP was scarcely changed.

**Discussion**

APAP is a widely used analgesic-antipyretic drug. A large dose of APAP causes the hepatotoxicity in human and experimental animals. The increased production of reactive metabolite, N-acetyl-p-benzoquinoneimine

![Graph](image)

**Fig. 5.** Comparison between RBP and albumin in plasma of rats treated with APAP.

RBP and albumin in plasma were measured at 0 and 12 hr after the APAP administration. Data represent the means ± S.D. of 3 rats and are expressed as the ratio (%) of RBP and albumin at 12 hr to those at 0 hr, respectively. *p<0.05, significantly different from the control rats.

![Graph](image)

**Fig. 6.** RBP and albumin in liver of rats treated with APAP.

(A) Western blot analysis of RBP and albumin in liver of rats treated with APAP at 12 hr after the APAP administration. The bands correspond to 20 kD for RBP and 68 kD for albumin. (B) Results are expressed as percent ± S.D. of RBP and albumin of control rats, respectively.

![Graph](image)

**Fig. 7.** Effect of APAP on the mRNA expression in liver.

(A) Northern blot analysis of RBP and albumin in liver at 0, 6, 12 and 24 hr after the APAP administration. (B) Relative intensity expressed as% of intensity of rats at 0 hr (control) (n = 3). *p<0.05, significantly different from the rats at 0 hr after the APAP administration.
(NAPQI) depletes the hepatic GSH pool and enhances the covalent binding of NAPQI to the thiol groups of cellular proteins, leading to the dysfunction of the hepatocytes. In addition, NAPQI binds directly to DNA and cytosolic and mitochondrial proteins, resulting in the necrotic cell death. Simultaneously, lipid peroxidation occurring during the APAP metabolism contributes to the APAP-induced hepatotoxicity.

LDH and ALT are indicative of the damage of liver and other organs. In particular, ALT is specific for the hepatotoxicity. In the present experiment, LDH and ALT in plasma of the APAP-treated rats were elevated (Figs. 1 and 2). On the other hand, APAP is known to cause the nephrotoxicity. RBP concentration in plasma will be affected by not only the hepatotoxicity but also the nephrotoxicity. Thus, BUN and creatinine in plasma of rats treated with APAP were examined. They were not affected by the APAP treatment (BUN: 13.38 ± 2.29 (mg/mL) at 0 hr after the APAP administration, 16.77 ± 6.32 (mg/mL) at 12 hr after the APAP administration; creatinine: 0.58 ± 0.13 (mg/mL) at 0 hr, 0.77 ± 0.19 (mg/mL) at 12 hr). Thus, the hepatotoxicity occurred at the large dose of APAP used here but the nephrotoxicity seemed not to occur.

Transferrin, TTR and RBP have short half-life times in blood and their concentrations in plasma are low. These proteins are classified as rapid turnover proteins and considered to be affected sensitively in patients with various diseases of liver and kidney. On the other hand, albumin is a representative plasma protein that is synthesized in the hepatocytes as well as RBP and is used for clinical test and monitoring the nutritional state. The half-life time in plasma of albumin (20 days) is much longer than that of RBP (16 hr). We previously reported the decrease of RBP in plasma of the D-galactosamine administered rats, suggesting that RBP in plasma is useful to monitor the drug-induced hepatotoxicity. Thus, the present study was intended to clarify whether RBP can monitor the APAP-induced hepatotoxicity by comparing RBP in plasma with the conventional markers such as LDH, ALT and albumin. Plasma RBP was shown to decrease significantly at 12 hr after the APAP administration (Fig. 4). However, the APAP treatment of rats did not affect the plasma albumin concentration so much (Fig. 5). This may possibly be due to the difference of the half-life times in plasma of both proteins.

RBP level in plasma is probably influenced by the physiological conditions of animals, for example, hormonal and nutritional factors. LDH and ALT are cytosol enzymes, and the increase of them in plasma suggests the cell membrane damage. On the other hand, the decrease of RBP in plasma suggests the damage in the processes of synthesis and/or export of RBP from hepatocytes. In order to clarify the mechanism for the change of RBP concentration in plasma, we measured the protein and mRNA levels of RBP in liver (Figs. 6 and 7). RBP content in liver of the APAP-treated rats decreased about 20% at 12 hr after the APAP administration, compared with the control rat, but albumin content was scarcely changed. mRNA expression of RBP in liver of the APAP-treated rats decreased about 31% at 12 hr after the APAP administration, compared with that at 0 hr, but mRNA expression of albumin was scarcely changed. The rapid recovery of RBP mRNA expression at 24 hr seemed to improve the impaired protein synthesis in the liver. On the other hand, albumin mRNA was shown to be scarcely changed. These results suggest that the decrease of RBP concentration in plasma of rats treated with APAP is due to the reduced synthesis of RBP in the liver and that RBP is useful to evaluate the change in function of liver in the drug-induced hepatotoxicity.

In conclusion, RBP in plasma was shown to monitor the APAP-induced hepatotoxicity from the different viewpoint from the conventional markers of hepatotoxicity such as LDH and ALT. Further, RBP was suggested to be more sensitive for the acute drug-induced hepatotoxicity than albumin. In addition, the decrease of RBP in plasma was suggested to be caused by the dysfunction of RBP synthesis in the liver.

References


