J. Hard Tissue Biology (Supplement). 14(4)359-360, 2005

A Cytoprotection of Rat Hepatocytes Mediated by the Intracellular Calcium

Toshihiro Tachibana , Akiko Kusumoto ,Yukari Ueda , Zenei Taira

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima 770-8514, Japan
(Accepted for publication, Junuary 20, 2006)

Abstract: The role of intracellular Ca^{2+} in CCl_4 hepatotoxicity in rats was clarified by measuring detailed changes in serum ALT values and intracellular Ca^{2+} after CCl_4 administration. Serum ALT activity clearly increased up to a peak value at 1 day after i.p. administration of CCl_4 at 0.2 ml/kg, and then decreased to the control level after 3 days. Intracellular Ca^{2+} responded to CCl_4 intoxication with a biphasic increase. The first intracellular Ca^{2+} peak increased up to 5.6-fold the control level at 1 hour, and the second Ca^{2+} peak 6.5-fold at 3.5 days. The results showed that the first Ca^{2+} increase might be attributable to a signal arising from the extent of hepatic injury. The second Ca^{2+} increase might act to activate cellular protective functions. We discussed that sustained intracellular Ca^{2+} and S100A4 increases may be involved in autoprotection or adaptive cytoprotection, namely susceptibility against a toxication.

Key words: Basic study, hepatotoxicity, intracellular Ca^{2+}

Introduction

The physiological roles of Ca^{2+} ions are to regulate a wide variety of cellular processes such as hormonal responses, enzymatic activation, cell division and cell death. The intracellular Ca^{2+} also plays a potential role in carbon tetrachloride (CCl4) induced liver injury. Schanne et al. (1979) concluded that intracellular accumulation of Ca^{2+} ion is the final common pathway by which toxic cell death occurs. However, we have found that the intracellular Ca^{2+} concentration ($[Ca^{2+}]$) increase induced by CCl_4 injury acts the roles of cytoprotectives (Taira et al., 2002). The serum ALT activity clearly increased up to a peak value of 29-fold the control value 1 day after i.p. administration of CCl_4 at 0.2 ml/kg, and then decreased to its control level after 3 days. Then, $[Ca^{2+}]_i$ in hepatocytes prepared from CCl_4 administered rats increased steeply to the first peak one hour after CCl_4 administration, and then decreased to the control level 2 hours later. The values gradually increased again to reach to the second peak 3 days after CCl_4 administration before again returning to the control level in 4 days. We have further concluded that the second Ca^{2+} increase acts to activate cellular protective functions, based on the comparative study used drugs, including nifedipine, verapamil, thioridazine, dantrolene, quinacrine, compound 48/80, baicalin, cimetidine, and cycloheximide.

In this study, we further investigated the role of $[Ca^{2+}]_i$ in CCl_4 hepatotoxicity in rats.

Materials and Methods

Chemicals

CCl_4, bovine serum albumin (BSA), sodium dodecyl sulfonate (SDS) and fura 2-AM were purchased from Wako Co. (Osaka, Japan). All other reagents were of the highest grade available from commercial sources.

Animal treatment

Male Donryu ratswere obtained from SLC Co. Ltd. (Shizuoka, Japan), and had free access to a commercial diet and water during the experimental period. Some rats were given an i.p. injection of 0.2 ml/kg of CCl_4 as 20% (v/v) CCl_4 in olive oil on the first day (day 0), and then blood was drawn daily from the tail vein of these etherized rats with a heparinized syringe. Serum ALT activity was measured spectroscopically using a diagnostic kit from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Isolation of hepatocytes

Hepatocytes were prepared from livers in normal or CCl4 fed rats by circulating collagenase perfusion of the liver. Cells were washed, and resuspended in Krebs-Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM $CaCl_2$. The hepatocytes were prepared to $3x10^6$ cells/ml. The cells were loaded with fura2-AM, and cells displaying high fluorescence were selected for sorting by flow cytometer

Assay of cell viability in hepatocyte suspension

Propidium iodide (PI) fluorescence was used to quantitate percent cell viability in suspensions of isolated hepatocytes. Freshly isolated hepatocytes (5,000 cells/ml) were incubated in Krebs-Ringers buffer (pH 7.4), containing 2 mg/ml BSA, 1 µM PI and 25 mM NaHEPES. Total fluorescence of PI was monitored with fluorometer using 520 nm excitation and 605 nm emission filters. After measuring basal fluorescence (A), 50 mM CCl4 was added, and total fluorescence was measured for 150 minutes. Individual experiments were terminated by adding 375mM digitonin to permeabilize all cells, and a final fluorescence measurement (B) was obtained 20 minutes later. Percent cell viability (V) at time points during the incubation was calculated by the formula where X is fluorescence at any given time: V(%) = 100 x (B-X) / (B-A).

Pharmacokinetics of salicylamide (SAM)

CCl_4 (0.2 ml/kg) was administered intraperitoneally at 20% (v/v) solution in olive oil. At 0-5 days after CCl_4-treatment, rats were anesthetized with diethyl ether, and polyethylene cannulas were inserted into the femoral artery and vein. SAM was intravenously administered and blood was collected by 90 min. SAM was extracted from blood samples and its fluorescence was measured at em. 415 nm and ex. 335 nm. Pharmacokinetic parameters of SAM were calculated by the moment analysis. AUC were calculated as follows: $AUC(0\text{-}\infty) = AUC(0\text{-}t) + C(t)/k_e$. Where t is the time of the last plasma concentration and k_e is the elimination rate constant calculated as the slope of the plasma concentration. AUC(0-t) was calculated using the trapezoidal rule with linear interpolation.

Assay of aniline metabolism

Aniline metabolism was measured by a 96-well microplate assay using the phenol–indophenol method. Each aliquot contained 0.3

M Tris chloride buffer pH 7.4, 0.15 M $MgCl_2$, microsomes, 5 mM NADPH and 5 mM NADH. The reaction was completed at 37°C with samling at some interval times by 9 min. The sample was treated to stop the reaction. After centrifugation, the supernatant was mixed with phenol (1% w/v) and 1 M Na_2CO_3, and allowed to stand at 37°C for 30 min. The absorbance of the solution was measured at 620 nm. The Michaelis constant, K_m, and the maximum velocity constant, V_{max}, were obtained from the Hofstee plot.

Results are expressed as mean±S.D. Statistical significance between two mean values was assessed using Student's *t*-test for paired data. A difference was considered significant when *P* was 0.05 or less.

Results

Cell viability in hepatocyte suspension

Cell viability in suspensions of hepatocytes was measured by total fluorescence of PI. Since fluorescence of PI is enhanced after nuclear binding, we used PI fluorescence to monitor the percent of nonviable cells in suspensions of hepatocytes.

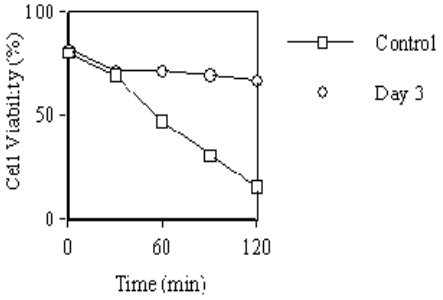

Fig. 1. Cell viability in hepatocyte suspension after i.p. CCl4 administration.

Loss of cell viability after PI began with a characteristic latency of about 30 minutes, and 50% cell killing occurred after about an hour (Fig. 1). Thus, the kinetics of cell killing for freshly isolated hepatocytes resembled closely for day 1 cultured hepatocytes. Using the PI assay, cell killing after exposure was determined.

Effect of CCl_4–induced hepatotoxicity on pharmacokinetics of SAM

Fig. 2(a) shows the time-courses of plasma concentrations of SAM, administered intravenously at a dose of 137 mg/kg to rats at various days after a single ip administration of CCl_4 (0.2 ml/kg), and then analyzed using the moment analysis. The results of AUC showed that the liver function in the rats decreases significantly at days one and three after the administration of CCl_4, that is, an increase of AUC.

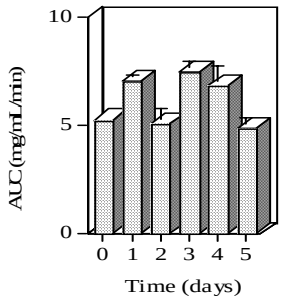

Fig. 2. Changes of AUC values of SAM in rats administered CCl_4.

Although AUC values at days three and four also increased up to 41.4 and 32.2% of the control value, respectively. These results may show that the decreased liver function at day one is directly attributable to damage by CCl_4-induced hepatic injury, but at later days may be attributed to the physiological changes effected by the prolonged hepatotoxication of CCl_4, such as regeneration.

Effect of Ca^{2+} on P450 activity

We studied the inhibitory effects of Ca^{2+} ion on metabolism of aniline using liver microsomes from acetone-treated rats. The apparent kinetic parameters were estimated using Hofstee plots, as shown in Fig.3.

The apparent kinetic parameters for aniline metabolism were determined to be a K_m 0.171 mM and V_{max} 1.595 mM/min. Furthermore, at 5 mM Ca^{2+}, V_{max} value decreased 54% less than that of Ca^{2+} free.

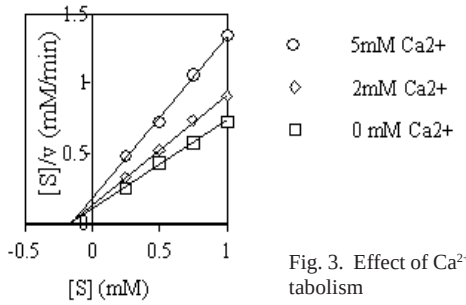

Fig. 3. Effect of Ca^{2+} on the aniline metabolism

Discussion

As mentioned above, Schanne et al. (1979) concluded that intracellular accumulation of Ca^{2+} ion is the final common pathway by which toxic cell death occurs. However, we showed that the intracellular Ca^{2+} increase acts the role of the cytoprotection after the CCl_4 intoxication. In this study, we conformed that the cells induced the intracellular Ca^{2+} increase have protective against PI intoxication. Mehendale et al. (1994) showed this phenomenon involved in autoprotection, whereby prior exposure to a small dose of a chemical results in protection against a subsequently administered lethal dose of the same compound; and further that such protection against CCl_4 toxicity is closely associated with active hepatocyte regeneration. We also showed, using pharmacokinetic measurements, in which the metabolic activity of SAM decreases in rats in such a regenerating period after CCl_4 administration at 0.2 ml/kg (Taira et al., 2004). Therefore, the present study is first demonstration in vivo that intracellular Ca^{2+} acts to protect cells or life from intoxication.

Acknowledgements.

This work was supported by MEXT.HAITEKU, 2003-2007.

References

1) Taira Z, Yabe K, Hamaguchi Y, Hirayama K, Kishimoto M, Ishida S, Ueda Y (2004) Effects of Sho-saiko-to extract and its components, baicalin, baicalein, glycyrrhizin and glycyrrhetic acid, on pharmacokinetic behavior of salicylamide in carbon tetrachloride intoxicated rats. Food Chem Toxicol 42, 803-807
2) Taira Z, Yabe K, Miyake S, Inui Y, Hashida M, Yamada S, Ueda Y. (2002)
3) Intracellular Ca2+ mediating biodefense signal on CCl4-induced Autoprotection. Nippon Yakurigaku Zasshi. 2002 120, 119P-121P.
3) Mehendale HM, Thakore KN, Rao CV (1994) Autoprotection: stimulated tissue repair permits recovery from injury. J Biochem Toxicol 9, 131- 139
4) Schanne FA, Kane AB, Young EE, Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. Science 206, 700-702