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A Protective Function Against Radical Stress Mediated by the Intracellular Calcium in Rat Hepatocytes

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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. 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. We further examined the effects of drugs on serum ALT activity and intracellular Ca^{2+} in rats administered at 1 hour prior to or 3 hours after CCl_4 administration. 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+} increases may be involved in autoprotection, namely susceptibility against a toxication.

Key words: Basic research, hepatic injury, intracellular Ca^{2+}, carbon tetrachloride

Introduction

Calcium is usually utilized as signal transduction molecules, as cells internalize these stimulatory signals from the extracellular environment across the plasma membrane, and also send intracellular signals to receptors. Cells normally maintain cytoplasmic Ca^{2+} at very low levels, such as 10^{-7} M, but the concentration briefly raises several folds in response to physiological stimuli. The role of Ca^{2+} ions in cells has been well established to regulate a wide variety of cellular processes, such as hormonal responses and cell death. Numerous studies have documented that intracellular Ca^{2+} plays a potential role in chemically induced liver injury, including that caused by carbon tetrachloride (CCl_4). CCl_4 is metabolized by cytochrome P450 to form highly reactive trichloromethyl radicals, $\bullet CCl_3$. Intracellular glutathione (GSH) is exhausted to neutralize these radicals, and thus depletion of GSH causes oxidative stress, and irreversible damage to hepatocytes as a result of the increased cytosolic Ca^{2+} concentration, and further lead to cell death and liver injury. Schanne et al. (1979) concluded that intracellular accumulation of calcium is the final common pathway by which toxic cell death occurs. However, we examined serum ALT values and intracellular Ca^{2+} in hepatocytes prepared from rat after administrations of both CCl_4 and a drug that affects intracellular Ca^{2+} homeostasis or CCl_4 metabolism, and deduced that the Ca^{2+} increase should act to activate cellular protective functions. Regarding the mechanism of the intracellular Ca^{2+} increase, CCl_4-induced inactivation of Ca^{2+}/Mg^{2+}-ATPase has been demonstrated in several studies; so that a pool of calcium may be released into the cytoplasm as a consequence of inhibition of the endoplasmic reticulum (ER) calcium pump Ca^{2+}/Mg^{2+}-ATPase by CCl_4, while the cytosolic concentration of Ca^{2+} in hepatocytes exposed to CCl_4 is elevated.

In this study, we further investigated the mechanism of intracellular Ca^{2+} increase in CCl_4 hepatotoxicity in rats.

Materials and Methods

Chemicals

Carbon tetrachloride (CCl_4), bovine serum albumin (BSA), sodium dodecyl sulfonate (SDS) and fura 2-AM were purchased from Wako Co. (Osaka, Japan). Quinacrine dihydrochloride, compound 48/80, sodium dantrolene, nifedipine, verapamil hydrochloride and cimetidine were purchased from Sigma (Missouri, USA). All other reagents were purchased from commercial sources and were of the highest grade available.

Animal treatment

Seven- to ten-week-old male Wistar rats, weighing 250–300g, were obtained from SLC (Shizuoka, Japan). Rats (230–330 g), fed on a standard laboratory diet and maintained at 22°C under a constant 12-h light/12-h dark cycle, were used throughout. Animals had free access to food and water during the experimental period.

Isolation of hepatocytes

Hepatocytes were prepared from fed rats by circulating collagenase perfusion of liver. Livers were perfused at a flow rate of 20 ml/min with 50 ml Ca^{2+}-free Krebs–Ringer bicarbonate buffer, pH 7.4, containing 5 mM glucose, 1 mM $CaCl_2$ and 0.2 mg/ml collagenase. The livers were then removed and gently agitated in 40–60 ml Krebs–Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM $CaCl_2$. Cells were washed free of collagenase by two cycles of centrifugation at 40 g for 5 min and resuspension in Krebs–Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM $CaCl_2$. Hepatocyte density was measured with a hemacytometer and adjusted to $3x10^6$ cells/ml.

Microspectrofluorometry system equipped HPLC

The intracellular Ca^{2+} in a hepatocyte was monitored using a microspectrofluorometry system (Nikon ECLIPSE TE 300; Nikon co.). The system was equipped with a perfusion chamber of 70 µl with a coverslip for attachment of the cells. The chamber was further bound to an HPLC pump to stream an on-line carrier PBS solution and to inject various cell treatment solutions from its sampling block. Fluorescence was monitored at an excitation wavelength of 340 or 380 nm and an emission wavelength of 500 nm. Hepatocytes ($5x10^5$ cells/ml) were incubated in minimal essential medium for 1 h and allowed to attach to collagen-coated coverslips on the bottom of the culture dish. The cells were incubated during the monitoring period in the perfusion chamber which was perfused by the HPLC pump with a stream of 1 ml/min PBS. The fluorescence was monitored for 10 min. The

fluorescence output was digitized and analyzed using the Nikon image analysis system, AQUACOSMOS ORCA-ER.

Results

Effect of CCl_4 on Ca^{2+} release from hepatocytes

To examine effect of CCl_4 on Ca^{2+} release from hepatocytes, we measured CCl_4 (0.5 mM) induced a $[Ca^{2+}]i$ transient in hepatocytes perfused with TBS solution involving Ca^{2+} or not. As shown in Fig. 1, CCl_4 was induced a transient $[Ca^{2+}]i$ increase in both conditions. This means that $[Ca^{2+}]i$ was mobilized within the cells mediated by IP3R or RyR, and by mitochondria.

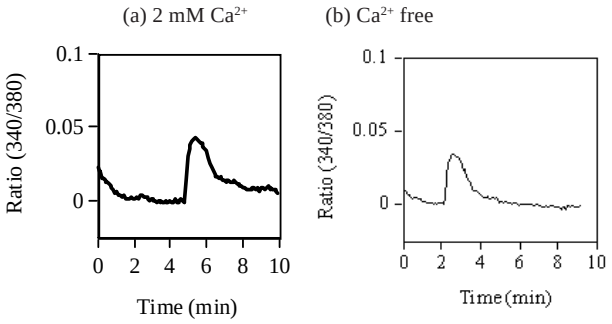

Fig. 1. CCl_4-induced Ca^{2+} release in hepatocytes

Ca^{2+} ion transport through the plasma membrane, the VDCC channel etc.

We further examined which channel in the plasma membrane uses to mobilize the $[Ca^{2+}]$. As shown in Fig. 2, the CCl_4-induced Ca^{2+} release was not blocked by the VDCC antagonist, verapamil and nifedipine, and further by the Ca^{2+} pump antagonist, Na_3VO_4.

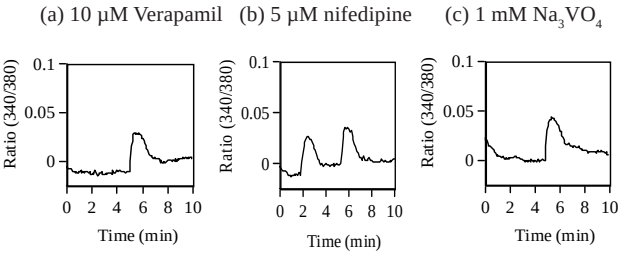

Fig. 2. Effect of Ca^{2+} ion transport throughthe plasma membrane on the CCl_4-induced Ca^{2+} release in hepatocytes

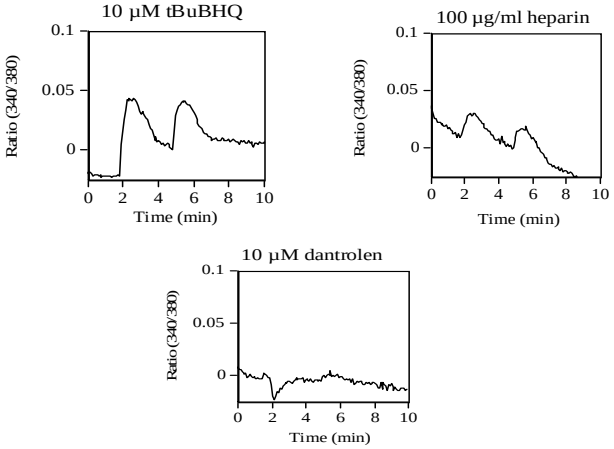

Fig.3. Effect of Ca^{2+} ion transport throughthe ER membrane on the CCl_4-induced Ca^{2+} release in hepatocytes

Ca^{2+} ion transport through ER membrane

The $[Ca^{2+}]i$ transient was not blocked by heparin (100 µg/ml) (IP3R inhibitor), but completely did by dantrolen (10 µM) (RyR inhibitor, as shown in Fig. 3. Thus, the $[Ca^{2+}]i$ transient was mobilized mediated by RyR channel. Furthermore, this means that the Ca^{2+} increase does not causes of cell membrane injury. We further examined that the Ca^{2+} increase causes mediated by IP3R from PLC activation.

PLA2 and calmoduline

We further examined effect of PLC and PL A2 inhibitors on the CCl4-induced Ca2+ release in hepatocytes.

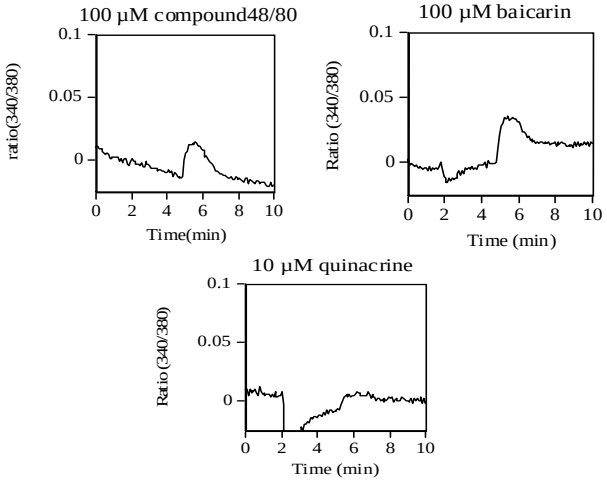

Fig. 4. Effect of PLC or PL A2 inhibitor on CCl4-induced Ca^{2+} release in hepatocytes

Discussion

We investigated the role of intracellular Ca^{2+} signals transmitted in regenerating cells during tissue injury and recovery after CCl_4 intoxication. As noted by Long and Moore (1986), serum ALT changes should be a signal from dead or dying cells, but the present study showed that intracellular Ca^{2+} might act as a signal in living cells isolated from damaged tissue after intoxication. Therefore, the first Ca^{2+} peak could be a signal produced from living cells immediately after CCl_4 intoxication, followed by the ALT and the second Ca^{2+} peaks in hepatocytes during the regenerating period.

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