**Abstract.** We have investigated the bio-defensive function of low-dose LPS pretreatment on CCl4-induced fulminant hepatic failure (FHF) in rats. In male Donryu rats treated with CCl4 at 1.0 ml/kg i.p., the serum alanine aminotransferase (ALT) increased up to a peak at 24 h. The increase in intracellular Ca2+ concentration was biphasic, i.e., a first peak at 1 h and a second at 3 days. However, when rats were treated daily with CCl4 at 1.0 ml/kg, serum ALT successively increased over several h after the second administration, but the rats died of FHF. The first Ca2+ peak occurred 1 h after CCl4 administration, but the concentration did not increase continually, until it reached the second peak. However, rats did not die when they were treated with lipopolysaccharide at 0.5 mg/kg 24 h prior to the initial dose of CCl4 at 1.0 ml/kg. The serum ALT value did not increase after administration of CCl4 at 1.0 ml/kg, but the second Ca2+ peak was sustained at >1,000 nM. We concluded that the second Ca2+ peak acts to protect the liver from injury.

Lipopolysaccharide (LPS) is a component of the outer wall of Gram-negative bacteria. While LPS directly causes liver injury by mechanisms involving inflammatory cells, such as Kupffer cells, and chemical mediators, such as superoxide, nitric oxide, tumor necrosis factor-α (TNF-α) and other cytokines, LPS also potentiates liver damage induced by ethanol (1) and D-galactosamine (2). Furthermore, Zhang et al. (3) estimated that calcium homeostasis disorder might be one of the causes or at least an important mediator of LPS-induced pancreatic acinar cell damage. Furthermore, pretreatment with low doses of LPS in the rat provided protection against subsequent challenge with injurious focal ischemia in the brain (4). The mechanisms involved in LPS pretreatment are not well understood; however, activation of inflammatory pathways appears to play a role. In particular, LPS-induced activation of TNF-α and its downstream signaling mediator ceramide is important for neuroprotection against ischemic injury. Beneficial roles for superoxide dismutase and endothelial NO synthetase have also been postulated, which support the critical involvement of inflammatory pathways in LPS preconditioning. The liver also responds to LPS and produces cytokines.

In general, cells normally maintain a cytoplasmic Ca2+ concentration at very low levels, such as 10⁻⁷ M, but the concentration briefly rises several-fold in response to physiological stimuli. The role of intracellular Ca2+ ions in regulating a wide variety of cellular processes is well established, such as hormonal responses and cell death. Numerous studies have documented that intracellular Ca2+ ions play a potential role in chemically-induced liver injury, including that caused by CCl4. Zhang et al. (3) estimated that calcium homeostasis disorder might be one of the causes, or at least an important mediator, of LPS-induced pancreatic acinar cell damage. Furthermore, Schanne et al. (5) concluded that intracellular accumulation of calcium is the final common pathway by which toxic cell death occurs. However, we showed that intracellular Ca2+ has a role in cytoprotection of hepatocytes (6). Serum alanine aminotransferase (ALT) activity increased up to a peak within 1 day after i.p. administration of 0.2 ml/kg CCl4 and then successively decreased to the control level after 3 days. The intracellular Ca2+ concentration then responded to the CCl4 intoxication with a biphasic increase, with a first phase that rapidly peaked at 1 h, and a second phase that gradually increased to a peak at 3 days, and recovered to control levels by 4 days. We showed that the second Ca2+ peak acts protectively against hepatic injury.

In this study, we investigated the effect of low-dose LPS pretreatment on CCl4-induced fulminant hepatic failure (FHF) in rats, and demonstrated the protective role of
increased intracellular Ca$^{2+}$ concentration in hepatocytes isolated from rats with CCl$_4$-induced FHF.

**Materials and Methods**

**Chemicals.** CCl$_4$, LPS, bovine serum albumin (BSA), sodium dodecyl sulfonate (SDS) and fura 2-AM were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were purchased from commercial sources and were of the highest grade available.

**Animal treatment.** Seven- to 10-week-old male Donryu rats, weighing 250-300 g, were obtained from SLC (Shizuoka, Japan). Rats had free access to food and water during the experimental period, and were maintained at 22°C under a constant 12 h light-dark cycle. Some rats were treated with CCl$_4$ 1.0 ml/kg by i.p. injection (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 etherized rats with a heparinized syringe. Serum ALT activity was measured spectroscopically using a diagnostic kit from Wako Pure Chemical Industries (Osaka, Japan).

**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 resuspended in Krebs-Ringer bicarbonate, pH 7.4, containing 5 mM glucose and 1 mM CaCl$_2$. The hepatocyte density was measured with a hemacytometer and adjusted to 3x10$^6$ cells/ml.

**Measurement of cytosolic Ca$^{2+}$ concentration with fura 2-AM.** The intracellular Ca$^{2+}$ concentration was measured using the fluorescent Ca$^{2+}$ indicator fura 2 as described previously (6). Hepatocytes were loaded with fura 2-AM (50 nM) under continuous shaking for 30 min. After washing and centrifugation (50 g for 2 min) twice in phosphate-buffered saline (PBS) at pH 7.4, the cells were incubated at 37°C for 5 min to hydrolyze the fura 2-AM to free fura 2. The cells were collected by centrifugation at 13,000 xg for 5 sec. The hepatocytes were resuspended in Krebs-Henseleit buffer (without supplements) at a final density of 1x10$^6$ cells/ml and transferred to a 10-mm quartz cuvette. Fluorometric measurements were performed using an MPF-44 fluorometer (Hitachi, Japan), with excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm. The intracellular Ca$^{2+}$ concentration, [Ca$^{2+}$], was calculated from the ratio (R) between the fluorescence intensities (F) at 500 nm after excitation at 340 and 380 nm. The calibration values for R$_{max}$ and R$_{min}$ were obtained by permeabilization of the hepatocytes with 20 nM SDS, followed by addition of 4 mM/ml EGTA (0.5 M in 2 M Tris-HCl, pH 7.4). The cytosolic Ca$^{2+}$ concentration was calculated as follows, assuming the dissociation constant (Kd) for Ca$^{2+}$-fura 2 of 115 nM:

$$[Ca^{2+}]_i = \frac{K_d \cdot (R - R_{min})(R_{max} - R)}{R}$$

**Statistical analysis.** The results are expressed as means±S.D. Significant differences between two mean values were assessed using the Student’s t-test for paired data. A difference was considered to be significant at $p<0.05$.

**Results**

**Time-course of serum ALT and intracellular Ca$^{2+}$ concentration in hepatocytes after administration of CCl$_4$ in rats.** As shown in Figure 1(a), the serum ALT activity clearly

![Figure 1](https://example.com/figure1.png)
increased to a peak 29-fold (539.7±12.8 IU/l) greater than that of the controls on 1 day after i.p. administration of 1.0 ml/kg CCl₄, and then rapidly decreased to the control level (10.7±1.1 IU/l) by 3 days (p<0.005). None of the rats died during the experimental period. As shown in Figure 1(b), the intracellular Ca²⁺ concentration in rat hepatocytes changed in a biphasic manner after CCl₄ administration. The concentration increased steeply to an initial peak (1060.8±80.7 nM) at 1 h after CCl₄ administration, and then had decreased to the control level (234.5±5.8 nM) within 2 h (p<0.001). However, the concentration gradually increased again to reach a second peak (1390.9±152.8 nM) at 3 days, before returning to the control level by 4 days. Therefore, the intracellular Ca²⁺ level was maintained at >500 nM in hepatocytes for 4 days.

Changes in serum ALT and intracellular Ca²⁺ increases after daily CCl₄ administration in rats. In rats treated with daily i.p. 1.0 ml/kg CCl₄, serum ALT continued increasing >714 IU/l after the second CCl₄ administration, but the rats died from FHF several hours later, as shown in Figure 2(a). On the other hand, as shown in Figure 2(b), intracellular Ca²⁺

Figure 2. Time-courses of serum ALT and intracellular Ca²⁺ concentration in rat livers after CCl₄ administration. Rats were given an i.p. injection of CCl₄ of 1.0 ml/kg/day (20% (v/v) CCl₄ in olive oil). Serum ALT (a) and intracellular Ca²⁺ concentration (b) were estimated using the method described in Figure 1. Data points represent means±S.D. of four rats.

Figure 3. Time-courses of serum ALT and hepatic intracellular Ca²⁺ concentration in rat livers with pretreatment of LPS after CCl₄ administration. Rats were given an i.p. injection of LPS of 0.5 mg/kg at day −1, followed by i.p. injection of CCl₄ of 1.0 ml/kg/day. Serum ALT (a) and intracellular Ca²⁺ concentration (b) were estimated using the method described in Figure 1. O, daily CCl₄ administration; ●, single LPS administration as a control. Data points represent means±S.D. of four rats.
increased to its first peak after every dose of 1.0 ml/kg CCl₄, there was no continued elevation after the first Ca²⁺ peak.

**Effect of LPS on serum ALT and intracellular Ca²⁺ increases in CCl₄-induced hepatic injury in rats.** If rats were injected with LPS at 0.5 mg/kg 24 h prior to the first dose of 1.0 ml/kg CCl₄, they did not die during the experimental period, even if CCl₄ was administered daily. In this case, as shown in Figure 3(a), serum ALT levels were not increased. As shown in Figure 3(b), intracellular Ca²⁺ concentration in hepatocytes increased to >1,000 nM 24 h after LPS pretreatment, at the time of the initial administration of CCl₄ at 1.0 ml/kg. Intracellular Ca²⁺ concentration was sustained at >1,000 nM by day 2, temporarily decreasing to 500 nM at day 4, and again increasing to >1,000 nM. However, the first Ca²⁺ peak was not seen 1 h after any administration of 1.0 ml/kg CCl₄.

**Discussion**

We have investigated the bio-defensive function of low-dose LPS pretreatment on CCl₄-induced FHF in rats. In the present study, we showed that serum ALT activity increased to a peak 24 h after a single administration of 1.0 ml/kg CCl₄, but none of the rats died during the experimental period. The intracellular Ca²⁺ concentration in hepatocytes prepared from the rats increased in a biphasic pattern involving peaks at 1 h and 3 days, respectively, similar to the pattern following administration of 0.2 ml/kg CCl₄ (7). However, when rats received daily i.p. injection of 1.0 ml/kg CCl₄, the serum ALT activity continued to increase up to 714 IU/l, and the rats died several hours after the second dose. The rats might be considered to have FHF, according to the criteria of Blitzer et al. (8). FHF was avoided when LPS of 0.5 mg/kg was injected i.p. 24 h prior to the initial administration of CCl₄ (1.0 ml/kg), and the serum ALT activity was maintained at the control level. As described above, prior exposure to a low dose of a chemical can result in protection against a subsequently administered lethal dose of the same compound. This tolerance phenomenon is known as autoprotection (9) or adaptive cytoprotection (10). Although the mechanism is not well understood, Mehendale et al. (9) showed that protection against CCl₄ toxicity is closely associated with active hepatocellular regeneration, and Miller et al. (10) further showed that the mechanism of adaptive cytoprotection may be mediated by maintenance of intracellular Ca²⁺ homeostasis. In a previous study, we demonstrated that intracellular Ca²⁺ concentration may increase during regeneration after hepatic injury. Thus, it has been proposed that autoprotection and adaptive cytoprotection are mediated by intracellular Ca²⁺ concentration, in that they represent a protective mechanism involving intracellular Ca²⁺ concentration and the calcium binding protein S100A4. Cai and Mehendale (11) showed that the protection against CCl₄ toxicity afforded by partial hepatectomy was closely associated with active hepatocellular regeneration. Using pharmacokinetic measurements, we further showed that the metabolic activity of salicylamide decreases in rats during the days after CCl₄ administration at 0.2 ml/kg (7). However, mechanism of the protective function of increased intracellular Ca²⁺ remains unclear.

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**References**


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