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CARBON TETRACHLORIDE-INDUCED CELL DEATH IN PERFUSED LIVERS FROM PHENOBARBITAL-PRETREATED RATS UNDER HYPOXIC CONDITIONS AND VARIOUS IONIC MILIEU

FURTHER EVIDENCE FOR CALCIUM-DEPENDENT IRREVERSIBLE CHANGES

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Abstract—The role of Ca^{2+} in the initiation of carbon tetrachloride (CCl_4) hepatotoxicity was studied using perfused livers isolated from phenobarbital-pretreated rats in a single-pass system. Krebs–Henseleit bicarbonate buffer containing 1.3 mM $CaCl_2$ (KHB) was the regular ionic milieu. In the liver perfused with fructose-supplemented regular KHB equilibrated with 95% N_2–5% CO_2, infusion of 0.5 mM CCl_4 caused an early uptake of Ca^{2+} coupled with K^+ leakage and Na^+ uptake within the infusion time of 30 min, which was followed by a marked lactic dehydrogenase (LDH) leakage into the effluent perfusate and further Ca^{2+} uptake by the liver. With Ca^{2+}-free medium, the prenecrotic K^+ leakage and the successive LDH leakage were suppressed markedly. However, a perfusate exchange from regular to Ca^{2+}-free KHB at the end of the prenecrotic stage did not protect against the LDH leakage, and the perfusate exchange conversely did not produce LDH leakage. Perfusion of the liver with high $K^+(Cl^-)$ medium under 20% O_2 markedly suppressed CCl_4-induced LDH leakage even in the presence of Ca^{2+}, whereas once CCl_4 had acted under regular KHB perfusion, changing the medium to high K^+ did not further prevent the LDH leakage. High K^+-lactobionic acid medium containing Ca^{2+} and supplemented with fructose also suppressed LDH leakage under 95% N_2 without the accompanying prenecrotic Ca^{2+} uptake. However, a change of the medium after CCl_4 infusion to regular KHB containing Ca^{2+} caused LDH leakage and K^+ leakage, with Ca^{2+} uptake. The prevention of LDH leakage in a different ionic milieu may not be due to suppression of CCl_4 bioactivation, since the liver cytochrome P450 content decreased to a similar extent. These findings suggest that entry of extracellular Ca^{2+} into hepatocytes coupled with K^+ leakage and Na^+ entry is a prerequisite for CCl_4-induced hepatocyte death and that association of Ca^{2+} with a CCl_4-derived radical-mediated process may be necessary for early and irreversible plasma membrane damage.

The mechanism of carbon tetrachloride (CCl_4) hepatotoxicity involves bioactivation of CCl_4 to reactive free radicals, which initiate lipid peroxidation on the one hand and covalently bind to cellular macromolecules on the other [1–4]. However, which of these free radical-induced events is more important in the development of hepatic cell necrosis is still controversial [2, 4], although lipid peroxidation has long been proposed as a major mechanism for CCl_4 hepatotoxicity [4]. Our previous studies with perfused livers also suggested that lipid peroxidation is not the major mechanism for acute cell death [5, 6]. In addition, the mechanism of how these initial events lead to final death remains obscure.

Ca^{2+} is now proposed as a toxic messenger. Various toxic stimuli are known to increase the intracellular Ca^{2+} concentration, which causes deleterious effects on the cellular mechanisms such as disruption of the plasma membrane cytoskelton organization and increased activities of cellular degradative enzymes: proteinase, phospholipases and ribonuclease [7–9].

CCl_4 is one of several hepatotoxic agents that cause accumulation of a large amount of calcium in the intoxicated liver [10, 11]. A requirement for extracellular Ca^{2+} in cultured hepatocytes [12, 13] and with perfused livers [5] has been reported. The microsomal Ca^{2+} sequestration mechanism is impaired early after CCl_4 administration *in vivo* [14] and *in vitro* [15]. Ca^{2+} pump activity of plasma membranes also decreases *in vivo* [16]. An increase in the intracellular Ca^{2+} concentration has been shown in isolated hepatocytes by monitoring glycogen phosphorylase *a* activity [17, 18] and by using fluorescent probes [18, 19]. Thus, the disturbance of calcium homeostasis in CCl_4 hepatotoxicity is well recognized. However, its causal relationship with irreversible cellular changes and final necrosis is still under debate [20].

In the present study, the role of extracellular Ca^{2+} and its entry into the hepatocytes was examined in relation to the development of necrosis, by using the liver perfusion system. The perfused liver is more suitable than isolated hepatocytes, since the former, besides being easy for manipulating the perfusate composition, can be spared the enzymatic

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and mechanical influences especially in hepatocyte plasma membranes, which are inevitable in the latter during preparation [21]. This is particularly important in toxicity experiments. For example, in the perfused liver, prenecrotic K^+ leakage can be separated from necrotic enzyme leakage [5], even though K^+ leakage, like lactic dehydrogenase (LDH*) leakage, is widely used as a parameter of toxic cell death in cultured or isolated cells [22].

Experimentally, CCl_4 was infused into livers from phenobarbital (PB)-treated rats perfused with media of various ionic compositions under low oxygen tensions. LDH activity, thiobarbituric acid reactive substances (TBARS) and ionic concentrations (K^+, Na^+ and Ca^{2+}) in the effluent perfusate were measured as indices of necrosis, lipid peroxidation and prenecrotic ionic changes, respectively. PB is reported to enhance CCl_4 hepatotoxicity [23] by inducing a specific form of cytochrome P450 [24] that activates CCl_4 in the perivenous zones of the liver lobules [25]. Low oxygen tension also enhances CCl_4 hepatotoxicity both *in vivo* [26, 27] and *in vitro* [5, 6, 27, 28], which is considered to be due to the enhanced metabolism of CCl_4 with an increased covalent binding of CCl_4 metabolites [27, 29]. Thus, perfusion of PB-pretreated rat livers under low oxygen tension may be appropriate for short-term studies without altering the basic mechanism of the necrotic action of CCl_4

MATERIALS AND METHODS

Animals. Male, SPF-grade Sprague–Dawley rats, weighing 80–100 g, were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Japan, and housed in an air-conditioned animal room (temperature 24 ± 1°, humidity 50–60%). PB (0.1% in drinking water) was administered for 5–6 days. Nourished animals weighing 150–160 g were used for the liver perfusion experiments.

Liver perfusion. The liver was isolated according to the usual method except that the larger lobes (lobus sinister, lobus sinister medialis and pars infraportalis) were perfused in a non-recirculating, constant flow (25 mL/min) system, whereas the smaller lobes (proc. papillaris, proc. caudatus and lobus dexter) were tied and cut off [5]. Krebs–Henseleit bicarbonate buffer (KHB, 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ and 5.6 mM glucose, saturated with a gas mixture containing 5% CO_2 at 37°) was the standard perfusion medium (regular KHB). The actual oxygen concentrations of the medium bubbled with 95% O_2–5% CO_2, 20% O_2–75% N_2–5% CO_2 or 95% N_2–5% CO_2 were about 0.6, 0.2 and 0.08 mM, respectively. The ionic composition of KHB was modified as follows: (1) under 95% N_2, glucose was replaced with 15 mM fructose. (2) In Ca^{2+}-free medium, $CaCl_2$ was omitted from KHB. (3) High K^+ medium contained 118 mM and 4.8 mM NaCl instead of 4.8 mM KCl and 118 mM NaCl. (4) High K^+-lactobionic acid (LBA) medium contained 118 mM K^+–99 mM LBA (pH 7.4) instead of 118 mM KCl in the high K^+ medium. CCl_4 solution (5 mM) was prepared as follows: 0.5 mmol of CCl_4 was added to 100 mL of ice-cold medium in a sealed 100-mL measuring flask with a magnetic rod inside, and CCl_4 was dispersed and dissolved by occasionally immersing the flask in a sonicating washer while stirring on ice for over 1 hr. This solution was infused exactly 30 min after cannulation of the portal vein for a period of 30 min at a rate of 2.5 mL/min through a warmed coiled tube. The final concentration of CCl_4, 0.5 mM, reportedly has little direct solvent effect on the plasma membranes in the isolated hepatocytes [19], and the total amount infused (375 μmol) was within the range of the doses used *in vivo*. After the experiments, some livers were infused with 0.2 mM trypan blue, fixed with 1% paraformaldehyde and sectioned to confirm the necrotized lobular areas [30].

Effluent monitoring and assays. Concentrations of oxygen and ions (K^+, Na^+ and Ca^{2+}) were monitored using a Clark type-oxygen electrode and ion-selective electrodes (Orion) connected to the venous outlet of the perfusion system. Calculations were performed as previously described [5]. The LDH activity of the effluent perfusate was assayed by the reduction of NAD^+ at 25° by a clinical assay [31]. TBARS were determined essentially as described by Ernster and Nordenbrand [32].

Hepatic assays. Twenty minutes after CCl_4 infusion, the liver was cooled instantly in cold 0.25 M sucrose–50 mM Tris–HCl buffer (pH 7.4), and portions were assayed as follows. The cytochrome P450 content in the left lobe was measured by the method of Matsubara *et al.* [33] and expressed as the percentage of that of the fresh lobus dexter which was dissected during surgery. The TBARS content in the median lobe was determined by the method described previuosly [34]; trichloroacetic acid extracts were reacted with thiobarbituric acid, the colored products were extracted into *n*-butanol, and the absorbance spectra of the extracts were recorded between 470 and 600 nm. The TBARS values were calculated from the O.D. at 535 nm after base line correction using malondialdehyde (MDA) as the standard. The calcium content of the left lobe after trichloroacetic acid extraction was assayed by means of a clinical orthocresolphthalein complexone method (Calcium kit, Wako Chemicals, Japan).

Statistical analysis was performed by means of Student's *t*-test, and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of oxygen concentration on CCl_4-induced necrosis, regular KHB. As reported previously [5], when 0.5 mM CCl_4 was infused for 30 min into livers perfused with regular KHB gassed with 95% O_2, little LDH leakage was observed. The LDH leakage increased markedly under 20% O_2, with the greatest

* Abbreviations: LDH, lactate dehydrogenase; PB, phenobarbital; KHB, Krebs–Henseleit bicarbonate buffer; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; and LBA, lactobionic acid.

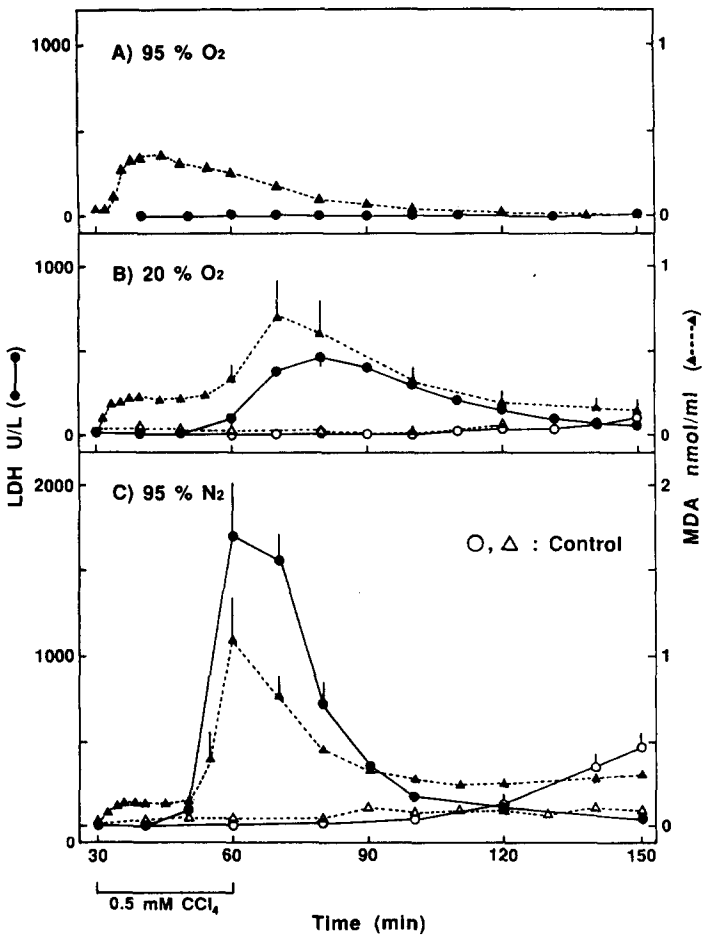

Fig. 1. Leakage of LDH and TBARS following infusion of CCl_4 (0.5 mM) in the isolated, PB-pretreated rat liver, perfused with regular KHB under (A) 95% O_2, adopted from our previous study [5] for comparison, (B) 20% O_2, and (C) 95% N_2. Values are means ± SEM (N = 5–6).

and earliest leakage under 95% N_2 (Fig. 1). Lobular uptake of trypan blue was nearly parallel with the LDH leakage, i.e. the stained lobular area being about 0, 50 and more than 90% with a decreasing O_2 concentration (data not shown). The TBARS release occurred biphasically; there was an early release soon after CCl_4 infusion, which was slightly greater under higher O_2 concentrations, and a greater release in parallel with LDH leakage.

Ionic movement following CCl_4 *infusion, fructose-supplemented KHB with or without* Ca^{2+} *under* 95% N_2. In regular KHB under 95% N_2, the LDH leakage of the control liver increased over time (Fig. 1C), probably due to a cellular energy deficiency caused by the limited oxygen supply, even though nourished animals were studied. To minimize such a CCl_4-independent deleterious effect, 15 mM fructose instead of glucose was added to the perfusion medium, which reportedly protects against anoxic liver damage [35] by maintaining hepatic ATP levels in the anoxic perfused liver [36]. In the present study, fructose-supplemented KHB protected the liver against LDH leakage under 95% N_2 for at least up to 3 hr. Under these conditions, CCl_4 caused a marked LDH leakage comparable to that observed without fructose, although with a later onset and peak time (Fig. 2A).

In accordance with our previous study under conditions of 20% O_2 [5], marked K^+ leakage and a nearly equivalent amount of Na^+ uptake occurred within 30 min of CCl_4 infusion preceding the LDH leakage. Ca^{2+} uptake during the prenecrotic phase, which was slight under a 20% O_2 supply [5], was detected simultaneously with K^+ leakage and Na^+ uptake. A much greater Ca^{2+} uptake slightly preceded or occurred nearly simultaneous with the LDH leakage.

In Ca^{2+}-free KHB supplemented with fructose (Fig. 2B), the control liver tolerated the hypoxic conditions well. CCl_4 produced much less K^+ leakage during the prenecrotic phase, and the succeeding LDH leakage was considerably suppressed and delayed.

The TBARS release was slight in the prenecrotic phase and was not affected by the presence or absence of Ca^{2+}, although it increased together with the LDH leakage.

Effects of medium exchange, fructose-KHB with

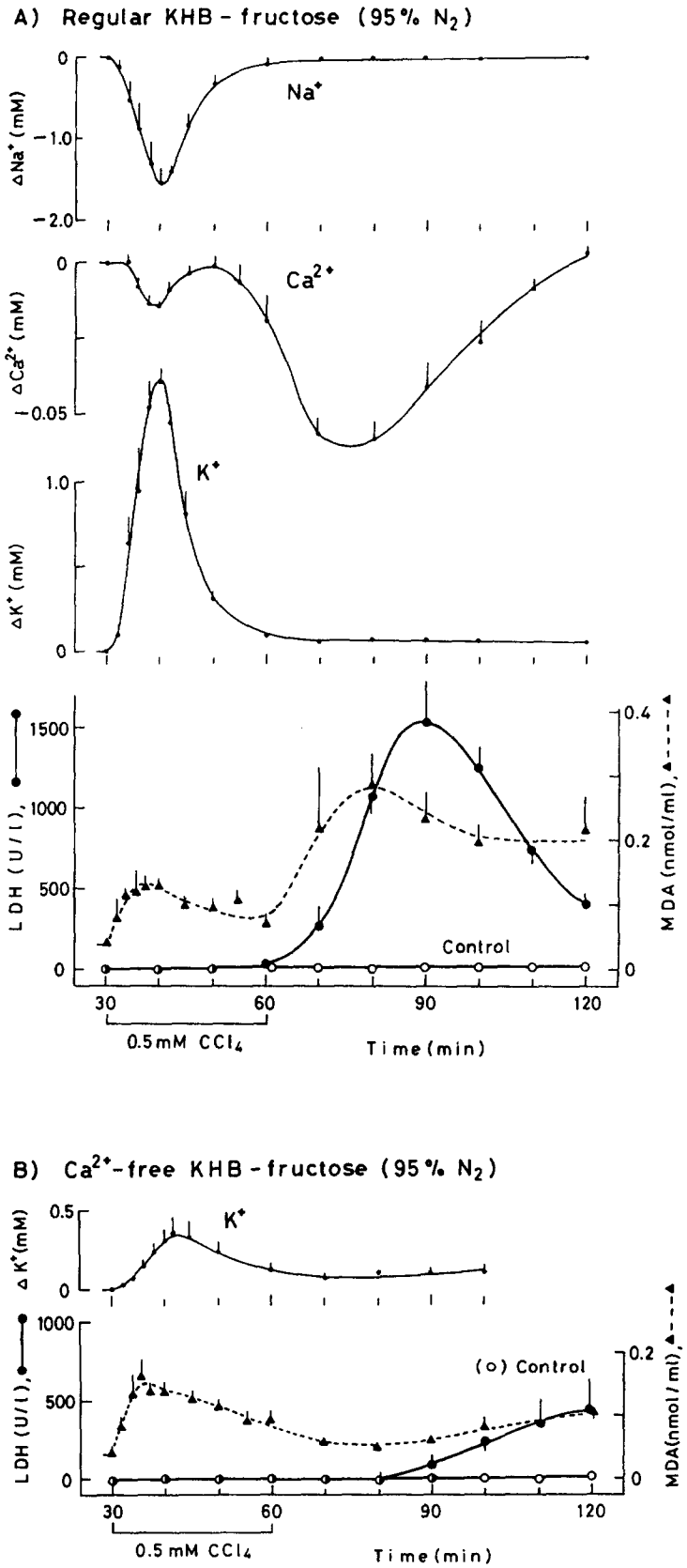

Fig. 2. Ionic movements and leakage of LDH and TBARS following infusion of CCl_4 in the isolated, PB-pretreated rat liver perfused with fructose-supplemented KHB under 95% N_2. (A) With Ca^{2+} in the perfusate. (B) Without Ca^{2+}. Values are means ± SEM (N = 6).

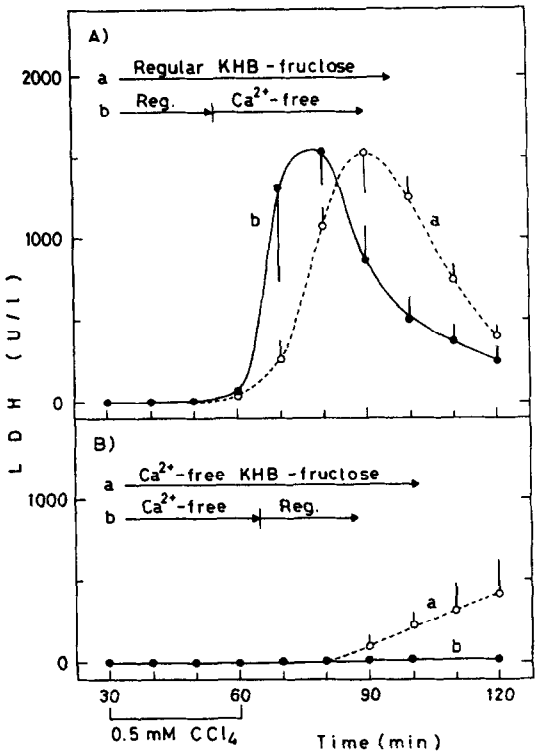

Fig. 3. Effects of perfusate exchange on the CCl_4-induced LDH leakage in the isolated, PB-pretreated rat liver, perfused with fructose-supplemented KHB under 95% N_2. (A) Ca^{2+}-containing perfusate was changed to Ca^{2+}-free perfusate after 55 min. (B) Ca^{2+}-free perfusate was changed to Ca^{2+}-containing perfusate after 65 min. The curves "a" in (A) and (B) denoting no medium exchange were adopted from Fig. 2 for comparison. Values are means ± SEM (N = 5–6).

95% N_2. Exchange of the perfusing medium from regular to Ca^{2+}-free KHB prior to the necrotic phase did not protect against but rather accelerated the LDH leakage (Fig. 3A). On the other hand, the change of Ca^{2+}-free KHB to regular KHB at the end of CCl_4 infusion did not further enhance LDH leakage but rather almost completely suppressed it (Fig. 3B). Such a delay and suppression of the LDH leakage by the presence of Ca^{2+} in the necrotic phase may be due to the membrane-stabilizing action of Ca^{2+}.

Hepatic changes during the prenecrotic stage, in the presence of fructose with 95% N_2. The gross appearance of the liver surface always turned from reddish to brownish after CCl_4 infusion. At 20 min of CCl_4 infusion, the hepatic cytochrome P450 content decreased by about 50% irrespective of the presence or absence of Ca^{2+} in the perfusate (Fig. 4A). The hepatic TBARS content increased slightly but with no significant difference between Ca^{2+}-plus and Ca^{2+}-free media (Fig. 4B). Such a small increase due to CCl_4 infusion may be due, in part, to a leakage into the perfusate as well as suppressed lipid peroxidation under hypoxic conditions. The calcium content was increased significantly by CCl_4 in the presence of Ca^{2+} (Fig. 4C).

Experiments with high $K^+(Cl^-)$ *medium and* 20% O_2. Since CCl_4 caused a marked K^+ leakage preceding the necrosis, we examined whether counteraction of the K^+ leakage by high K^+ perfusion medium could prevent necrosis. This was first tested under conditions of 20% O_2 using a high $K^+(Cl^-)$ medium containing Ca^{2+}, in which the concentrations of NaCl and KCl in the regular KHB were reversed. As shown in Fig. 5A, the control liver perfused with high $K^+(Cl^-)$ medium showed somewhat greater LDH leakage than that observed with regular KHB (Fig. 1B): the activity increased gradually, reaching a plateau level of about 100 U/L at 120 min. Under such conditions, CCl_4-induced LDH leakage that occurred under regular KHB perfusion was suppressed markedly even below the control level. Furthermore, high $K^+(Cl^-)$-medium perfusion only during the prenecrotic phase was sufficient to prevent the LDH leakage (Fig. 5C). On the contrary, exchange from regular KHB to high $K^+(Cl^-)$ medium at the end of the prenecrotic phase did not further prevent LDH leakage (Fig. 5B). TBARS released during the prenecrotic phase were not significantly different among the experimental groups, although higher TBARS values were observed during the necrotic stage.

Experiments with high K^+ *(*LBA^-*)-fructose medium with* 95% N_2. The control liver perfused with high $K^+(Cl^-)$ medium became swollen with accompanying gradual LDH leakage, especially under conditions of 95% N_2 and in the presence of fructose. Since this swelling may have been caused by passive entry of Cl^- accompanying the entry of Na^+ and K^+, Cl^- was replaced with the impermeable anion lactobionic acid (LBA^-), which is prescribed as a tissue preservation medium for transplantation, known as UW solution [37]. By using high $K^+(LBA^-)$-fructose medium containing Ca^{2+}, the control liver tolerated the hypoxic conditions under 95% N_2 for at least up to 2 hr without accompanying swelling and LDH leakage. Under these conditions, CCl_4 infusion caused no LDH leakage (Fig. 6A). Perfusate exchange was achieved in two ways. First, as shown in Fig. 6B, CCl_4 was infused in high $K^+(LBA^-)$-fructose medium containing Ca^{2+}; then the perfusate was changed to regular KHB-fructose medium, under which conditions LDH leakage occurred after some delay. The Ca^{2+} concentration of the effluent perfusate did not change during 30 min of CCl_4 infusion, but K^+ leakage and Ca^{2+} uptake were observed after the medium exchange. Second, when CCl_4 was perfused in Ca^{2+}-free high $K^+(LBA^-)$-fructose medium, which was changed to regular KHB-fructose medium, the LDH leakage remained suppressed (Fig. 6C). Virtually no LDH leakage occurred in the control livers without CCl_4 in these medium exchange experiments during the experimental period (data not shown).

At 20 min after CCl_4 infusion, the hepatic cytochrome P450 content of the livers perfused with regular KHB, Ca^{2+}-free KHB and high $K^+(Cl^-)$ medium under 95% N_2 decreased to 56.8 ± 0.5, 57.4 ± 5.0 and 49.6 ± 3.4% of the fresh liver (mean ± SEM, N = 5), respectively. The calcium

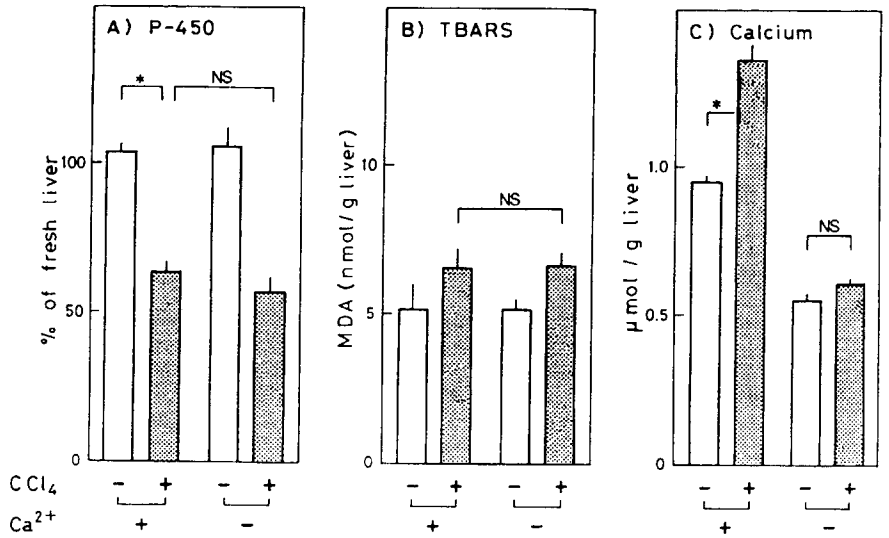

Fig. 4. Effects of CCl_4 on hepatic cytochrome P450, TBARS and calcium contents in the isolated, PB-pretreated rat liver, perfused with fructose-supplemented KHB under 95% N_2 in the presence and absence of Ca^{2+}. Measured 20 min after CCl_4 infusion had been started. Values are means ± SEM (N = 5–6). *P < 0.01.

content of the liver perfused with a calcium-containing high $K^+(Cl^-)$ medium did not increase after CCl_4 infusion (89 ± 4.7% of the fresh liver). In the high $K^+(LBA^-)$-fructose medium with Ca^{2+}, CCl_4 decreased the hepatic cytochrome P450 to 45 ± 6.8% (mean ± SEM, N = 3) without an increase in the calcium content (92 ± 1%, N = 3).

Figure 7 shows the relationship between cellular ionic movements and LDH leakage under various ionic environments.

DISCUSSION

Oxygen has dual effects on the hepatotoxic mechanism of CCl_4. Reductive metabolism of CCl_4 by microsomal cytochrome P450 to reactive trichloromethyl radicals and their covalent binding to cellular macromolecules are competitively inhibited by oxygen [27, 29], whereas propagation of the lipid peroxidation initiated by the radicals is definitely an oxygen-dependent process [38, 39]. In agreement with previous experiments *in vivo* [26, 27] and *in vitro* [26, 27, 28], CCl_4 hepatotoxicity was enhanced under low oxygen tension in perfused livers (Fig. 1). Although covalent binding of CCl_4 metabolites was not examined in the present study, LaCagnin *et al.* [28] reported that metabolism of CCl_4 in the perfused rat liver, as measured by carbon dioxide anion radical production, is much faster during perfusion with a nitrogen-saturated medium, than with an oxygen-saturated medium, accompanying a more rapid LDH release. On the other hand, lipid peroxidation may not have a major role in the development of necrosis under the present hypoxic conditions, since the early TBARS release following CCl_4 infusion was not enhanced as compared with that under 95% O_2. In addition, in our previous studies with isolated livers under 20% O_2, the antioxidants diphenyl-*p*-phenylene diamine and promethazine markedly suppressed TBARS release but not LDH leakage [5]. However, the participation of lipid peroxidation *in vivo* may not be ruled out.

In the present study, the process of CCl_4-induced cell death was separated into two phases: a prenecrotic phase (during CCl_4 infusion) characterized by ionic movements, namely leakage of K^+ from and entry of Na^+ and Ca^{2+} into the liver cells, and the following necrotic phase (after termination of CCl_4 infusion) of LDH leakage and further Ca^{2+} uptake. This biphasic nature of Ca^{2+} uptake is in accord with the report of Reynolds [11] that calcium biphasically accumulated in the CCl_4-intoxicated liver and also with the report of Agarwal and Mehendale [40] that $^{45}Ca^{2+}$ uptake by the livers isolated from rats treated with chlordecone, followed by CCl_4, increased before obvious necrosis developed. However, the simultaneous passive movement of K^+ and Na^+ during the prenecrotic phase has not been demonstrated in other experimental systems, although the necrotic liver has an altered ionic composition [10]. Furthermore, the following observations with medium-exchange experiments may indicate that the entry of extracellular Ca^{2+}, linked with movements of Na^+ and K^+, during the prenecrotic phase is causally related to hepatic cell death: (1) with regular KHB containing Ca^{2+}, once the ionic movements had occurred during the prenecrotic phase, perfusate exchange to Ca^{2+}-free or high K^+ medium had no protective effect (Fig. 7A); (2) in Ca^{2+}-free medium, CCl_4 caused much less K^+ leakage, followed by a delayed and suppressed LDH leakage, and addition of Ca^{2+} after the prenecrotic phase could no longer

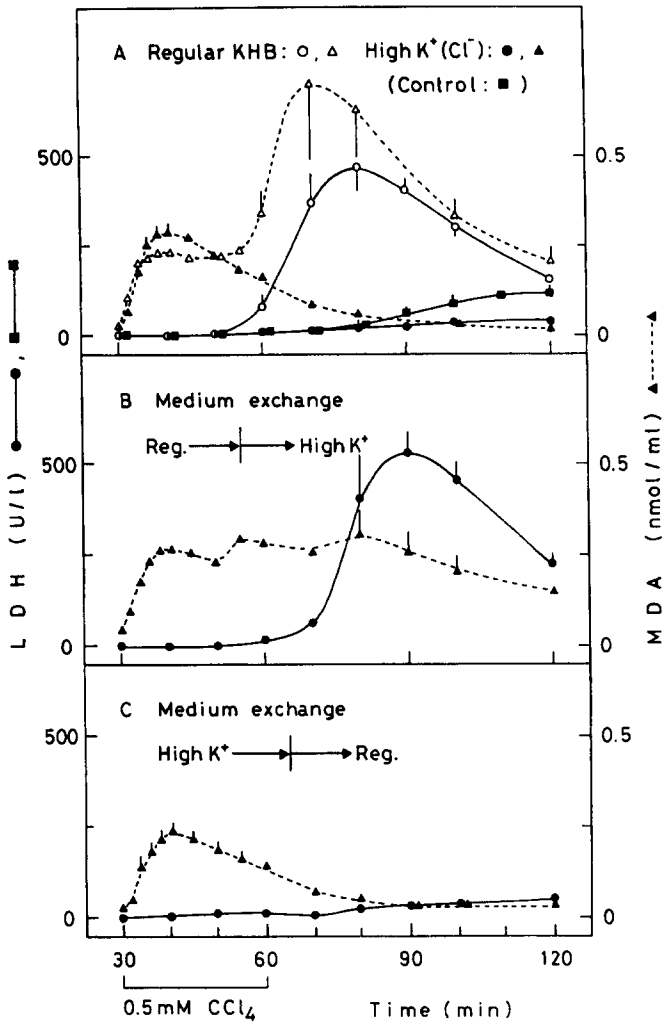

Fig. 5. Effects of high $K^+(Cl^-)$ medium on CCl_4 hepatotoxicity in the isolated, PB-pretreated rat liver, perfused under 20% O_2 in the presence of Ca^{2+}. (A) Comparison with regular KHB. The data on regular KHB were adopted from Fig. 1B for comparison. (B) Perfusate exchange from regular KHB to high $K^+(Cl^-)$ medium at 55 min. (C) Perfusate exchange from high $K^+(Cl^-)$ to regular KHB at 65 min. Values are means ± SEM (N = 5).

provoke LDH (Fig. 7B); and (3) in high K^+ medium containing Ca^{2+}, CCl_4 caused neither initial Ca^{2+} uptake nor cell death (Fig. 7, C and D).

In the present study, we applied rather unphysiological experimental conditions, i.e. hypoxia, addition of a high concentration of fructose in the perfusate, high K^+ ionic milieu, and their combinations. Although the perfusing conditions were set up so that the control livers did not show significant LDH leakage, the possibility that they affect CCl_4-dependent cell death exists.

For example, it is well known that depletion of ATP due to hypoxia by itself is fatal to hepatocytes [41–43], and the increase of intracellular free Ca^{2+} is considered one of the causative linking events between ATP depletion and cell death which results from an arrest of the energy-dependent ion transport system [41, 43]. Insufficiency of the plasma membrane Na^+–K^+ pump causes an influx of extracellular Ca^{2+} which occurs by means of the reversed Na^+–Ca^{2+} antiportor system on the one hand, and the cellular free Ca^{2+} concentration is also increased by suppression of intracellular Ca^{2+} sequestration by mitochondria and endoplasmic reticulum on the other. The elevated cellular Ca^{2+}, by activating plasma membrane phospholipases and proteases and by acting on cytoskeletal systems, produces functional and structural disintegration of plasma membranes such as formation and rupture of blebs and leakage of intracellular enzymes [7–9, 42], finally resulting in cell death. Therefore, the cell death induced by CCl_4 under such conditions may also involve a CCl_4-independent process (Fig. 1C). However, the fructose-supplemented perfusate completely prevented hypoxic LDH leakage of the control livers (Fig. 2), as already reported by other investigators

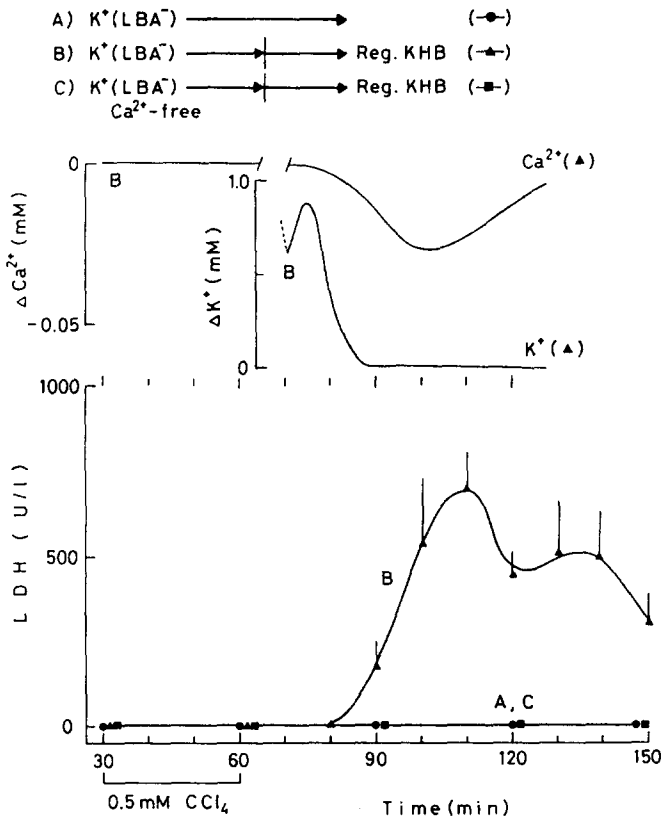

Fig. 6. Effects of high $K^+(LBA^-)$ medium on CCl_4 hepatotoxicity in the isolated, PB-pretreated rat liver, perfused under 95% N_2 with fructose addition. Regular KHB-fructose medium was used for the initial 15 min after cannulation of the liver and then changed to high $K^+(LBA^-)$ medium. Curve A: Perfused with high $K^+(LBA^-)$ medium containing Ca^{2+} throughout experimental period. Curve B: Perfusate exchange from high $K^+(LBA^-)$ medium containing Ca^{2+} to regular KHB at 65 min. Curve C: Perfusate exchange from Ca^{2+}-free high $K^+(LBA^-)$ to regular KHB at 65 min. Values are means ± SEM (N = 4). Virtually no LDH leakage occurred in the control livers in experiments A, B and C.

[35, 36]. Fructose can maintain the physiological functions of the cells and the oxidative phosphorylation capacity of mitochondria during anoxia by the enhancement of glycolytic production of ATP [35, 36] and, in this sense, CCl_4 may exhibit a more specific action in the presence of fructose. However, other metabolic changes such as marked lactate production [35, 36] and enhancement of the anoxia-induced cellular pH decrease [44], which is also considered as the mechanism of the protection against anoxic cell death [45], could affect CCl_4-dependent cell death. Thus, various undetermined cellular factors that alter the development of CCl_4-induced cell death could be involved. However, the basic triggering mechanism, i.e. bioactivation of CCl_4 by cytochrome P450, is probably not altered much, since the decrease of hepatic cytochrome P450 and TBARS content and release of TBARS into the perfusate during the prenecrotic phase, which are the indices of production of active radical metabolites, were not much different between regular and Ca^{2+}-free ionic milieu. This is partly in agreement with the report of Casini and Farber [12] that the extracellular concentration of Ca^{2+} had no effect on the extent of covalent binding of CCl_4 metabolites and on the extent of lipid peroxidation in cultured hepatocytes. In addition, we would like to point out that there is a fundamental difference between various physiological modifications used in the present study and the toxic chemical insult by CCl_4, i.e. the former usually causes reversible cellular changes if the exposure time is short, whereas the latter substantially causes irreversible cellular changes, magnified under conditions of hypoxia and cytochrome P450 induction. For example, it is unlikely that such rapid ionic changes observed soon after CCl_4 infusion in the presence of Ca^{2+} could result from enhancement of CCl_4-independent physiological degenerative processes. Thus, we prefer to consider that the observed reactions due to CCl_4 infusion are basically intrinsic to CCl_4.

The biphasic feature of the cellular degenerative process may be explained as follows. The initial phase may be triggered by the trichloromethyl radicals. The radical-mediated process is considered to be very rapid and irreversible, involving covalent binding with cellular constituents [1–4], and may finish within the period of CCl_4 infusion under the

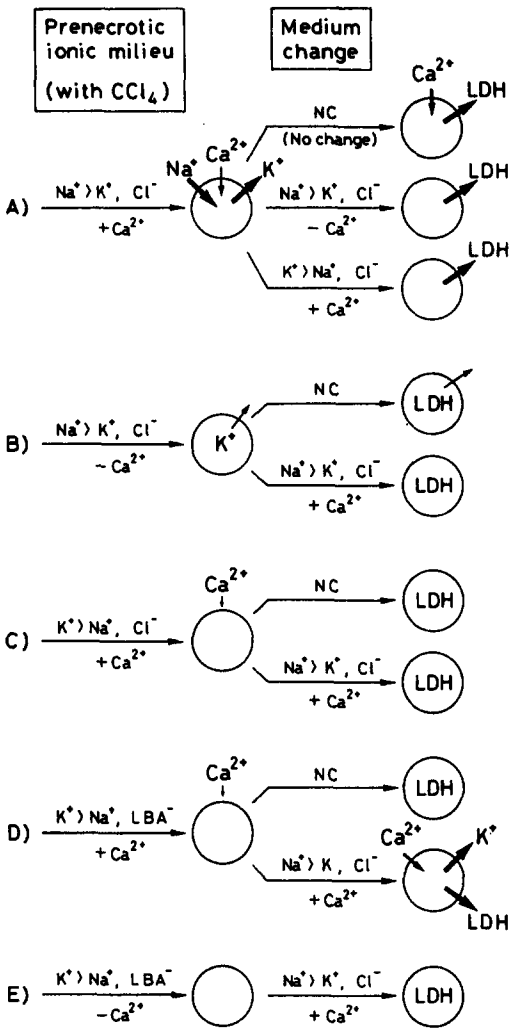

Fig. 7. Summary of the relationship between the perfusate ionic milieu and the development of necrosis by CCl_4 in the isolated, PB-pretreated rat liver under hypoxia. (A) from Figs. 1C, 2A, 3A and 5B; (B) from Figs. 2B and 3B; (C) from Fig. 5, A and C; (D) from Fig. 6, A and B; and (E) from Fig. 6C.

present experimental conditions of enhanced CCl_4 bioactivation. Cooperation of Ca^{2+} with the events evoked by CCl_4 is necessary for the prenecrotic change, since in the absence of Ca^{2+} the prenecrotic changes and necrosis were suppressed markedly (Fig. 7B). Although various early intracellular disturbances involving inhibition of endoplasmic reticular Ca^{2+} sequestration [14, 15] may be provoked, plasma membrane permeability changes for smaller ions (premature but irreversible membrane damage at this stage) may be a crucial event for subsequent cellular damage. During the initial 20 min of CCl_4 infusion, total K^+ leakage amounted to about 70 μEq/g liver, which means about 60% of the intracellular K^+ (115 mEq/L) had already leaked out during the initial phase. The intracellular K^+ loss was compensated for by the entry of a nearly equivalent amount of Na^+. Ca^{2+} entry during the initial phase amounted to about 0.4 μmol/g liver, even though this cation is rather impermeable compared with Na^+ and K^+. The occurrence of transient recovery of Ca^{2+} entry upon cessation of the Na^+/K^+ movement suggests not only a passive transport but also a reversed Na^+/Ca^{2+} antiport. The exact mechanism of the ionic movement remains to be clarified. Since the intracellular free Ca^{2+} concentration is normally less than 10^{-6} M, entry of this amount of Ca^{2+} in the initial phase may cause a marked increase of intracellular free Ca^{2+} concentration in cooperation with a defect in Ca^{2+} sequestration by the endoplasmic reticulum, followed by Ca^{2+}-dependent degenerative processes similar to that proposed for hypoxic cell death. Thus, at the end of the initial phase, because of the altered intracellular ionic composition and Ca^{2+}-dependent disintegration of cellular membrane structures particularly in plasma membranes (mature plasma membrane damage), hepatocytes are ready to die. Large amounts of Ca^{2+} enter the hepatocytes down the concentration gradient and may accumulate in the mitochondria and other intracellular organelles (about 10 μmol calcium/g liver finally taken up), and cellular macromolecules such as LDH start to leak out. Accumulation of Ca^{2+} in the mitochondria, with mitochondrial loss of function, is a well-known late phenomenon in CCl_4-intoxicated animals [10, 11].

Ca^{2+} entry during the necrotic phase by itself may not be a direct cause of cell death, since the perfusate exchange from the regular medium containing Ca^{2+} to the Ca^{2+}-free medium did not prevent the LDH leakage but rather enhanced it (Fig. 3), indicating that causative changes had already been produced during the prenecrotic phase.

The prevention of Ca^{2+} entry and cell death by the high K^+ medium (Fig. 7, C and D, NC) is probably due to a counteracting action against the passive efflux of K^+ and the influx of Na^+, since the response to CCl_4 was suppressed irrespective of the counter anion, permeable Cl^- or impermeable LBA^-, each of which may have different cellular effects as described below. Inhibition of the bioactivation of CCl_4 is also unlikely since hepatic cytochrome P450 decreased as in the case with regular medium. The difference observed between high $K^+(Cl^-)$ and $K^+(LBA^-)$ media was that only the perfusate exchange from the latter to regular KHB produced cell death accompanying K^+ leakage and Ca^{2+} entry (Fig. 7D). With a high $K^+(Cl^-)$ medium, passive influx of Cl^- may occur due to a loss of membrane potential accompanied by an influx of K^+ and Na^+ [46], thus causing liver swelling. The liver cell swelling has been shown to have various effects on liver functions, e.g. inhibition of proteolysis [47, 48] and glycogenolysis [49]. Such ionic and metabolic effects could modify Ca^{2+} movement as well as the toxic action of CCl_4. However, with high $K^+(LBA^-)$, no influx of Cl^- occurs and the intracellular ionic milieu may remain relatively unchanged without causing liver swelling, under which conditions the liver may be ready to respond to the medium change to regular KHB. Thus, preceding the dynamic ionic movements, latent and irreversible plasma membrane damage may have been produced by CCl_4 in the presence of Ca^{2+} (but not in the absence of Ca^{2+}, Fig. 7E) by utilizing a

small amount of extracellular (or glycocalyx) or intramembrane Ca^{2+} without accompanying detectable Ca^{2+} entry. This has some resemblance to the two-step killing of cultured hepatocytes reported by Casini and Farber [12]; hepatocytes incubated in low Ca^{2+}, then with CCl_4, increased the loss of viability after exposure to high Ca^{2+}.

Plasma membrane damage has long been proposed as a mechanism of CCl_4 hepatotoxicity. Activation of phospholipase A_2 [50, 51] and phospholipase C [52, 53] by CCl_4 is a reasonable hypothesis that explains the disruption of the structural and functional integrity of the plasma membranes, although its causal relationship with cell death is still argued [54]. Further studies are necessary to determine the mechanisms of the irreversible plasma membrane damage triggered by cooperation of Ca^{2+} with reactive metabolites of CCl_4.

In conclusion, extracellular Ca^{2+} is required in the development of CCl_4-induced hepatocyte death. Plasma membranes may be the initial and critical site of irreversible damage, and the Ca^{2+} entry linked with K^+ leakage and Na^+ entry may be a critical event that finally leads to cell death.

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