Histological Evidence for Dissociation of Lipid Peroxidation and Cell Necrosis in Bromotrichloromethane Hepatotoxicity in the Perfused Rat liver

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ABSTRACT — Bromotrichloromethane (CBrCl₃)-induced hepatic lipid peroxidation and cell necrosis were studied histologically and biochemically, using isolated perfused livers from phenobarbital-pretreated rats. Lipid peroxidation was assessed by fuchsin staining of the liver slices and release of thiobarbituric acid reactive substances (TBARS) into the perfusate; necrosis was assessed by trypan blue uptake and lactate dehydrogenase (LDH) leakage. A good correlation was observed between the Schiff-positive reaction and TBARS release under various experimental conditions, supporting the validity of the fuchsin staining method for histological detection of lipid peroxidation. Lobular localization of lipid peroxidation and necrosis was as follows: Under high oxygen supply (95% O₂-saturated buffer), infusion of CBrCl₃ caused the Schiff-positive reaction in the pericentral to midzonal hepatocytes, irrespective of the direction of perfusion, but did not produce necrosis. Under low oxygen supply (20% O₂) with retrograde perfusion, dissociation of lipid peroxidation and necrosis was observed, i.e., trypan blue uptake in the periportal zones and Schiff-positive staining in the pericentral hepatocytes. Thus, lipid peroxidation by itself may have a relatively minor role in the development of CBrCl₃-induced acute hepatic cell death.

It has long been hypothesized that carbon tetrachloride (CCl₄) and bromotrichloromethane (CBrCl₃) are metabolized by microsomal cytochrome P-450 to active free radicals, which trigger peroxidation of membrane lipids and bind covalently to cellular constituents; this process finally leads to hepatocellular necrosis (1—5). However, the relative importance of lipid peroxidation and covalent binding in the development of halogenomethane-induced hepatic necrosis is still controversial (6). Our recent studies with isolated perfused livers have also suggested that lipid peroxidation may not be a crucial factor for necrosis in the isolated perfused liver (7).

Liver cell necrosis following halogenomethane administration is histologically restricted to centrilobular areas of the liver lobules (2). Therefore, if lipid peroxidation is a prerequisite for the development of necrosis, it should at least occur in the centrilobular zones and precede necrosis, although occurrence of lipid peroxidation and cell necrosis in the same area does not always prove a definite causal relationship. Biochemical methods have clearly characterized lipid peroxidation as a very early event in halogenomethane hepatotoxicity (3, 8, 9), whereas the lobular localization of lipid peroxidation is still unclear. On this point, Taper et al. (10) developed a histochromic method for determining the topography of cellular aldehydes in the liver...
lobules by the Schiff reaction, i.e., using fuchsin staining of cellular aldehydes. They observed that Schiff-positive foci appeared in the liver sections as early as 1 hr after administration of CCl₄ to the rats; these foci spread over the whole lobular area within 24 hr. Since aldehydes are formed in the in vitro peroxidation system of microsomal lipids and also in vivo early after intoxication of rats with CCl₄ and CBrCl₃ (11–14), the Schiff-positive reaction may be considered as one piece of evidence for lipid peroxidation in halogenomethane hepatotoxicity. In the previous study (15), by using tert-butyl hydroperoxide and ADP-iron complex as lipid peroxidation stimulators, we have shown that this staining method is useful for the identification of peroxidized lobular zones in isolated perfused livers.

One of the purposes of the present study is to confirm the validity of this staining method in the liver perfusion system using CBrCl₃, another lipid peroxidation stimulant having different mechanisms. By applying this method, we attempted to determine if lipid peroxidation is causally related to necrosis in halogenomethane hepatotoxicity. Livers were perfused under conditions of both high and low oxygen supply and under both antero- and retrograde perfusion. Localization of necrosis was examined by trypan blue uptake. Leakage of thiobarbituric acid reactive substances (TBARS) and lactate dehydrogenase (LDH) into the perfusate were simultaneously monitored as biochemical indices.

MATERIALS AND METHODS

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

Liver perfusion
The method of surgery and the perfusion apparatus were described elsewhere (7, 15). The perfusion was conducted in a non-recirculating, constant flow rate (25 ml/min) system in either an antero- or retrograde manner. Krebs-Henseleit bicarbonate buffer (KHB) (118 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 5.6 mM glucose, saturated with 95% O₂–5% CO₂ or 20% O₂–75% N₂–5% CO₂ gas mixtures at 37°C, hereinafter referred to as 95% O₂-KHB or 20% O₂-KHB, respectively) was used as the perfusion medium. Experiments commenced exactly 30 min after cannulation of the portal vein, and this time was defined as zero minutes. CBrCl₃ was well dispersed in cold KHB (not gassed) to a concentration of 5 mM by stirring and occasional sonication in a sealed flask. This preparation was infused at 0 min for a period of up to 30 min at a rate of 2.5 ml/min through a warmed coiled tube. N,N’-Diphenyl-p-phenylene diamine (DPPD), dissolved in dimethylsulfoxide (DMSO), was infused at 0.025 ml/min.

Histological examination
At the end of perfusion, the livers were immediately pre-fixed with 1% paraformaldehyde solution (in 20 mM phosphate-balanced saline, pH 7.4) for 8 min, weighed, and kept on ice for about 1 hr in order to complete fixation and to fasten the tissue. Some livers were infused with 0.2 mM trypan blue solution for 10 min, and then with 1% paraformaldehyde for examination of necrosis (16). Small blocks of the pre-fixed liver were cut into approximately 50 μm-thick slices in cold 0.1 M phosphate buffer (pH 7.4) containing 20% glycerin, by using a tissue microslicer (Dosaka EM, Japan). Fuchsin staining was conducted essentially according to the previously described method (7, 15). Necrotic areas of the liver lobules were directly examined in 50 μm-thick slices of trypan blue-stained and pre-fixed livers, with or without fuchsin co-staining.
Biochemical assays

The TBARS content and LDH activity in the effluent perfusate were measured as described previously (7, 15).

RESULTS

Under high oxygen supply

In the livers perfused under 95% O₂-KHB (oxygen concentration, 0.62 ± 0.02 mM) in an anterograde direction, the outlet (venous) oxygen concentration was 0.157 ± 0.039 (S.D.) mM, with a post-fixed liver weight of 6.5 ± 0.6 g (n = 24). In the retrograde perfusion, the outlet (portal) oxygen concentration was 0.143 ± 0.047 mM, with a liver weight of 7.1 ± 0.4 g (n = 20). In such perfused livers, infusion of 0.5 mM CBrCl₃ further increased oxygen consumption as observed in the case of CCl₄ infusion (7) (data not shown).

In an anterograde perfusion system, effluent TBARS levels started to increase soon after infusion of 0.5 mM CBrCl₃; reached a peak at 20–25 min; and returned to a very low level at 1 hr (Fig. 1A, top). LDH leakage was quite slight at 1 hr (Fig. 1A, bottom). In our pre-

![Fig. 1. Time-course of the release of thiobarbituric acid reactive substances (TBARS) and lactate dehydrogenase (LDH) following infusion of bromotrichloromethane (CBrCl₃) in isolated livers from phenobarbital-pretreated, fed rats under antero- and retrograde perfusion systems. (A) The livers were perfused with 95% O₂–5% CO₂-saturated Krebs Henseleit bicarbonate buffer (KHB). Infusion of N,N'-diphenyl p-phenylene diamine (DPPD, 10⁻³ M) was started at 10 min before zero time and continued for 40 min. (B) 20% O₂–75% N₂–5% CO₂-KHB was used as the perfusing medium. In both (A) and (B), perfusion was stopped at 15, 30 and 60 min (n = 3 to 6 for each time point), and the livers were histologically examined. Each point represents a mean ± S.D.](image-url)
Fig. 2. Histological observations on lobular lipid peroxidation and necrosis in isolated livers following infusion of bromotrichloromethane (CBrCl$_3$). All liver samples shown in Fig. 1 were stained with fuchsin or trypan blue, or both, as described in the Methods section. Schiff-positive areas and necrotic areas were stained pink and dark blue respectively. Typical specimens are shown. A: Anterograde infusion of CBrCl$_3$ under 95% O$_2$, 15 min. B: Same as in A, 30 min. C: Same as in A, 60 min. D: Simultaneous infusion of N,N'-diphenyl p-phenylene diamine in B, 30 min. E: Retrograde infusion of CBrCl$_3$ under 95% O$_2$, 30 min. F: Retrograde infusion of CBrCl$_3$ under 20% O$_2$, 15 min. G: Same as in F, 60 min, trypan blue uptake. H: Same as in G, co-staining with fuchsin. The scale shown in A denotes 1 mm (same magnification through A to H).
vious study, little LDH leakage occurred during a 2-hr perfusion under the same experimental conditions (7). Histologically, the size of the Schiff-positive areas, which stained pink with fuchsin, roughly paralleled the TBARS release: i.e., at 15 min, some pericentral and midzonal (ring-like) areas of the liver lobules were stained; the pericentral staining area reached a maximum at 30 min, and at 1 hr, the Schiff positive reaction faded concomitantly with a decrease in TBARS release (Fig. 2, A, B and C). In the presence of DPPD (10^{-5} M), a lipid peroxidation inhibitor (17), the CBrCl₃-induced TBARS release was considerably suppressed (Fig. 1A, top), and entire lobular areas were Schiff-negative (Fig. 2D). DMSO alone, the solvent for DPPD, had no effect on CBrCl₃-induced TBARS release or fuchsin staining.

Retrograde infusion of CBrCl₃ also released TBARS to an extent comparable to that observed under anterograde perfusion, with no accompanying LDH leakage (Fig. 1A, top and bottom). Histologically, a time-course pattern similar to that observed under anterograde perfusion was observed. Pericentral areas were mainly Schiff-positive, with the peak at 30 min (Fig. 2E).

Under low oxygen supply
When the livers were perfused under 20\% O₂-KHB (oxygen concentration, 0.204 ± 0.006 mM) in anterograde and retrograde directions, the outlet oxygen concentrations were 0.029 ± 0.009 (n = 15) and 0.031 ± 0.008 (n = 16), respectively. No further increase in oxygen uptake was detectable after CBrCl₃ infusion.

As compared with the case of the high oxygen supply, infusion of CBrCl₃ produced a much earlier increase of effluent TBARS levels, which reached a peak within 10 min and gradually decreased after cessation of CBrCl₃ infusion (Fig. 1B, top). In the anterograde perfusion, centrilobular to midzonal areas were Schiff-positive at 15–30 min, but the stained areas were sporadic and small (data not shown). LDH leakage started to increase after 40 min, and considerable leakage occurred at 60 min (Fig. 1B, bottom). At this time, pericentral areas were necrotic, as evidenced by trypan blue uptake, and pericentral to midzonal hepatocytes were Schiff-positive to a variable degree (data not shown).

In the retrograde perfusion, CBrCl₃ produced an instantaneous and significantly greater TBARS release than that observed in the anterograde perfusion, followed by earlier and greater LDH leakage (Fig. 1B, top and bottom). The livers at 15 min of CBrCl₃ infusion, when no LDH leakage occurred, exhibited a strong Schiff positive reaction at all pericentral areas (Fig. 2F). At 60 min, marked necrosis developed in midzonal to periportal hepatocytes as evidenced by trypan blue uptake, whereas pericentral hepatocytes survived (Fig. 2G). At this time, fuchsin staining alone revealed Schiff-positive hepatocytes, in a ring-form at the midzonal area, leaving hepatocytes just around the central vein unstained (data not shown). Co-staining of trypan blue-infused livers with fuchsin showed that necrosis and Schiff-positive reactions occurred in separate zones of the liver lobules (Fig. 2H).

DISCUSSION
First, in agreement with our previous study with γ-butyro hydroperoxide and ADP-iron complex (15), a good correlation was observed between the histological Schiff-positive reaction and the TBARS release in the CBrCl₃-infused isolated livers under various experimental conditions. This further supports the validity of the fuchsin staining method for detecting peroxidized lobular zones in perfused liver systems.

In CCl₄-infused livers, in which TBARS release was much lower than that in CBrCl₃-infused livers (7), the Schiff reaction was very weak (data not shown). The sensitivity of the staining method, therefore, appears low compared with the biochemical method, and this is the reason why we used CBrCl₃, which has greater peroxidative and necrogenic activity than CCl₄ (3, 18), and the reason why we
used phenobarbital-pretreated rats, in which halogenomethane hepatotoxicity is known to be enhanced (19).

Next, we will discuss the causal relationship between lipid peroxidation and necrosis in CBrCl\textsubscript{3} hepatotoxicity. As in the previous study with CC\textsubscript{14} (7), CBrCl\textsubscript{3} caused necrosis only under a 20% O\textsubscript{2} supply to efferent zones of the lobules, depending on the direction of perfusion, as evidenced by trypan blue uptake and LDH release. Anoxia by itself may not be the cause of necrosis in our present study with the livers from fed animals, since the control livers well-tolerated the low oxygen supply during a 2 hr-perfusion period (7), although the livers from fasted rats were sensitive to the low oxygen supply, releasing LDH at around 40 min (data not shown). In addition, the perfused liver is reported to survive under the anoxic state as far as ATP is supplied intracellularly by glycogen through the glycolytic pathway or extracellularly by supplementing the perfusate with an appropriate substrate, e.g., fructose (20, 21). On the other hand, the Schiff-positive reaction following CBrCl\textsubscript{3} infusion was always restricted to pericentral to midzonal hepatocytes, irrespective of a high or low oxygen supply or the direction of perfusion, as schematically shown in Fig. 3. This is probably due to the higher concentration of cytochrome P-450 in the pericentral hepatocytes (22). Isolated pericentral hepatocytes were also noted to be more sensitive than the periportal cells to CCl\textsubscript{4}-induced lipid peroxidation, as assessed by TBARS production (23).

Thus, from two lines of evidence that peroxidized (pericentral) and necrotic (periportal) areas were distinctly dissociated under conditions of low oxygen tension and retrograde infusion, and that no necrosis developed under a 95% O\textsubscript{2} supply despite the occurrence of considerable lipid peroxidation under such conditions, lipid peroxidation may not be causally related to CBrCl\textsubscript{3}-induced acute cell death. However, auxiliary roles of lipid peroxidation, such as membrane disorganization and a range of enzymatically damaging consequences (5), can not be ruled out in vivo, in which both lipid peroxidation and necrosis are expected to occur in the centrilobular area.

The degree of lipid peroxidation is complicated by the dual effects of oxygen: oxygen is definitely required for lipid peroxidation, while it competitively inhibits bioactivation of halogenomethanes, thus suppressing lipid peroxidation (23, 24). For example, De Groot et al. (23), using an oxystat system and hepatocyte suspension, demonstrated that lipid peroxidation was preferentially induced by CC\textsubscript{14} under low Po\textsubscript{2} (3-70 mmHg), but not under anaerobic conditions or at Po\textsubscript{2} above 100 mmHg. Although the exact relationship between oxygen concentration and lipid peroxidation is difficult to assess in the isolated livers because of the marked oxygen gradient across the liver lobule (25, 26), in our perfusion system, the size and staining intensity of the Schiff positive areas appear to decrease in the following order: retrograde perfusion (20% O\textsubscript{2}) > antero (95% O\textsubscript{2}) > retro (95% O\textsubscript{2}) > antero (20% O\textsubscript{2}), roughly in parallel with the extent of TBARS release into the

![Fig. 3. Schematic presentation of the lobular zone-specific occurrence of lipid peroxidation and necrosis following bromotrichloromethane (CBrCl\textsubscript{3}) infusion in isolated perfused livers of phenobarbital-pretreated rats under different conditions. The vertical scale indicates the approximate oxygen concentration gradient between the portal and central veins; calculated from influent and effluent oxygen concentrations. P: portal vein, C: central vein. □: Schiff-positive area, □: necrotic area.](image-url)
efficient perfusate. It seems that there exists an optimal oxygen concentration for maximal lipid peroxidation and a roughly similar tendency exists in both experimental systems. Formation of the ring-like staining pattern around the central vein may partly be due to such an oxygen dependency of the lipid peroxidation.

The occurrence of necrosis only under low oxygen supply and in the most anoxic lobular zones in the present perfusion system is in agreement with studies on isolated hepatocytes in vitro (23) and also with animal studies with CCl₄ (27, 28).

According to current concepts, low oxygen tension enhances the reductive metabolism of CCl₄ and CBrCl₃ to trichloromethyl free radicals by microsomal cytochrome P-450. Thus, the present results support the idea that accumulation of reactive trichloromethyl free radicals and their covalent binding to cellular constituents in the anoxic lobular area may have greater significance in the development of hepatic necrosis.

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