Histological Detection of Lipid Peroxidation Following Infusion of Tert-Butyl Hydroperoxide and ADP-Iron Complex in Perfused Rat Livers

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ABSTRACT—Lipid peroxidation was assessed histologically and biochemically in hemoglobin-free perfused rat livers using two different types of stimulators. The Schiff reaction of fuchsin with cellular aldehydes was used as a histological index for lipid peroxidation. t-Butyl hydroperoxide (BHP, 0.8 mM) infusion caused a rapid and sustained release of thiobarbituric acid reactive substances (TBARS) into the effluent perfusate for up to 60 min, which was accompanied by lactate dehydrogenase (LDH) leakage after 30 min. The Schiff positive foci were initially restricted to periportal zones and spread with time to whole areas, accompanied by periportal necrosis. Co-infusion of diphenyl-p-phenylenediamine suppressed the TBARS release, with negative fuchsin staining, but the LDH leakage was unaffected. Under retrograde perfusion, BHP produced pericentral staining and necrosis. With 2.5 mM ADP-100 μM Fe³⁺, little TBARS was released up to 60 min, even though the hepatic TBARS levels increased considerably by this time. By 90 min, marked TBARS release occurred, but LDH leakage remained low. Irrespective of the direction of perfusion, pericentral hepatocytes became Schiff positive after 30 min. The fuchsin staining method may be useful for detecting peroxidized zones of the liver lobules.

Lipid peroxidation of biological membranes has been considered to be an important pathological process in various degenerative tissue injuries, including the earliest example of carbon tetrachloride hepatotoxicity (1–4). Although a number of biochemical methods have been developed for detecting lipid peroxidation (5), histological methods must be developed to determine the localization of lipid peroxidation within tissues or organs.

Taper et al. (6) applied a fuchsin staining method, which is based on the Schiff base formation via the reaction of fuchsin with cellular aldehydes, to livers from CCl₄-intoxicated rats; and they demonstrated that the Schiff positive areas in the liver lobules progressively increased after the administration of CCl₄. CCl₄ and related halogenomethanes are well-known to induce lipid peroxidation (1, 4). Furthermore, a large amount of aldehydic compounds are reported to be produced during lipid peroxidation in various biological systems including a halogenomethane-induced peroxidation system (7–12). Therefore, it appears that the histologic Schiff positive reaction may be applied for the detection of peroxidized lobular zones. This, however, remains to be established, since this staining method is not specific for lipid peroxidation and depends on cellular aldehyde levels which may possibly increase under various conditions without accompanying lipid peroxidation.
In the present study, we examined the validity of the fuchsin staining method for the detection of peroxidized zones using an isolated liver perfusion system infused with two different types of lipid peroxidation stimulators, t-buty1 hydroperoxide (BHP) and ADP-Fe$^{3+}$ complex. For this purpose, the release of thiobarbituric acid reactive substances (TBARS) into the effluent perfusate or hepatic TBARS content was measured concurrently with the histological method. Leakage of lactate dehydrogenase (LDH) was simultaneously monitored to see the relationship between lipid peroxidation and cell death.

MATERIALS AND METHODS

Materials
The reagents were obtained from the following sources: 2-thiobarbituric acid (TBA), N,N'-diphenyl-p-phenylene diamine (DPPD) and fuchsin basic, Wako Pure Chemicals; t-buty1 hydroperoxide (BHP), Katayama Chemicals; ADP and NAD$^+$, Oriental Yeast; trypan blue, Chroma Gesellschaft; and malonaldehyde bis(dimethylacetal), Aldrich. All other chemicals were of the highest grades commercially available.

Animals and treatment
Male, SPF-grade, Sprague Dawley rats 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%). Untreated rats with body weights of 170–180 g and phenobarbital (PB)-pretreated rats (0.1% PB in drinking water for 5 days) weighing 155–165 g were used for the liver perfusion experiments.

Liver perfusion
Liver 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 CaCl$_2$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25 mM NaHCO$_3$, and 5.6 mM glucose, saturated with 95% O$_2$–5% CO$_2$ at 37°C, hereinafter referred to as 95% O$_2$-KHB) was used as the perfusion medium. Details of the surgical procedure and the liver perfusion system are described elsewhere (13). Experiments commenced exactly 30 min after cannulation of the portal vein, which was defined as time zero. BHP (final concentration, 0.8 mM) and ADP-iron complex (final concentration, 2.5 mM ADP and 100 μM FeCl$_3$) was infused through a syringe pump at 0 min for appropriate periods. DPPD (final concentration, 10 μM), dissolved in dimethylsulfoxide, was infused at 0.025 ml/min.

After the experiments, the livers were immediately pre-fixed with 1% paraformaldehyde solution (in 20 mM phosphate-buffered saline, pH 7.4) for 8 min, weighed, and kept on ice for fuchsin staining. Some livers were infused with 0.2 mM trypan blue solution for 10 min, and then with 1% paraformaldehyde for examination of necrosis (14).

Histological examination
All livers except those used for the hepatic TBARS assay were subjected to histological examination. For fuchsin staining, the perfused liver, pre-fixed with 1% paraformaldehyde, was kept in an ice bath for 1 hr in order to complete fixation and to fasten the tissue. Small blocks of the liver were cut into approximately 50 μm-thick slices in cold 0.1 M phosphate buffer (pH 7.4) containing 20% glycerin, by a tissue micro-slicer (Dosaka EM, DTK-1000, Japan). Fuchsin staining was conducted essentially according to the method described by Taper et al. (6): The slices were stained with fuchsin in a small beaker for 2 hr, followed by washing with sodium bisulfite solution for 4 min; then they were washed with water, mounted on a slide glass with a drop of glycerin-phosphate buffer, and examined under a microscope at low magnification. Necrotic areas of the liver lobules were examined on trypan blue-stained and paraformaldehyde-fixed livers after fuchsin co-staining.
Biochemical assays

TBARS in the effluent perfusate was measured essentially according to the method of Ernster and Nordenbrand (15) using malondialdehyde (MDA) as the standard. Hepatic TBARS contents were assessed as follows: the control and ADP-Fe\(^{3+}\)-infused livers, after washing out the agent for 2 min, were cooled in ice-cold KHB; and then 2.5 g of each type of liver, in duplicate, was homogenized in 7.5 ml of 10% trichoroacetic acid (TCA). To 5 ml of each TCA extract was added 0.5 ml of 5 N HCl and 5 ml of 0.75% TBA. The mixture was kept in boiling water for 15 min, and the colored products were then extracted into 5 ml of n-butanol-pyridine (15 : 1). The absorbance spectra of these extracts were recorded between 470 - 600 nm.

LDH activity was assayed by the reduction of NAD\(^+\) at 25°C using the clinical assay method (16). One unit was defined as 1 \(\mu\)mol of NAD\(^+\) reduced/min/l of the perfusate.

RESULTS

Infusion of BHP

Normal rat livers were infused with a final concentration of 0.8 mM of BHP for 15 to 60 min, in which the concentration of BHP and duration of infusion exceeded the hydroperoxide removing capacity of the liver as reported in a similar liver perfusion system (17). As shown in Fig. 1A, the infusion of BHP in the anterograde perfusion system caused a rapid increase in the effluent TBARS levels and marked LDH leakage after 30 min. At 15 min, the periportal zones of the liver lobules were stained with fuchsin (Fig. 2B). The Schiff-positive areas spread with time to pericentral areas; and at 60 min, when considerable LDH leakage had occurred, hepatocytes around the periportal zones became necrotic as evidenced by trypan blue uptake (Fig. 2E). In the liver simultaneously infused with DPPD, a lipid peroxidation inhibitor (15), the TBARS release and Schiff-positive reaction were suppressed throughout the 60 min of BHP infusion, but marked LDH leakage and periportal necrosis occurred (Fig. 1B and Fig. 2D). With retrograde infusion of BHP, similar patterns of TBARS and LDH leakage were observed (Fig. 1C). However, the Schiff-positive foci were initially localized in the pericentral zones, which thereafter eventually became necrotic (Fig. 2, C and F). The control livers showed neither TBARS release nor LDH leakage and were Schiff-negative in both anterograde and retrograde perfusion systems (Fig. 2A and Fig. 5A).

Infusion of ADP-Fe\(^{3+}\)

In this study, we infused 2.5 mM ADP - 100 \(\mu\)M FeCl\(_3\), the concentration being nearly the same as that used by Poli et al. (12) and Sandy et al. (18) in experiments with isolated hepatocytes. In the preliminary experiments, normal rat livers were tested for fuchsin staining after infusion of the activator for up to 90 min, but the staining was only weakly and sporadically positive. Simultaneous infusion of Tween 80, a membrane permeabilizing agent (19), evidently increased TBARS release, but the liver became too soft to make slices. Therefore, in the following experiments, livers from PB-pretreated rats were used, in which microsomal Fe\(^{2+}\) + ADP + NADPH-dependent lipid peroxidation is reported to be enhanced (20).

In such livers, infusion of ADP-Fe\(^{3+}\) for 30 min did not increase the effluent TBARS levels, and an apparent TBARS release occurred after 60 min (Fig. 3A). Hepatic TBARS contents, however, increased considerably by 60 min (Fig. 4). At 90 min, a marked TBARS release occurred, but LDH leakage still remained low. Pericentral zones became Schiff positive at 30 min (Fig. 5B), when hepatic TBARS content had already increased, and the staining was more demarcated at 60 min (Fig. 5C) and spread over the entire lobule at 90 min. In a few specimens, periportal zones
also tended to be stained; the reason for this is unknown at present, but constriction of capillary blood vessels may change hepatic microcirculation, since influent hydraulic pressure was increased during ADP-Fe³⁺ infusion.

Retrograde infusion of ADP-Fe³⁺ for 60 min also resulted in a demarcated Schiff reaction at pericentral hepatocytes (Fig. 5D), without accompanying TBARS release (Fig. 3B).

DISCUSSION

A good correlation was obtained between TBARS production and positive fuchsin staining in the isolated livers infused with BHP, ADP-Fe³⁺ and BHP plus DPPD. We have also observed that the infusion of CBrCl₃ into perfused livers results in marked TBARS release and positive fuchsin staining, both of which are suppressed in the presence of DPPD (21). Thus, the parallelism between biochemical and histological observations satis-
Fig. 2. Histology of the isolated perfused livers infused with t-butyl hydroperoxide (BHP) (normal rats).  
(A) Control liver, 90 min, anterograde perfusion.  
(B) BHP (0.8 mM) infusion, 15 min, anterograde.  
(C) BHP, 15 min, retrograde.  
(D) BHP plus N,N'-diphenyl-p-phenylene diamine (10 μM), 60 min, anterograde.  
(E) BHP, 60 min, anterograde.  
(F) BHP, 60 min, retrograde.  
A–C: Fuchsin staining.  
D–F: Fuchsin staining of trypan blue infused livers.  
All liver samples used in the experiments shown in Fig. 1 were subjected to histological examination; and among them, typical examples are shown.  
P: Portal vein.  
C: Central vein.
Fig. 3. Leakage of thiobarbituric acid reactive substances (TBARS) and lactate dehydrogenase (LDH) following the infusion of ADP-Fe$^{3+}$ in the perfused livers isolated from phenobarbital-pretreated rats. (A) Anterograde perfusion. (B) Retrograde perfusion. Infusion of 2.5 mM ADP-100 μM Fe$^{3+}$ was started at 0 min. The number of livers used in experiments A and B were 13 and 3, respectively, and the number of livers subjected to histological examination at 30, 60 and 90 min are given in parentheses. Each point represents the mean ± S.D.

Fig. 4. Increase in hepatic thiobarbituric acid reactive substances (TBARS) content following the infusion of ADP-Fe$^{3+}$ in perfused livers from phenobarbital-pretreated rats. TBARS in the liver was reacted with 2-thiobarbituric acid, the colored products were extracted into n-butanol, and the absorbance spectra of the extracts were recorded. For details see the Methods section. Dotted line: Standard, equivalent to 4 nmol malondialdehyde per g liver. a: Control perfused livers (n = 6). b: ADP-Fe$^{3+}$ infusion, 30 min (n = 4). c: ADP-Fe$^{3+}$ infusion, 60 min (n = 5). Each point represents the mean ± S.D. of optical density at 535 nm. Note that because of base line deviation in the liver samples, accurate calculation of TBARS content is difficult.
fies the minimum requirements for the fuchsin staining method to be used as a histological method for the detection of lipid peroxidation.

Detection of hepato-lobular localization of lipid peroxidation may be of pathological significance. In an attempt to examine if fuchsin positive loci really represent sites of lipid peroxidation, we selected two different types of lipid peroxidation stimulators and changed the direction of perfusion. First, hydroperoxides are considered to stimulate lipid peroxidation autocatalytically through the formation of lipid peroxide and alkoxy radicals in the presence of transition metals (3, 22). BHP, in addition, is lipid soluble. Sies et al. (17) reported that BHP is readily extracted by the isolated liver up to certain amounts: e.g., only 3% of the BHP appears in the effluent perfusate under infusion of 0.6 mM BHP and the maximum extraction rate by the liver is calculated to be 1.5 μmol/g liver/min, although the intralobular distribution of BHP still remains to be determined. Therefore, in the perfusion system, hepatocytes in the inlet zones are expected to be exposed to higher concentration of BHP as well as higher oxygen tension and eventually to be more peroxidized than the outlet hepatocytes. This agrees with our present results. Second, enhancement of microsomal lipid peroxidation by ADP-Fe$^{3+}$ requires reduction of the iron by NADPH-cytochrome P-450 reductase and is thought to be initiated by hydroxy radicals (3, 5, 22, 23). This enzyme is more concentrated in the pericentral hepatocytes and is inducible by PB treatment (24). This appears to be in agree-
ment with our observations that PB-pretreated rat livers exhibited a much stronger Schiff-positive reaction than normal rat livers, and that the Schiff-positive reaction under anterograde perfusion appeared in pericentral to midzonal hepatocytes even though the hepatocytes in these zones should be exposed to lower concentrations of oxygen and ADP-Fe$^{3+}$ than the periportal hepatocytes. The delay in the onset of lipid peroxidation with ADP-Fe$^{3+}$ may be partly due to limited absorption of ADP-Fe$^{3+}$, which is less lipid soluble than BHP, since co-infusion of Tween 80 accelerated lipid peroxidation. In isolated hepatocytes, a similar concentration of the iron complex was reported to cause rapid lipid peroxidation (18); however, in this study, the plasma membrane permeability of the hepatocytes may have been somewhat altered during the isolation of cells by collagenase digestion.

Third, we have also observed that infusion of CBrCl$_3$, irrespective of the direction of perfusion, to the PB-pretreated rat livers causes Schiff-positive staining preferentially in the pericentral zones (21), where cytochrome P-450, which is required for reductive activation of CBrCJ$_3$ (4), is also enriched (24).

Thus, in view of considerations such as concentration gradient and liposolubility of the lipid peroxidation stimulators, lobular compartmentation of metabolic processes and oxygen concentration gradient, we would like to conclude that the Schiff-positive foci and the peroxidized areas are topologically identical. It should be mentioned, however, that positive fuchsin staining is based on the Schiff base formation of fuchsin with cellular aldehydes that are produced as a result of microsomal lipid peroxidation (7–12) and is not specific for lipid peroxidation. It is possible that cellular aldehyde levels may increase under various metabolic disturbances such as in the process of cellular degeneration, without accompanying lipid peroxidation. Therefore, at present, this staining method alone cannot determine the occurrence of lipid peroxidation. A number of factors should be also taken into consideration in this histological method: e.g., aldehydic compounds produced during lipid peroxidation may vary qualitatively and quantitatively under various conditions, and cellular concentration of aldehydes may not be kept constant due to further metabolization in the hepatocytes and leakage from them.

Another point concerns the relationship between lipid peroxidation and cell death. Sies et al. (17) and Sies and Summer (25) reported that an infusion of 0.3–0.6 mM BHP for 5 min into hemoglobin-free perfused livers leads to only reversible changes, accompanied by cellular oxidation of GSH through GSH peroxidase and the resultant release of GSSG into the perfusate. In the present study, we infused 0.8 mM BHP for much longer periods. Under such conditions, the peroxidized areas became necrotic at a later stage as evidenced by marked LDH leakage and trypan blue uptake. Co-infusion of DPPD considerably suppressed lipid peroxidation, but not necrosis. Ca$^{2+}$ is considered to play an important role in toxic cell injury (26), and omission of Ca$^{2+}$ from the perfusate markedly suppressed CCl$_4$-induced necrosis (13). However, the Ca$^{2+}$ omission reduced neither lipid peroxidation nor necrosis induced by BHP. These observations suggest that the necrotic action of BHP may not be simply due to its lipid peroxidizing action but rather to its direct deleterious effects on protein components, such as protein fragmentation (27), particularly in plasma membranes. In the case of ADP-Fe$^{3+}$ moderate LDH leakage occurred only after 90 min of infusion, while the hepatic TBARS content increased considerably by this time. Sandy et al. (18), using isolated hepatocytes, also demonstrated that a similar concentration of ADP-Fe$^{3+}$ produced rapid enhancement of lipid peroxidation, accompanied with only a slight loss of cell viability up to 180 min. The resistance of hepatocytes to death under ADP-Fe$^{3+}$-induced lipid peroxidation conditions may be due to localization of lipid peroxidation within or near the endoplasmic reticulum where ADP-Fe$^{3+}$ is activated, and plasma membranes may remain rather intact up to longer times. Through the actions of BHP and
ADP-Fe$^{3+}$ in perfused livers, it appears that lipid peroxidation itself may not always be the direct cause of acute cell death, although various deleterious effects on cellular functions may be involved (2).

In conclusion, this fuchsins staining method may be useful for the topological detection of lipid peroxidation in liver lobules in mechanistic studies of hepatotoxicity, even though there are a number of variables that affect such histological methods.

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