

EFFECT OF LINOLEIC ACID HYDROPEROXIDE ON LIVER MICROSOMAL ENZYMES IN VITRO

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Abstract—Rat liver microsomes incubated with linoleic acid hydroperoxide (LAHPO) lost cytochrome P-450 specifically among the enzymes of microsomal electron transport systems. The loss of cytochrome P-450 content and glucose-6-phosphatase activity by LAHPO was accompanied by an increase in malondialdehyde (MDA) production. Turbidity of microsomal suspensions was decreased with increasing MDA production, but not proportionately. Diethyldithiocarbamate (DTC), N,N'-diphenyl-p-phenylenediamine and α -tocopherol inhibited almost completely the LAHPO-induced MDA production of microsomes, however no perfect protection against the loss of cytochrome P-450 content and glucose-6-phosphatase activity was observed. The decrease of microsomal turbidity by LAHPO was little affected in the presence of DTC. Purified cytochrome P-450 was destroyed by LAHPO, with minimal protection by the compounds described above. These results suggest the possibility that the loss of microsomal enzyme activities during lipid peroxidation may be attributed largely to a direct attack on enzyme proteins by lipid peroxides rather than indirectly to a structural damage of microsomal membranes resulting from peroxidative breakdown of membrane lipids.

Biochemical and structural alterations of endoplasmic reticulum membranes induced by a peroxidative breakdown of membrane lipids are considered to play an important role in the hepatotoxicity of carbon tetrachloride, chloroform and halothane (1–4). Several lines of experiments *in vitro* show that microsomal drug-metabolizing enzymes, cytochrome P-450 and glucose-6-phosphatase are particularly sensitive to lipid peroxidation (5–9). However, the mechanisms involved are not well-defined. Since these membrane-bound enzymes are lipid-dependent and lose the activities in the absence of lipids (10, 11), they may be susceptible to the degradation of membrane lipids caused by lipid peroxidation (7).

On the other hand, lipid peroxidation process is a complex free radical chain reaction involving various reactive intermediates and end-products, some of which may be responsible for further degradation of membrane lipids and inactivation of membrane-bound enzymes as well (12). Among the reactive intermediates produced in an early step of lipid peroxidation process may be peroxides. Fatty acid peroxides and other organic peroxides are known to propagate a peroxidative breakdown of lipids (12, 13) as well as to inactivate various cell components and enzymes by means of oxidation of the SH-group (14–16). In this study, effects of LAHPO-induced lipid peroxidation on microsomal enzymes and structure were examined in the absence and presence of inhibitors of lipid peroxidation, in an attempt to gain insight into the mechanisms of the loss of enzyme activities during the lipid peroxidation process.

MATERIALS AND METHODS

Wistar rats weighing 200–250 g were used throughout the experiments. Microsomes were isolated from well-perfused livers of female rats: liver homogenate (20%, W/V) in 0.15 M KCl-10 mM EDTA (pH 7.5) was centrifuged at $15,000 \times g$ for 15 min, the supernatant fraction was then centrifuged at $125,000 \times g$ for 30 min, and the precipitated microsomal fraction was washed once with 0.1 M potassium phosphate buffer (pH 7.5). Linoleic acid hydroperoxide (LAHPO) was prepared according to the method of O'Brien (17) and the concentration was determined from the absorbance at 233 nm using a molar extinction coefficient $25.25 \text{ cm}^{-1}\text{M}^{-1}$. Microsomal lipids were extracted from liver microsomes by the method of Folch et al. (18), and liposomes ($20 \mu\text{moles Pi/ml}$) were prepared by sonicating the solvent-free lipids in 0.15 M KCl-25 mM Tris-HCl buffer (pH 7.5). Cytochrome P-450 was purified from phenobarbital-pretreated male rats according to the method of Imai and Sato (19). The final preparation contained 7.9 nmoles of cytochrome P-450 per mg protein. The degree of lipid peroxidation was estimated by measuring malondialdehyde (MDA) production using a thiobarbituric acid method (20). Glucose-6-phosphatase activity was measured by the method of Swanson (21). Other enzyme assays of microsomal electron transport systems have been described elsewhere (9). Protein was determined by the method of Lowry et al. (22). The regular experimental procedure was as follows. Microsomes (1 mg protein/ml) suspended in 10 ml of 0.15 M KCl-25 mM Tris-HCl buffer (pH 7.5) were incubated with various concentrations of LAHPO at 37°C for 10 min. The reaction ceased within 10 min. The mixture was then cooled in an ice bath and aliquots were assayed for MDA production, cytochrome P-450 content and glucose-6-phosphatase activity. When inhibitors were added, microsomes were preincubated with these inhibitors for 5 min at 37°C prior to the addition of LAHPO.

Turbidity change of microsomal suspensions (1 mg protein/ml of 50 mM Tris-HCl buffer, pH 7.5) was measured independently according to the method of Robinson (23) by recording a decrease in optical density at 520 nm, for which a spectrophotometer attached with a thermo-regulated cell-holder (37°C) was used. The absorbance change after addition of LAHPO was so rapid that more than 90% of overall optical density change occurred within a few minutes. Regularly, a decrease in absorbance after 10 min was adopted as a $\Delta \text{O.D.}$ value.

RESULTS

Microsomal MDA production by LAHPO

As hydroperoxides are intermediates of the peroxidative process of unsaturated fatty acids (12, 13), it is expected that LAHPO is incorporated into biological membranes and provoke lipid peroxidation. Microsomes incubated with LAHPO exhibited a rapid MDA production, which reached a plateau between 5 and 10 min at 37°C . Fig. 1(A) shows MDA production of microsomes 10 min after incubation with LAHPO, the rate being increased as the concentration of microsomes or LAHPO was raised. LAHPO alone exhibited minimal MDA production.

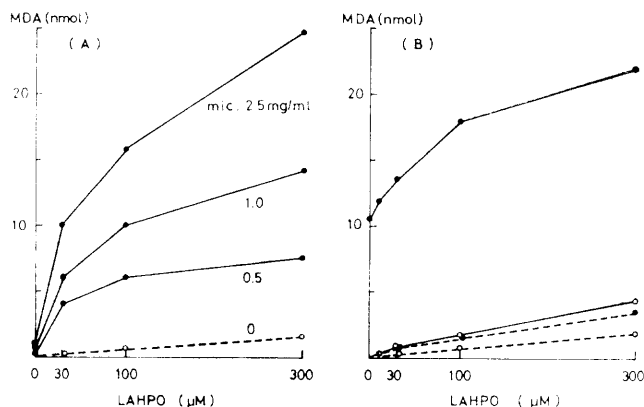


FIG. 1. Malondialdehyde (MDA) production of microsomes and liposomes by linoleic acid hydroperoxide (LAHPO). (A): MDA production of microsomes. Microsomes and LAHPO were mixed with various concentrations as indicated in the figure in a total volume of 1.0 ml of 0.15 M KCl-25 mM Tris-HCl buffer (pH 7.5) and after incubation at 37 °C for 10 min, MDA produced in the mixture was determined. (B): MDA production of liposomes. Liposomes (1 μ mole Pi/ml) were mixed with LAHPO in the absence and presence of activator (20 μ M sodium pyrophosphate-100 μ M ferric chloride-50 μ M EDTA) and processed as described in (A). (○—○): LAHPO alone, (○—○): LAHPO with activator, (●—●): liposomes plus LAHPO, (●—●): liposomes plus LAHPO with activator.

On the other hand, liposomes (1 μ mole Pi/ml) prepared from microsomal lipids showed only a small increment in MDA production even in the presence of LAHPO. In this experiment, microsomes contained approx. 0.8 μ moles of lipid phosphorus per mg protein. Addition of an activator (20 μ M sodium pyrophosphate-100 μ M ferric chloride-50 μ M EDTA), however, markedly enhanced liposomal MDA production, and the enhancement by LAHPO was more obvious (Fig. 1(B)). These observations indicate that microsomal lipid peroxidation by LAHPO might be accelerated by intrinsic microsomal components as catalyzers—probably some iron compounds including hemoproteins—and by their degradation products.

Effect of LAHPO on enzymes of microsomal electron transport systems

Although it is reported that organic hydroperoxides destroy various cell components (14–16), apparently no systematic experiment with microsomal enzymes has been done with LAHPO. Effects of LAHPO on enzyme activities and hemoprotein contents of microsomal electron transport systems are shown in Fig. 2(A). Among the enzymes examined, cytochrome P-450 was particularly sensitive to LAHPO. The loss of cytochrome P-450 content was also observed when LAHPO was added to microsomes solubilized with cholate and Emulgen 913 (data not shown). Fig. 2(B) shows the effect of LAHPO on partially purified cytochrome P-450. Soret-peak at 418 nm of the hemoprotein spectrum disappeared with the addition of LAHPO, confirming the destruction of the heme portion of the enzyme molecule (6).

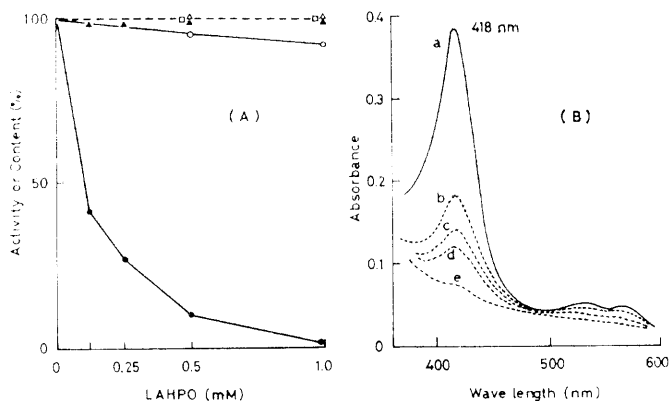


FIG. 2. Destruction of cytochrome P-450 by linoleic acid hydroperoxide (LAHPO). (A): Microsomal membrane-bound cytochrome P-450. Microsomes (2 mg protein/ml) were mixed with various concentrations of LAHPO in 0.2 M potassium phosphate buffer (pH 7.5) and incubated at 37 °C for 10 min. The mixture, after being diluted with cold water to double the volume, was assayed for enzyme contents (cytochrome P-450 and b_5) and activities (NADPH-cytochrome c reductase, NADH-ferricyanide and -cytochrome c reductase). (●—●): cytochrome P-450, (▲—▲): NADPH-cytochrome c reductase, (○—○): cytochrome b_5 , (△—△): NADH-ferricyanide reductase, (□—□): NADH-cytochrome c reductase. (B): Partially purified cytochrome P-450. Cytochrome P-450 was dissolved in 0.1 M potassium phosphate buffer (pH 7.5) containing 20% glycerol and 0.2% Emulgen 913, and the spectral change was followed after addition of LAHPO. Curve a: before addition of LAHPO, b: 3 min after addition of LAHPO (0.33 mM), c: 9 min, d: 18 min, and e: 5 min after further addition of LAHPO (final concentration, 0.66 mM).

Simultaneous monitoring of MDA production, cytochrome P-450 content, glucose-6-phosphatase activity and turbidity change of microsomes after addition of LAHPO (Fig. 3, solid lines)

To determine a possible relationship between LAHPO-induced lipid peroxidation and the loss of enzyme activity, simultaneous monitoring of these phenomena was attempted. In this case, glucose-6-phosphatase activity and turbidity of microsomes were also examined. As shown in Fig. 3(A, B and C), the loss of microsomal cytochrome P-450 content and glucose-6-phosphatase activity was inversely proportional to the MDA production. These observations and those shown in Fig. 2(A) are quite consistent with the results obtained when microsomes were peroxidized by other methods (5–9). Thus, the loss of microsomal lipid-dependent enzyme activities appears to be coupled with the peroxidative breakdown of membrane lipids. In addition, turbidity of microsomal suspension, another indicator of microsomal structural alteration, was also decreased with increasing MDA production, however such was not proportional: the turbidity change was more resistant to lipid peroxidation (Fig. 3(D)). If the microsomal changes induced by LAHPO were mediated through its lipid peroxidative action, inhibitors of lipid peroxidation would be expected to protect against these microsomal alterations. This point was examined in the following experiments.

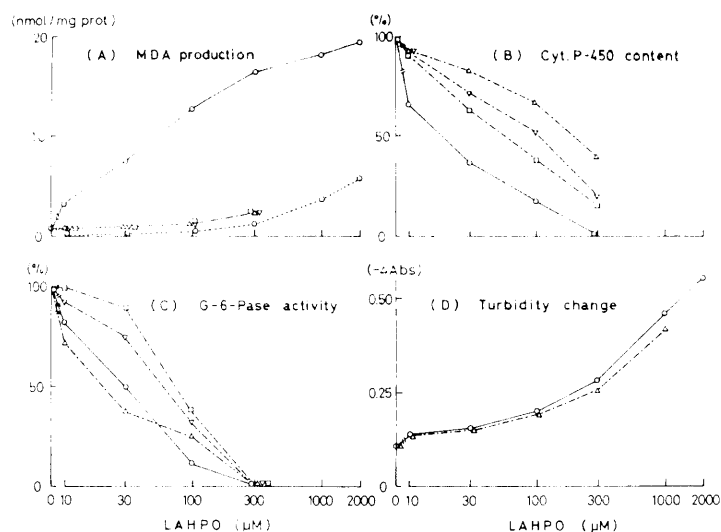


FIG. 3. Effect of linoleic acid hydroperoxide (LAHPO) on malondialdehyde (MDA) production, cytochrome P-450 content, glucose-6-phosphatase activity and turbidity of liver microsomes in the absence and presence of inhibitors of lipid peroxidation. Experimental procedures are given in Materials and Methods. Symbols and concentrations of inhibitors are as follows: (○—○): without inhibitor, (○—○): MDA value of LAHPO alone, (Δ — Δ): diethyldithiocarbamate (DTC) 1 mM, (∇ — ∇): N,N'-diphenyl-p-phenylenediamine (DPPD) 0.1 mM, and (\square — \square): α -tocopherol 1 mM. Each point represents the average of 2 experiments.

Effects of diethyldithiocarbamate (DTC), N,N'-diphenyl-p-phenylenediamine (DPPD) and α -tocopherol on LAHPO-induced microsomal changes (Fig. 3, broken lines)

Among the compounds tested for inhibition of the MDA production induced by 300 μM of LAHPO, DTC (1 mM <), DPPD (0.1 mM <) and α -tocopherol (1 mM <) were strong inhibitors. These compounds are also known to inhibit microsomal lipid peroxidation induced by other methods (9, 20). No inhibition was observed with EDTA (1–10 mM) or glutathione (1–10 mM). Complete inhibition of MDA production by the former three compounds is shown in Fig. 3(A). However, the loss of microsomal cytochrome P-450 content was only partially protected in the presence of these inhibitors in spite of the complete suppression of lipid peroxidation (Fig. 3(B)). Similar results were obtained with glucose-6-phosphatase activity (Fig. 3(C)): in this case, however, protective action of these compounds was weak as compared with the case of cytochrome P-450, and DTC rather enhanced the loss of the activity with lower concentrations of LAHPO. Nature of the inactivation of these enzymes by LAHPO may be different. Furthermore, LAHPO-induced turbidity change was little affected in the presence of DTC (Fig. 3(D)).

These findings suggest that LAHPO itself can act on microsomal proteins and inactivate some of the enzymes even without an accompanying peroxidative breakdown of microsomal lipids.

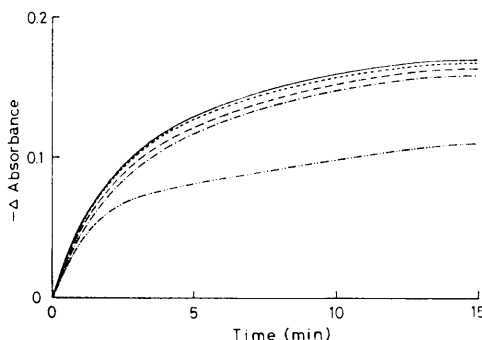


FIG. 4. Effects of inhibitors of lipid peroxidation on linoleic acid hydroperoxide (LAHPO)-induced destruction of partially purified cytochrome P-450. The reaction mixture contained cytochrome P-450 ($1.7 \mu\text{M}$), LAHPO ($250 \mu\text{M}$) and inhibitors in the same buffer as described in the legend of Fig. 2(B). The change in absorbance at 418 nm was followed after addition of LAHPO. Each curve is the mean of 2 to 3 experiments. (—): Without inhibitors, (---): Diethyldithiocarbamate (DTC) 1 mM, (---): DTC 10 mM, (----): N,N'-diphenyl-p-phenylenediamine (DPPD) 0.1 mM, (-·-·-·-): α -Tocopherol 1 mM.

Effects of lipid peroxidation inhibitors on LAHPO-induced destruction of purified cytochrome P-450 (Fig. 4)

In this experiment, a decrease in absorbance at 418 nm (Soret-peak of cytochrome P-450, see Fig. 2(B)) was measured after addition of LAHPO in the presence of inhibitors in order to assess the breakdown of the hemoprotein. DTC (1 mM), DPPD (0.1 mM) and α -tocopherol (1 mM) exhibited only a minimal protective effect against the breakdown of the hemoprotein by LAHPO. Obvious partial protection by a high concentration of DTC might be partly due to a decrease of actual LAHPO concentration owing to the interaction of LAHPO with DTC (data not shown). These observations may add further support for the direct action of LAHPO on the microsomal enzymes.

DISCUSSION

Present results with LAHPO as a peroxidizing agent show a good coincidence with experimental data obtained when microsomes were peroxidized enzymatically (with NADPH), non-enzymatically (with ascorbic acid) or in the presence of NADPH and carbon tetrachloride (5-9), i.e. glucose-6-phosphatase activity and cytochrome P-450 content were decreased and such was accompanied by a concomitant increase in MDA production, whereas NADH-ferricyanide reductase, NADH- and NADPH—cytochrome c reductase activities and cytochrome b_5 content were little affected under the present experimental conditions. Turbidity change of microsomal suspensions was also observed, though such did not parallel the MDA production. These observations indicate that lipid peroxides generated in microsomal membranes by any route in an early stage of lipid peroxidative process can be responsible for further propagation of lipid peroxidation reaction as well as enzyme inactivation and structural damage of microsomes. It is difficult to explain from

these data alone, however, how the lipid peroxidative breakdown of microsomal lipids can be related to the functional changes.

The following reasons may be considered for the inactivation of microsomal enzymes: firstly, direct action of hydroperoxides which inactivate enzymes or denature proteins by way of oxidation of the SH-group (14-16), and secondly, disturbance of hydrophobic milieu of the membrane due to lipid peroxidation. In connection with the latter point, glucose-6-phosphatase and cytochrome P-450 are highly lipid-dependent enzymes (10, 11).

To examine these points, effects of inhibitors of lipid peroxidation on microsomal enzymes were studied. DTC, DPPD and α -tocopherol, structurally quite different free radical scavengers which are known to inhibit lipid peroxidation (9, 20), suppressed the LAHPO-induced MDA production completely, but protected the enzyme inactivation only partially. These observations are in favor of the first notion. Destruction of partially purified cytochrome P-450 by LAHPO, with minimal protection by the lipid peroxidation inhibitors, may be further supporting evidence. In addition, the structural change of microsomes may also result from denaturation of membrane proteins, since the LAHPO-induced change in turbidity was little affected in the presence of DTC.

Thus, in conclusion, it appears that lipid peroxides produced by any route in the lipid layer of microsomal membranes may attack neighboring proteins, causing loss of some enzyme activities and structural alterations, and that this process may be promoted by further production of peroxides through the free radical-mediated propagation reaction on the one hand and the attendant breakdown of membrane lipids on the other. Microsomal lipid peroxidation is currently considered to be an important initiating factor in the hepatotoxicity of carbon tetrachloride which is accompanied by a considerable loss of cytochrome P-450 and glucose-6-phosphatase (1, 2, 9, 24). Involvement of the mechanism proposed herein is worthy of consideration.

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