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Oxidation of Rat Liver Phospholipids: Comparison of Pathways in Homogeneous Solution, in Liposomal Suspension and in Whole Tissue Homogenates[1]

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The oxidation of equivalent concentrations of phospholipids in homogeneous solution, in multilamellar liposomal suspension, and in rat liver homogenate was carried out under aerobic conditions at 37°C in order to examine the biochemical fate of oxidized phospholipids. Rat liver phospholipids were extracted with chloroform and methanol, and oxidation in this homogeneous solution was initiated with a lipid-soluble radical initiator. The oxidation products were phosphatidylcholine hydroperoxide (PC-OOH) and phosphatidylethanolamine hydroperoxide (PE-OOH), which were quantified by HPLC separation using a hydroperoxide-specific chemiluminescence detector. Co-extracted α-tocopherol and ubiquinol-9 suppressed the formation of PC-OOH and PE-OOH until oxidatively exhausted. The oxidation of extracted rat liver phospholipids in multilamellar liposomal suspension initiated with the lipid-soluble initiator gave similar results, but with slower rates of antioxidant depletion and phospholipid hydroperoxide formation due to a lower efficiency of free radical production in liposomal membranes. In contrast, the oxidation of rat liver homogenate containing active tissue enzymes initiated by the addition of either free radical initiators or *tert*-butyl hydroperoxide gave phosphatidylcholine hydroxide, phosphatidylethanolamine hydroxide, and free fatty acid hydroxides as oxidation products. Exogenous PC-OOH added to the rat liver homogenate was reduced to phosphatidylcholine hydroxide with subsequent hydrolysis to its free fatty acid hydroxide. These results suggest that peroxidase and phospholipase enzymes play important roles in the repair of oxidatively damaged phospholipids in biomembranes.

Key words: peroxidase, phospholipase, phospholipid hydroperoxide, α-tocopherol, ubiquinol-9.

Lipid peroxidation has received much attention in connection with its pathological effects and possible contributions to aging and degenerative diseases such as heart attack, diabetes, cancer, and others (*1*). The most susceptible substrates of cellular oxidation are phospholipids, since they contain considerable amounts of polyunsaturated fatty acids (PUFA). The oxidation of biomembranes proceeds by a free radical chain mechanism (*2*) to form phospholipid hydroperoxides (PL-OOH) as primary products. This was demonstrated by the oxidation of phosphatidylcholine (PC) liposomal membrane to yield its hydroperoxide (PC-OOH) as the major product (*3-5*).

A very sensitive method for the detection and characterization of lipid hydroperoxides at picomole levels by HPLC was previously developed by Yamamoto *et al.* (*6*). The method was first applied to human plasma, and was able to detect 3 nM cholesteryl ester hydroperoxide (*7*). However, we were unable to detect PC-OOH in human and rat plasmas (*8, 9*). This may be ascribed to the fact that cholesteryl ester hydroperoxide is stable in plasma while PC-OOH is not (*10*). We have shown that plasma glutathione peroxidase reduces PC-OOH, but not cholesteryl ester hydroperoxide (*11*), and these results are consistent with the presence of CE-OOH and the absence of PC-OOH in human and rat plasmas (*9*).

We have also applied this method to the analysis of lipid hydroperoxides in solid tissues but failed to detect PC-OOH in rat liver and heart (*12*). In order to clarify this result we examined the oxidation of phospholipids extracted from rat liver in homogeneous solution, as well as

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Abbreviations: 18:2-OH, linoleic acid hydroxide; AAPH, 2,2′-azobis-(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, 2,6-di-*tert*-butyl-4-methylphenol; BOOH, *tert*-butyl hydroperoxide; FFA, free fatty acid; FFA-OH, free fatty acid hydroxide; FFA-OOH, free fatty acid hydroperoxide; PC, phosphatidylcholine; PC-OH, phosphatidylcholine hydroxide; PC-OOH, phosphatidylcholine hydroperoxide; PE, phosphatidylethanolamine; PE-OH, phosphatidylethanolamine hydroxide; PE-OOH, phosphatidylethanolamine hydroperoxide; PI, phosphatidylinositol; PI-OOH, phosphatidylinositol hydroperoxide; PL-OH, phospholipid hydroxide; PL-OOH, phospholipid hydroperoxide; PLPC, 1-palmitoyl-2-linoleoyl-phosphatidylcholine; PLPC-OH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroxide; PLPC-OOH, 1-palmitoyl-2-linoleoyl-phosphatidylcholine hydroperoxide; PS, phosphatidylserine; PS-OOH, phosphatidylserine hydroperoxide; PUFA, polyunsaturated fatty acid; UQ-9, ubiquinol-9; VC, ascorbate; VE, α-tocopherol.

liposomal suspension, and tissue homogenate. The formation of phospholipid hydroxides (PL-OH) and free fatty acid hydroxides (FFA-OH) by the oxidation of the liver homogenate, the absence of these products in the case of oxidation in solution or liposomal suspension, and the rapid decomposition of PC-OOH added to the liver homogenate suggest that peroxidase and phospholipase enzymes play important roles in the repair of oxidative damage to membrane phospholipids.

MATERIALS AND METHODS

Materials—Isoluminol, microperoxidase, 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), lipoxygenase, and monodansylcadaverine were purchased from Sigma (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), *tert*-butyl hydroperoxide (BOOH), and diethyl phosphorocyanidate were obtained from Wako Pure Chemical (Osaka) and used as received. Ubiquinone-9 was a generous gift from Eisai (Tokyo) and ubiquinol-9 (UQ-9, reduced form of ubiquinone-9) was prepared by its reduction with sodium borohydride. Solvents and other reagents were of the highest grade commercially available. All HPLC analytical columns (5 μm, 250×4.6 mm i.d.) were obtained from Supelco Japan (Tokyo).

PLPC hydroperoxide (PLPC-OOH) was prepared by the aerobic oxidation of PLPC with lipoxygenase (*13*). PLPC (6 μmol) was dissolved in 20 ml of 0.1 M sodium borate buffer (pH 9) containing 3 mM sodium deoxycholate and mixed with 2 mg of soybean lipoxygenase (Sigma, Type 1B). The solution was stirred for 45 min at room temperature and was passed through a solid phase extraction tube (LC-18, 1 g; Supelco Japan) to remove bile acid and other components. The eluate was concentrated and PLPC-OOH was purified by HPLC (Superiorex ODS column; 20×250 mm, Shiseido, Tokyo) using 0.02% triethylamine in methanol as the mobile phase with a flow rate of 8 ml/min. The corresponding alcohol (PLPC-OH) was purified by solid-phase extraction and HPLC separation as described above after reduction with sodium borohydride. The extinction coefficients of PLPC-OOH and PLPC-OH should be similar, as observed for methyl linoleate hydroperoxides and hydroxides (*14*), and their concentrations were determined by comparing their absorbance at 234 nm with that of authentic methyl linoleate hydroperoxide (*6*). PC-OOH, PE hydroperoxide (PE-OOH), PS hydroperoxide (PS-OOH), and PI hydroperoxide (PI-OOH) were also prepared by the autoxidation of the parent phospholipids at room temperature for several days. Concentrations of these phospholipid hydroperoxides were estimated by the use of a hydroperoxide-specific chemiluminescence detection system (*6, 12*) as described below.

Oxidation of Rat Liver Phospholipid—Male Fisher rats (5 weeks old) were obtained from Charles River Japan (Atsugi). Lipids and oil-soluble antioxidants were extracted directly from 0.5 g of rat liver with 5 ml of chloroform/methanol (2 : 1, v/v) using a Teflon homogenizer. Aerobic oxidation of the rat liver extract in chloroform/methanol solution was initiated with 5 mM AMVN at 37°C.

Multilamellar liposomal suspension was prepared by removing solvents from 5 ml of the above extract under reduced pressure, followed by the addition of 5 ml of water containing 100 μM EDTA with vigorous shaking. Oxidation of multilamellar liposomal suspension was initiated by adding 50 μl of 505 mM AMVN in methanol (final concentration; 5 mM) under aerobic conditions at 37°C.

Rat liver homogenate was prepared by homogenizing 0.5 g of minced rat liver in 5 ml of Chelex 100-treated Tris-HCl (5 mM, pH 7.4) containing 0.25 M sucrose using a Teflon homogenizer and solids were discarded after centrifugation at 3,000 rpm for 10 min. Oxidation of the homogenate was initiated by the addition of either 20 mM AAPH, 20 mM AMVN, or BOOH (0.25, 0.5, 1, or 2 mM) under aerobic conditions at 37°C. PLPC-OOH in methanol (final concentration; 26 μM) was also added to the rat liver homogenate and the mixture was incubated under aerobic conditions at 37°C.

Product Analysis—PL-OOH and PL-OH were analyzed by HPLC with a UV detector and a hydroperoxide-specific chemiluminescence detector as previously described (*6, 12*) on an aminopropylsilyl column (5 μm, 250×4.6 mm i.d.) with a silica gel guard column (5 μm, 20×4.6 mm i.d.) using methanol/*tert*-butyl alcohol/40 mM aqueous monobasic sodium phosphate (6 : 3 : 1, v/v/v) as the mobile phase. Since PL-OOH and PL-OH have identical retention times under these conditions, the amount of PL-OOH was determined by the chemiluminescence detector and the combined amount of PL-OOH and PL-OH was determined by UV detection at 234 nm; the amount of PL-OH was calculated as the difference. Samples (20 μl) from phospholipid oxidation in solution and in multilamellar liposomal suspension were injected directly onto the HPLC. Homogenate samples were mixed with two volumes of chloroform/methanol (2 : 1, v/v) containing 100 μM 2,6-di-*tert*-butyl-4-methylphenol (BHT; to prevent oxidation) and centrifuged at 12,000 rpm for 3 min. Aliquots (20 μl) of the organic phase were analyzed by HPLC. Levels of phospholipids were estimated from the absorption at 210 nm.

The endogenous lipid-soluble antioxidants, α-tocopherol (VE) and UQ-9, present in rat liver were separated by HPLC on an octylsilyl column (5 μm, 250×4.6 mm i.d.) with 50 mM sodium perchlorate in methanol/*tert*-butyl alcohol (9 : 1, v/v) as the mobile phase and quantified by electrochemical detection on a carbon plate electrode operating at 800 mV (*15*).

Levels of water-soluble ascorbate (VC) in the rat liver homogenate was also determined by HPLC (aminopropylsilyl column; 5 μm, 250×4.6 mm i.d.) with UV-detection at 265 nm, using methanol/40 mM monobasic sodium phosphate (9 : 1, v/v) as the eluent (*8*). Prior to the injection, homogenate samples were mixed with 4 volumes of methanol containing 100 μM BHT, followed by centrifugation at 12,000 rpm for 3 min.

Concentrations of free fatty acid hydroperoxides (FFA-OOH), FFA-OH, and free fatty acid (FFA) were determined by a precolumn fluorescence derivatization method (*16*). Homogenate samples were mixed with two volumes of chloroform/methanol (2 : 1, v/v) containing 100 μM BHT and centrifuged at 12,000 rpm for 3 min. Aliquots (50 μl) were mixed with 500 pmol of tridecanoic acid and 5 nmol of margaric acid (internal standards), and dried under a stream of N_2, then the residue was dissolved in 50 μl of

N,N-dimethylformamide containing monodansylcadaverine (1 mg/ml) and 1 μl of diethyl phosphorocyanidate. The solution was left to stand for 15 min at room temperature in the dark, then 5 μl was injected onto a pKb-100 column (5 μm, 150×4.6 mm i.d., Supelco Japan). Derivatized FFA-OOH, FFA-OH, and FFA components were separated by isocratic elution with 60% methanol at a flow rate of 1.3 ml/min (15 min), followed by a linear gradient to 70% methanol (10 min), isocratic elution with 70% methanol (25 min), and a 5 min linear gradient to 85% methanol; the derivatized products were determined by fluorescence detection (excitation, 340 nm; emission, 518 nm).

All experiments were conducted at least twice to verify the reproducibility of data.

RESULTS AND DISCUSSION

Figure 1 shows the separation and detection of 10 pmol each of PC-OOH, PE-OOH, PS-OOH, and PI-OOH standards by HPLC equipped with a hydroperoxide-specific, chemiluminescence detection system (*6, 12*). This sensitive method provided the means to study free radical oxidation of biomembrane phospholipids at the initial stages and its inhibition by antioxidants (*12*).

The rates of aerobic oxidation of rat liver extracts in chloroform/methanol (2 : 1, v/v) solution and in multilamellar liposomal suspension, respectively, initiated with 5 mM AMVN at 37°C, were measured using the above method (Fig. 2, A and B). Figure 2, C and D, also shows the corresponding decreases of endogenous lipid-soluble antioxidants (VE and UQ-9) during the oxidation. The initial concentrations of PC, PE, PS, and PI were estimated as 2.3, 1.0, 0.1, and 0.3 mM, respectively, from the published data

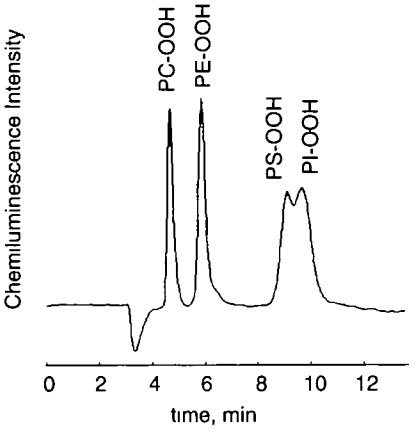

Fig. 1. **Separation and detection of 10 pmol each of PC-OOH, PE-OOH, PS-OOH, and PI-OOH by HPLC equipped with a hydroperoxide-specific, isoluminol chemiluminescence detection system.**

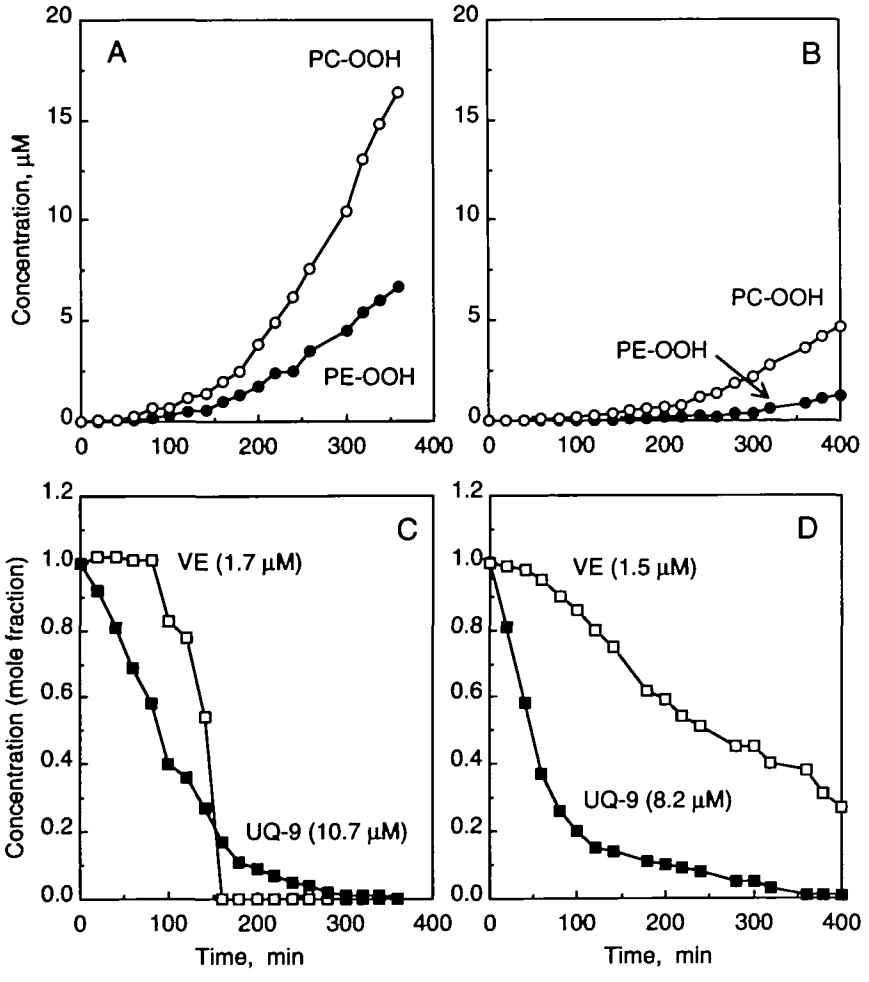

Fig. 2. **Formation of PL-OOH and the decrease of VE and UQ-9 during the oxidation of rat liver extracts in (A, C) chloroform/methanol (2 : 1, v/v) solution and (B, D) in multilamellar liposomal suspension under aerobic conditions at 37°C initiated with 5 mM AMVN.** Numbers in parentheses are the initial concentrations. Estimated initial concentrations (from Ref. *17*) of PC, PE, PS, and PI are 2.3, 1.0, 0.1, and 0.3 mM, respectively.

(*17*). The oxidation products were PC-OOH and PE-OOH in both oxidation systems, while the formation of PS-OOH and PI-OOH was not observed, probably due to the lower concentrations of PS and PI, in rat liver tissues. The formation of PC-OOH and PE-OOH was efficiently suppressed (Fig. 2A) as long as UQ-9 and VE were present (Fig. 2C). Since ubiquinol can reduce VE radical to VE (*18*), UQ-9 decreased while VE remained unchanged for the first 80 min (Fig. 2C) during the oxidation in homogeneous solution. Similar results were obtained in the oxidation of rat liver extracts in multilamellar liposomal suspension (Fig. 2D). The rates of PL-OOH formation (Fig. 2B) and antioxidant consumption (Fig. 2D) were slower than those in solution because the efficiency of free radical production from the lipid-soluble initiator is much lower in liposomal membranes than in solution (*19*). It is noteworthy that no formation of PC hydroxide (PC-OH) and PE hydroxide (PE-OH), FFA, FFA-OOH, and FFA-OH was observed in these hepatocyte enzyme-free systems (data not shown).

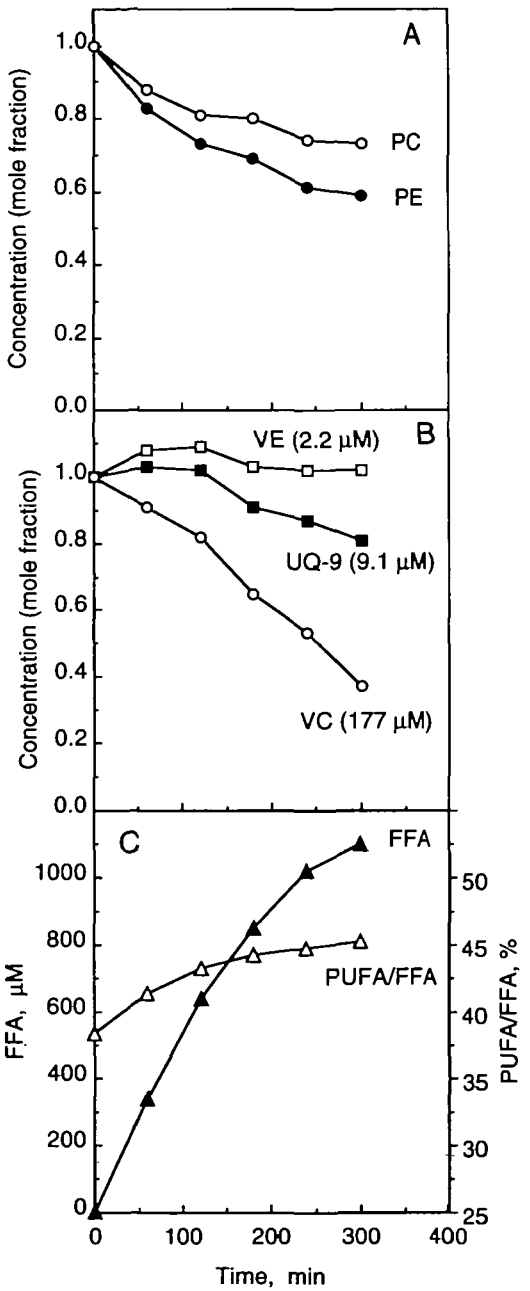

Fig. 3. **Decrease of (A) phospholipids (PC and PE), (B) antioxidants (VE, UQ-9, and VC) and (C) the formation of FFA during the incubation of rat liver homogenate under aerobic conditions at 37°C (Control).** The ratios of PUFA to FFA are also shown in (C). Numbers in parentheses are the initial concentrations. Estimated initial concentrations of phospholipids are the same as given in Fig. 2.

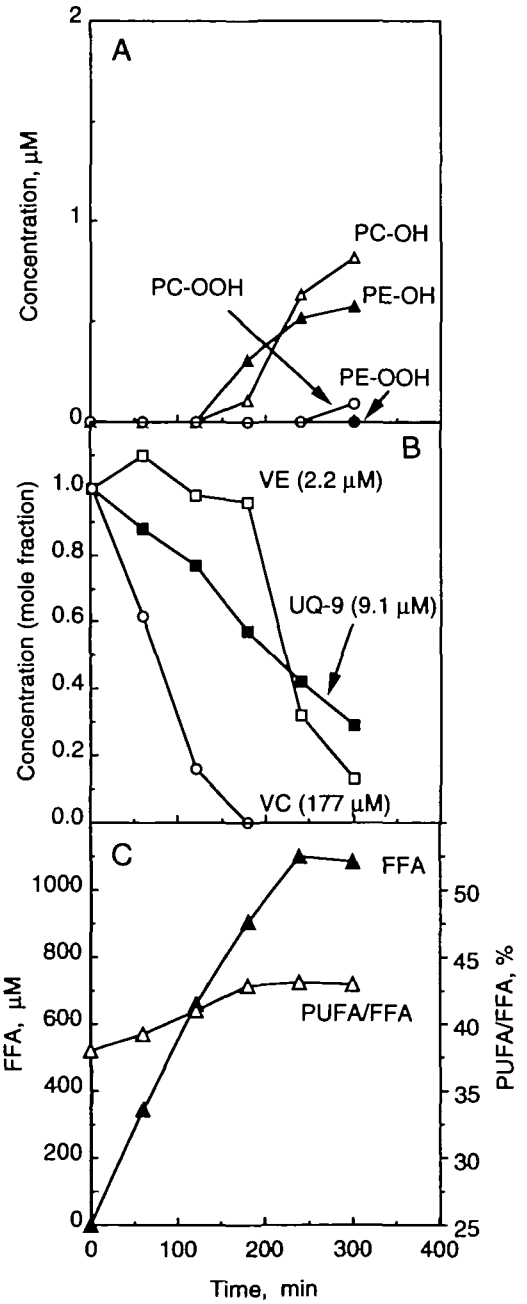

Fig. 4. **Formation of (A) PL-OOH and PL-OH, (B) the decrease of VE, UQ-9, and VC, and (C) the formation of FFA during the aerobic oxidation of rat liver homogenate initiated with 20 mM AAPH at 37°C.** The ratios of PUFA to FFA are given in (C). Numbers in parentheses are the initial concentrations. Estimated initial concentrations of phospholipids are the same as given in Fig. 2.

Next, we examined the changes in mole fraction levels of PC, PE, VE, UQ-9, VC, and FFA concentrations during the incubation of rat liver homogenate (without radical initiators) exposed to air at 37°C (Fig. 3). No formation of PC-OOH, PE-OOH, PC-OH, and PE-OH was observed (data not shown) although VC and UQ-9 slowly decreased (Fig. 3B). VE remained unchanged (Fig. 3B). Decrease of PC and PE (Fig. 3A) is probably due to the phospholipase activity in homogenate, since concomitant formation of FFA was observed (Fig. 3C). Increase in the ratio of PUFA to FFA (Fig. 3C) suggests that phospholipase A_2 is the major phospholipase activity, since PUFA are attached at the 2 position of phospholipids (*17*).

The oxidation of rat liver homogenate initiated with 20 mM water-soluble radical initiator (AAPH) under aerobic conditions at 37°C gave PC-OH, PE-OH, and PC-OOH as products (Fig. 4A). The formation of PC-OH and PE-OH (Fig. 4A) was significant only after the depletion of VC (Fig. 4B) and the amount of PC-OOH formed was much smaller than that of PC-OH (Fig. 4A). PE-OOH was not detected during 300 min of oxidation. VE remained unchanged for 180 min while VC was present. After 180 min incubation, VE decreased at a faster rate than UQ-9. This was not the case in the oxidation in solution or in multilamellar liposomal suspension (Fig. 2, B and D), suggesting that VE and UQ-9 may reside at different locations within the homogenate. VE is likely to be located on the outer surface of plasma and organelle membranes, being readily accessible to water-soluble peroxyl radicals produced from AAPH. On the other hand, UQ-9 may be located on the inner membrane surface, being less accessible to the water-soluble peroxyl radicals. It is well known that UQ-9 and ubiquinone-9 within the mitochondrial inner membrane play important roles in oxidative phosphorylation. It is noteworthy that the rate of FFA formation and the increase in the PUFA/FFA ratio (Fig. 4C) in the oxidation initiated by AAPH were identical with those in the control incubation (Fig. 3C), suggesting that the production of water-soluble peroxyl radicals did not interfere with the phospholipase A_2 activity in the aqueous phase.

The results of the oxidation of rat liver homogenate initiated with 20 mM AMVN, a lipid-soluble radical initiator, under aerobic conditions at 37°C are shown in Fig. 5. The initial disappearance rates of the antioxidants decreased in the order of VE>UQ-9>VC (Fig. 5B). This order is opposite to the one observed with the oxidation initiated by AAPH (Fig. 4B), and is consistent with the idea that AAPH produces free radicals in the water phase, while AMVN is active in the lipid phase. The rapid decay of VE and slower decrease of UQ-9 suggest that most VE is

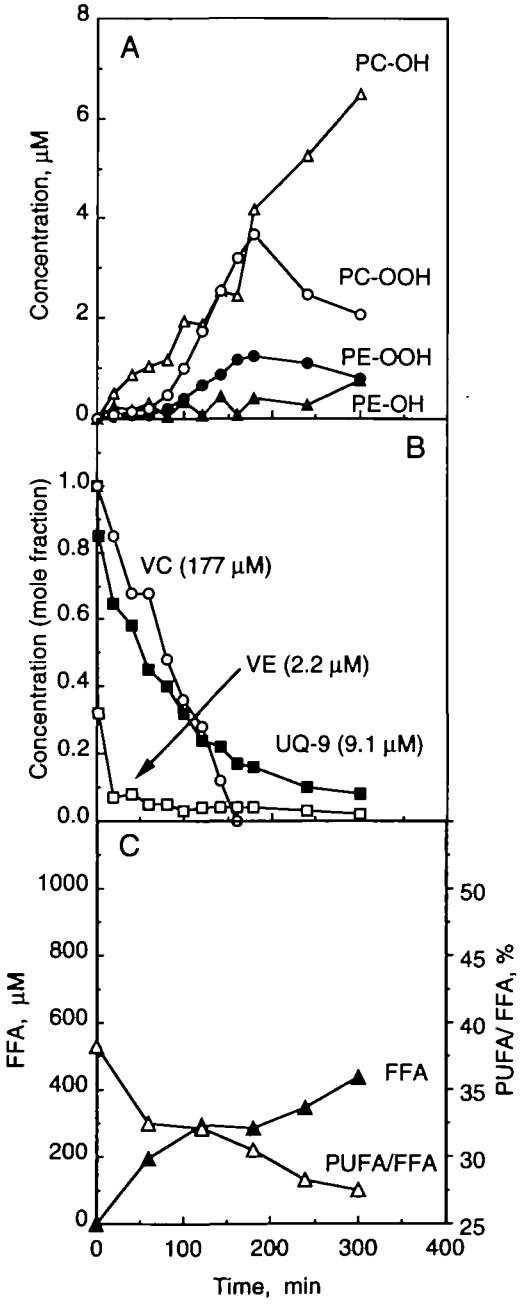

Fig. 5. **Formation of (A) PL-OOH and PL-OH, (B) the decrease of VE, UQ-9, and VC, and (C) the formation of FFA during the aerobic oxidation of rat liver homogenate initiated with 20 mM AMVN at 37°C.** The ratios of PUFA to FFA are given in (C). Numbers in parentheses are the initial concentrations. Estimated initial concentrations of phospholipids are the same as in Fig. 2.

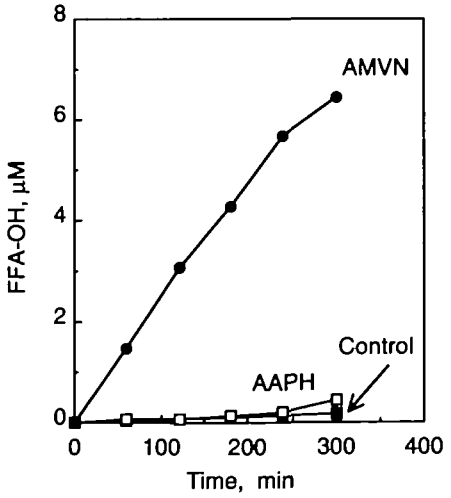

Fig. 6. **Formation of FFA-OH during the aerobic oxidation of rat liver homogenate in the absence (Control), or the presence of 20 mM AAPH or AMVN at 37°C.**

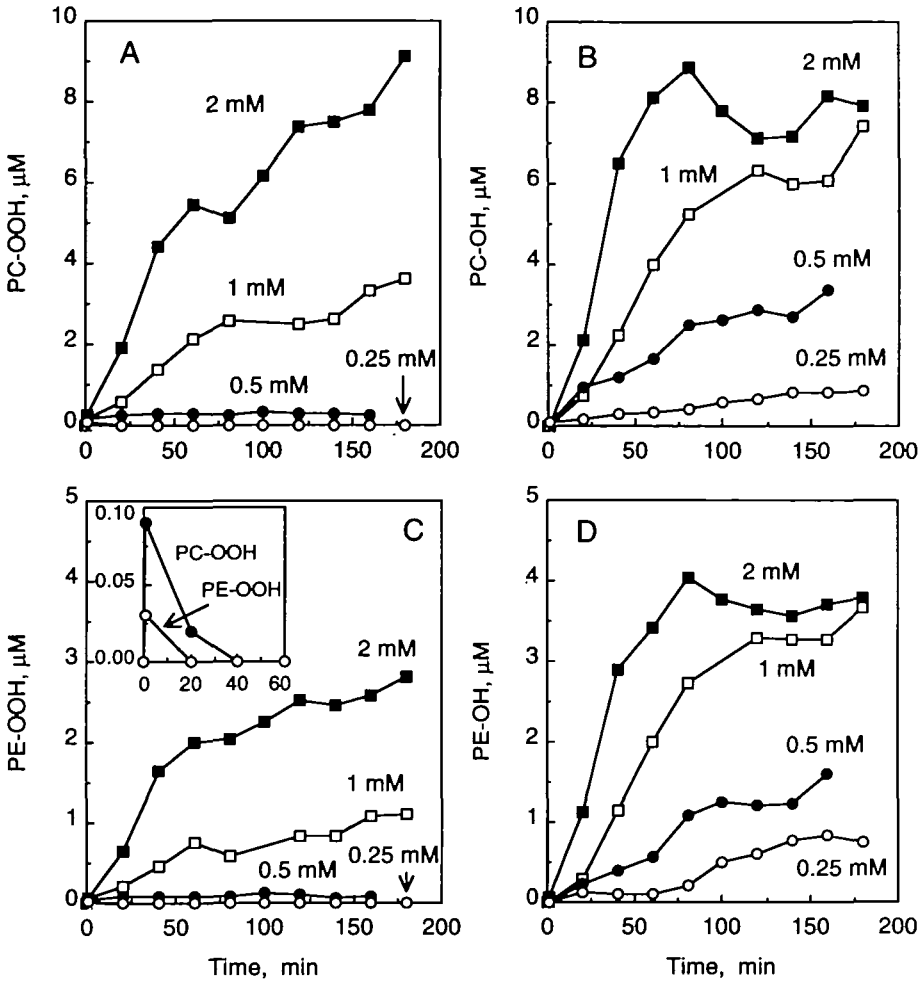

Fig. 7. **Changes in levels of (A) PC-OOH, (B) PC-OH, (C) PE-OOH, and (D) PE-OH after the addition of 0.25, 0.5, 1, and 2 mM BOOH to the rat liver homogenate exposed to air at 37°C.** Panel insert (C) shows the time course of the formation of PC-OOH and PE-OOH after the addition of 0.25 mM BOOH to rat liver homogenate under identical conditions. Estimated initial concentrations of phospholipids are the same as given in Fig. 2.

located in plasma and external membranes, while UQ-9 is located mainly in the interior mitochondrial membranes.

Greater rates for the formation of PC-OOH, PE-OOH, PC-OH, and PE-OH were observed in the AMVN-initiated oxidation of rat liver homogenate when compared to AAPH-initiated oxidation (Figs. 4A and 5A), despite the lower rate of free radical production from AMVN in the lipid phase than that from AAPH in the aqueous phase (*19*). These results would suggest that a higher proportion of antioxidant activity is located in the aqueous phase than in the lipid phase. In fact, it is well established that glutathione (and protein thiol groups) present in cytosol at millimolar concentration can inhibit the oxidation of liposomal membranes initiated with AAPH (*20*). Although the phospholipase activity was retarded during the AMVN-initiated oxidation as judged from the formation of FFA (compare Figs. 3C and 5C), considerable amounts of FFA-OH were formed, as shown in Fig. 6, indicating that phospholipase preferentially cleaves oxidized phospholipids, as previously proposed (*21, 22*). However, it is also possible that PUFA was oxidized to give FFA-OH during the AMVN-initiated oxidation since the ratio of PUFA to FFA decreased during the oxidation (Fig. 5C).

The oxidation of rat liver homogenate induced by 0.25, 0.5, 1, and 2 mM BOOH under aerobic conditions at 37°C also gave PC-OOH, PE-OOH, PC-OH, and PE-OH as products (Fig. 7). The formation of free radicals induced by a metal-catalyzed decomposition of BOOH is likely to be responsible for the initiation of phospholipid oxidation in rat liver homogenate (*1*). Levels of PC-OH and PE-OH

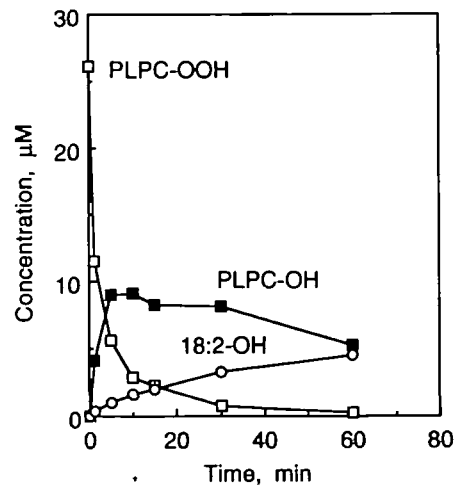

Fig. 8. **Changes in levels of PLPC-OOH, PLPC-OH, and 18:2-OH after the addition of 26 μM PLPC-OOH to the rat liver homogenate under aerobic conditions at 37°C.**

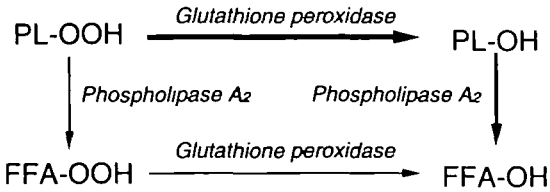

Fig. 9. **Possible pathways to the decomposition of lipid peroxidation products in rat liver.**

increased with increasing incubation time and initial BOOH concentration (Fig. 7, B and D). On the other hand, PC-OOH and PE-OOH were detected only at early stages (Fig. 7C, insert) when the oxidation was initiated with 0.25 mM BOOH. The rate of PC-OOH and PE-OOH formation increased with increasing initial BOOH concentration when it was greater than 0.5 mM (Fig. 7, A and C). These results and the formation of PC-OH and PE-OH during the oxidation of the homogenate strongly suggest that rat liver homogenate has the ability to reduce hydroperoxides and its capacity is about 0.5 mM under the present experimental conditions.

We further investigated the fate of exogenously added PLPC-OOH (26 μM) in the rat liver homogenate. PLPC-OH and linoleic acid hydroxide (18:2-OH) were the major products and the rate of PLPC-OH production was greater than that of 18:2-OH formation (Fig. 8), indicating that the reduction of PLPC-OOH by phospholipid hydroperoxide glutathione peroxidase (*21*) is faster than hydrolysis by phospholipase A_2 (*22, 23*), as summarized in Fig. 9. It is noteworthy that we could not detect linoleic acid hydroperoxide (data not shown).

In summary, the oxidation products of the rat liver phospholipids in homogeneous solution and multilamellar liposomal suspension were PC-OOH and PE-OOH. In addition, the oxidation of rat liver homogenate gave PC-OH, PE-OH, FFA, and FFA-OH, indicating that peroxidase reduces PL-OOH and phospholipase A_2 hydrolyzes PL-O(O)H. In fact, exogenously added PLPC-OOH was converted to PLPC-OH and 18:2-OH in the homogenate. These results are consistent with the previous observation that PC-OOH was undetectable in rat liver and heart (*12*). Furthermore, it is suggested that glutathione peroxidase and phospholipase A_2 may function as repair enzymes for oxidatively damaged phospholipids in biomembranes.

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