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Formation of the Aldehydic Choline Glycerophospholipids in Human Red Blood Cell Membrane Peroxidized with an Azo Initiator[1]

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The production of phospholipid hydroperoxide and aldehydic phospholipid was examined in human red blood cell (RBC) membranes after peroxidation with 2,2-azobis(2-amidinopropane)dihydrochloride (AAPH) or xanthine/xanthine oxidase (XO/XOD/Fe^{3+}). Both radical-generation systems caused a profound decrease in the amount of polyunsaturated fatty acid (PUFA) in choline glycerophospholipid (CGP) and induced formation of peroxidized CGP in RBC membranes to different extents. No consistent generation of peroxidized lipids from CGP was evident after peroxidation with XO/XOD/Fe^{3+}, which caused the apparent decomposition of phospholipids and the formation of large amounts of thiobarbituric acid-reactive substance (TBARS). On the other hand, CGP hydroperoxide was formed as a primary product of peroxidation with AAPH. Aldehydic CGP was also detected as a secondary product of hydroperoxide decomposition in AAPH-peroxidized RBC membranes. Aldehydic CGP was preferentially generated from arachidonoyl CGP rather than from linoleoyl CGP in AAPH-peroxidized membranes. AAPH mainly oxidized CGP to hydroperoxide and aldehydic phospholipids. The sum of hydroperoxide and aldehyde of CGP corresponded to the loss of CGP due to peroxidation by AAPH. This result indicates that CGP was mainly converted into these two oxidized phospholipids in AAPH-peroxidized RBC membranes.

Key words: aldehyde, hydroperoxide, oxidized phospholipids, peroxidation, red blood cell.

The development of various pathological states due to the dysfunction of cellular membranes has been proposed to be a result of oxidative injury, such as ischaemia-reperfusion injury (*1*). The major target molecules of oxygen radicals in biomembranes are known to be the polyunsaturated fatty acids (PUFA) of phospholipids. The initial step in the peroxidation of phospholipid is the oxidation of PUFA at the 2-position of the glycerol backbone to yield phospholipid hydroperoxides (*2*). Phospholipid hydroperoxides, as primary products, are unstable and decompose to secondary products through a number of pathways. The decomposition is accompanied by the generation of a variety of short-chain aldehydes (*3-5*) and oxidized phospholipids with carbonyl residues.

Extensive studies of lipid peroxidation have been performed *in vitro* and *in vivo*. However, these studies have been based on the determination of the water-soluble products of decomposition, such as the short-chain aldehydes. Lipid peroxidation is usually assessed by the thiobarbituric acid (TBA) assay, but complex problems are associated with this assay, such as the specificity of substrates and the absence of a method for specific quantitation (*6*). To characterize the way in which phospholipids are oxidized and decomposed in biomembranes, a specific assay for peroxidized lipids is necessary.

Specific and sensitive methods for the detection of phospholipid hydroperoxides has been developed that involve chemiluminescence-HPLC (CL-HPLC) (*7*). Phospholipid hydroperoxides have been found in human blood plasma and other biological samples (*8, 9*). However, little information is available about the secondary products, such as choline glycerophospholipids (CGP) containing aldehyde moieties (aldehydic CGP), that result from the decomposition of phospholipid hydroperoxides. Aldehydic CGP have attracted attention because of their effects on the activation of neutrophils through the receptor of platelet-activating factor (PAF) (*10*). However, the lack of an efficient method

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Abbreviations: AAPH, 2,2-azobis(2-amidinopropane)dihydrochloride; aldehydic CGP, CGP containing an aldehyde moiety at the 2-position; arachidonyl CGP, CGP containing arachidonic acid at the 2-position; BHT, butyl hydroxytoluen; CGP, choline glycerophospholipid;CGP-5CHO, 1-acyl-2-(5-oxopentanoyl)glycerophosphocholine; CGP-9CHO, 1-acyl-2-(9-oxononanoyl)glycerophosphocholine; CL, chemiluminescence; DBD-H, 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole; DNPH, dinitrophenylhydrazine; GC, gas chromatography; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; MDA, malondialdehyde; PAF; platelet activating factor; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SGP, serine glycerophospholipid; SM, sphingomyelin; TBA, thiobarbituric acid; TBARS, TBA-reactive substance; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin-layer chromatography XO, xanthine; XOD, xanthine oxidase.

for analysis of aldehydic phospholipids has made it difficult to demonstrate their generation during the peroxidation of biomembranes. Recently, we have developed a highly sensitive method for detection of aldehydic CGP that involves derivatization with a fluorescent probe (*11*). This method allows us to monitor small amounts of secondary oxidized phospholipids in peroxidized biomembranes.

A little quantitative and kinetic information about the peroxidation of phospholipids in biomembranes is available. It seems important now to clarify the chemical mechanisms of the metabolism of the primary and secondary products of phospholipids in peroxidized biomembranes at the molecular species level. In this study, we focused on the metabolic fate of phospholipid hydroperoxides as the primary products of peroxidation and on the formation of aldehydic phospholipids as the secondary products in human red blood cell membranes that had been peroxidized with two different radical-generation systems, the AAPH system for the generation of alkyl radicals and the XO/XOD/Fe^{3+} system for the generation of hydroxyl radicals.

MATERIALS AND METHODS

Materials—Luminol, butylated hydroxytoluene (BHT), 2,2-azobis(2-amidinopropane)dihydrochloride (AAPH), choline chloride, trifluoacetic acid (TFA), cyclohexanedione (CHD), and sodium methoxide were purchased from Wako Pure Chemical (Osaka). Xanthine (XO), xanthine oxidase (XOD), and cytochrome *c* were obtained from Sigma (St. Louis, MO, USA). 4-(*N,N*-Dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) was obtained from Tokyo Kasei Industry (Tokyo).

Preparation of Human Red Blood Cell (RBC) Membranes—Human RBC membranes from fresh human blood were prepared as described previously (*12*). In brief, packed RBCs were washed twice with saline, then resuspended in 20 volumes of 5 mM phosphate buffer (pH 8.0) for lysis. The lysate was centrifuged at 22,000×*g* for 10 min to precipitate RBC membranes. RBC membranes were washed 4-5 times to obtain white ghosts with 5 mM phosphate buffer. Washed RBC membranes were resuspended in 50 mM of Tris-HCl buffer (pH 7.4) at a concentration of 3-5 mg/ml and stored at −80°C.

Peroxidation of RBC Membranes with AAPH and XO/XOD/Fe^{3+}, and Extraction of Lipids—RBC membranes (1 mg) were incubated at 37°C for appropriate times in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 ml with or without 80 mM AAPH. In the XO/XOD/Fe^{3+} peroxidation system, RBC membranes (1 mg) were incubated in 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 1 ml in the presence of 0.4 U of XOD, 0.5 mM hypoxanthine, ADP/$FeCl_3$ complex (2.8 mM ADP and 0.2 mM $FeCl_3$), and EDTA/$FeCl_3$ complex (0.2 mM EDTA and 0.2 mM $FeCl_3$). Reactions were stopped by addition of an equal volume of organic solvent (chloroform and methanol, 1:2, v/v) containing 0.2 mM BHT. Total lipids were extracted by the method of Bligh and Dyer (*13*). Extraction was performed with cooling on ice. Extracted lipids were evaporated to dryness under a stream of nitrogen gas and dissolved in chloroform. Part of the sample was analyzed for phospholipid hydroperoxides, and the remainder was spotted on a TLC plate for the separation of phospholipids, with chloroform, methanol, and 25% NH_4OH, (65:35:8, v/v) as the moble phase. The band of CGP was scraped from the plate, and CGP was extracted by the method of Bligh and Dyer (*13*). CGP was quantitated by determination of the amount of phosphorus, as described by Rouser *et al.* (*14*). Fatty acids of CGP were quantitated by gas chromatography (GC; GC14A; Shimadzu, Kyoto) after transmethylation with 0.5 N sodium methoxide. Fatty acid methyl esters were analyzed at 240°C by GC on a capillary column (Spelcowax 10; Spelco Japan, Tokyo) and quantitated against the methyl ester of heptadecanoic acid as an internal standard.

Analysis of Phospholipid Hydroperoxides by Chemiluminescence-HPLC (CL-HPLC)—Phospholipid hydroperoxides were analyzed by CL-HPLC (*7*). Total lipids were injected into an HPLC system (model 10A; Shimadzu) with an aminopropyl column (Microbondsphere, 5 μ-NH_2; 150 mm×3.9 mm i.d.; Waters, Milford, MA, USA). The mobile phase was a mixture of acetonitrile, methanol, and 0.2% triethylamine (TEA) (82:10:8, v/v, pH 6.0), and the flow rate was 1 ml/min. Lipids were monitored at 205 nm with a UV detector (SPD-10A; Shimadzu). After passage through the UV detector, the eluate was combined with a chemiluminescence reagent consisting of 10 mg of cytochrome *c* and 3 mg of luminol dissolved in 1 liter of 50 mM borate buffer (pH 10.2), which was fed into the post-column system at a flow rate of 1 ml/min. Chemiluminescence was monitored with a chemiluminescence detector (model 825-CI; Japan Spectroscopic, Tokyo). Amounts of phospholipid hydroperoxides were determined by reference to the chemiluminescence of authentic CGP hydroperoxide, prepared by the photooxidation of egg yolk CGP (*15*).

Analysis of Aldehydic CGP by Fluorescence HPLC—The procedure was essentially as described in the previous report (*11*). Total lipids from RBC membranes were fractionated into individual phospholipids by TLC with chloroform, methanol, and 25% NH_4OH (65:40:8, v/v) as the mobile phase. Under these conditions, aldehydic CGP migrated more slowly than CGP and partially overlapped sphingomyelin (SM). The band of aldehydic CGP was scraped off the plate, and aldehydic CGP was extracted from the silica gel by the method of Bligh and Dyer (*13*).

Aldehydic CGP dissolved in acetonitrile was derivatized to fluorescent lipid by the addition of 0.1% of trifluoroacetic acid (TFA) and 0.5 mM 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H). The reaction mixture was incubated at room temperature for 60 min in darkness. Then the solvent was evaporated under a stream of nitrogen, and the product was purified by TLC with chloroform, methanol, and water (10:5:1, v/v) as the mobile phase. Purified fluorescent derivatives were fractionated and quantitated by reverse-phase HPLC. The fluorescent derivatives of aldehydic CGP were eluted at a flow rate of 1.0 ml/min and monitored with excitation at 450 nm and the emission at 560 nm with a spectrofluorometric detector (model RF-550; Shimadzu). The mobile phase was a mixture of methanol, water, and acetonitrile (90.5:7.0:2.5, v/v) containing 20 mM choline chloride. Aldehydic phospholipids were quantitated by reference to an authentic aldehydic CGP, which had been prepared chemically from arachidonoyl CGP by ozone degradation (*11*).

Determination of TBARS—After the peroxidation of RBC membranes with AAPH or XO/XOD/Fe^{3+} for 1 h,

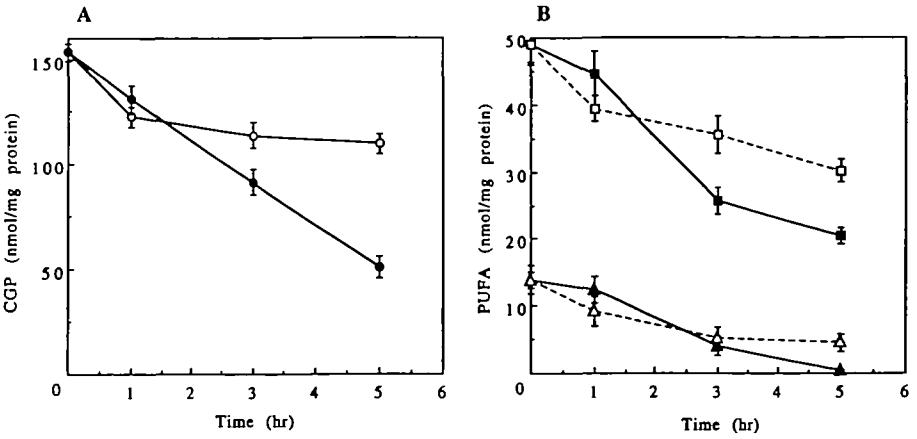

Fig. 1. **Degradation of CGP and its constituent polyunsaturated fatty acids during peroxidation with AAPH or XO/XOD/Fe^{3+}.** Human RBC membranes were peroxidized with AAPH (80 mM) or XO/XOD/Fe^{3+}. (A) Changes in the amounts of CGP in RBC membrane peroxidized with AAPH (open circles) and with XO/XOD/Fe^{3+} (closed circles). (B) Degradation of linoleic acid (squares) and arachidonic acid (triangles) in CGP in RBC membranes peroxidized with AAPH (open squares and triangles) and with XO/XOD/Fe^{3+} (closed squares and triangles). Each point and bar represent the mean ± SE of triplicate results from separate preparations of RBC membranes.

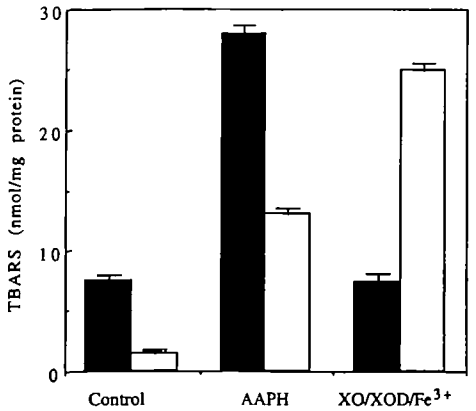

Fig. 2. **Distribution of water-soluble and lipid-soluble TBARS produced by peroxidation of RBC membranes with AAPH or XO/XOD/Fe^{3+}.** RBC membranes were peroxidized for 1 h. Equal volumes of chloroform, methanol, and water were added to the incubation mixture for the separation of the water-soluble and lipid-soluble phases. Amounts of TBARS in water-soluble phase (open columns) and in the lipid-soluble phase (closed columns) were determined by the TBA assay. Each column and bar represent the mean ± SD of results from triplicate determinations.

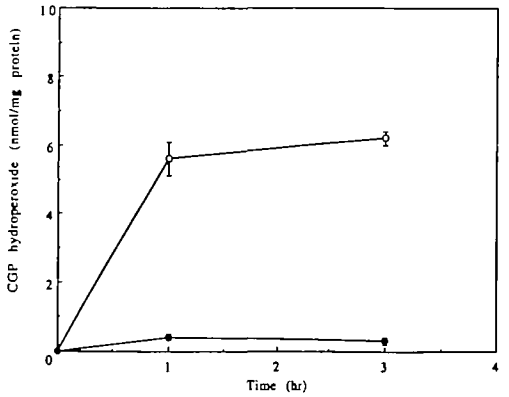

Fig. 3. **Time course of production of CGP hydroperoxides in RBC membranes during peroxidation with AAPH (open circles) or XO/XOD/Fe^{3+} (closed circles).** Each point and bar represent the mean ± SE of results of triplicate experiments with separate preparation of RBC membranes.

equal volumes of chloroform, methanol, and water were added to the incubation mixture for the separation of water-soluble and lipid-soluble fractions. The solution was separated into two phases, namely, the water-soluble (upper) phase and the lipid-soluble (lower) phase. Aliquots from both phases were used for the determination of TBARS by TBA assay (*16*).

RESULTS

The predominant phospholipids in human RBC membranes were choline glycerophospholipids (CGP), serine glycerophospholipids (SGP), and ethanolamine glycerophospholipids (EGP), accounting for 38, 23, and 24% of the total, respectively. Changes in the net amounts of CGP and polyunsaturated fatty acids (PUFA) in RBC membranes upon peroxidation with AAPH or with XO/XOD/Fe^{3+} are shown in Fig. 1. The extent of degradation of CGP by AAPH differed from that by XO/XOD/Fe^{3+}. CGP was rapidly degraded, in a time-dependent manner, and its level decreased to one-third of the original value during the peroxidation with XO/XOD/Fe^{3+} for 5 h (Fig. 1A). By contrast, AAPH caused more limited decomposition of CGP, although the amount of CGP decomposed during the first 1 h of peroxidation under our conditions, namely, 25 nmol/mg protein, was almost same as that with the XO/XOD/Fe^{3+} system. Linoleic acid and arachidonic acid are the predominant polyunsaturated fatty acids in human RBC membranes. Significant decompositions of these fatty acids in CGP were observed when RBC membranes were peroxidized with AAPH or XO/XOD/Fe^{3+} (Fig. 1B). Arachidonic acid was more susceptable to peroxidation than linoleic acid, and the level of the former was almost undetectable after peroxidation for 5 h. Peroxidation of RBC membranes for 1 h was chosen for comparison of the formation of oxidative phospholipids induced by AAPH or XO/XOD/Fe^{3+}, since CGP was degraded to the same extent with both systems during this period.

The amounts of water-soluble and lipid-soluble thiobarbituric acid-reactive substances (TBARS) in RBC membranes were determined after peroxidation with AAPH or XO/XOD/Fe^{3+} (Fig. 2). The incubation mixture containing peroxidized RBC membranes was separated into aqueous and organic phases by the addition of water, methanol, and

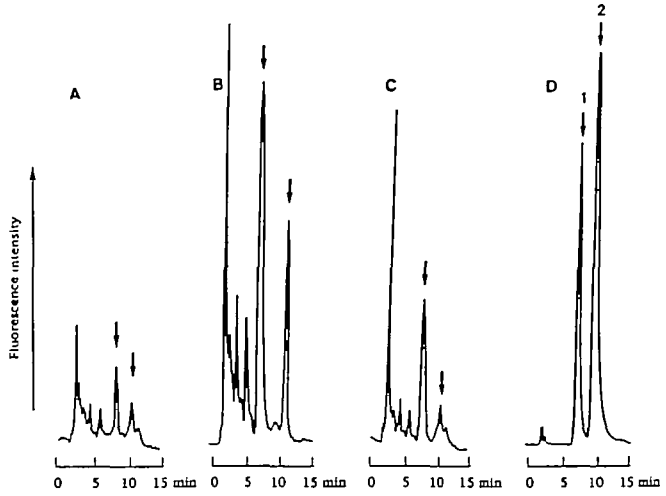

Fig. 4. **Separation of aldehydic CGP by reverse-phase HPLC.** RBC membranes were peroxidized with AAPH or XO/XOD/Fe^{3+} for 1 h. CGP was separated from total lipids by TLC and was derivatized with a fluorescent probe (DBD-H). Fluorescent derivatives of aldehydic CGP were separated and quantitated by reverse-phase HPLC. (A) Control, (B) AAPH peroxidation, (C) XO/XOD/Fe^{3+} peroxidation, (D) authentic standards. 1, CGP-5CHO; 2, CGP-9CHO.

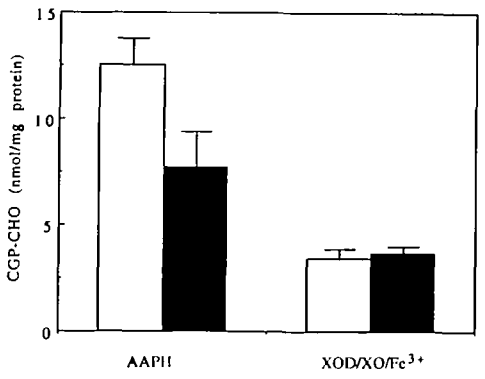

Fig. 5. **Amounts of aldehydic CGP produced in RBC membranes during peroxidation with AAPH or XO/XOD/Fe^{3+}.** Aldehydic CGPs were separated into the constituent molecular species, CGP-5CHO (open columns) and CGP-9CHO (closed columns), by reverse-phase HPLC. Each column and bar represents the mean ± SE of results of triplicate analysis.

chloroform for the quantitation of TBARS. Lipid-soluble TBARS, including lipids with carbonyl residues, was produced at significant levels in RBC membranes upon peroxidation with AAPH, but at low levels peroxidation with XO/XOD/Fe^{3+}. Conversely, the amount of water-soluble TBARS, which consisted predominantly of short-chain aldehydes, formed from AAPH-peroxidized RBC membranes was much lower than that formed from XO/XOD/Fe^{3+}-peroxidized RBC membranes.

Phospholipid hydroperoxides, as primary products of the peroxidation of phospholipids, were analyzed by chemiluminescence-HPLC (CL-HPLC), and the amount of CGP hydroperoxide was determined as a function of time by reference to an authentic standard (Fig. 3). The amount of CGP hydroperoxide increased sharply during peroxidation with AAPH for 1 h and at a lower rate up to 3 h. However, no significant production of CGP hydroperoxide was detected in RBC membranes during peroxidation with XO/XOD/Fe^{3+}.

Aldehydic phospholipids prepared from oxidized RBC membranes were derivatized with 4-(*N,N*-dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H) and quantitated by fluorescence HPLC. A typical chromatogram of the DBD-H derivatives of aldehydic CGPs is shown in Fig. 4. Clear peaks of 1-acyl-2-(5-oxopentanoyl)glycero-phosphocholine (CGP-5CHO) and 1-acyl-2-(9-oxononanoyl)glycero-phosphocholine (CGP-9CHO) were found in AAPH-peroxidized RBC membranes (Fig. 4B), whereas smaller peaks of both compounds were detected in XO/XOD/Fe^{3+}-peroxidized membranes (Fig. 4C). The amounts of the molecular species of aldehydic CGP were determined by fluorescence HPLC with authentic standards (Fig. 5). AAPH induced the production of CGP-5CHO, which originated from arachidonoyl CGP, and of CGP-9CHO, from linoleoyl CGP, in RBC membranes to levels of 12.5 and 7.5 nmol/mg protein, respectively. The amount of arachidonoyl CGP (4.5% of the total fatty acid of CGP) was approximately one-fifth of that of linoleoyl CGP (22.8%) in human RBC membranes. The ratio of the amount of CGP-5CHO to that of arachidonyl CGP (12.5/4.5=2.8) was 9 times higher than the ratio of the amount of CGP-9CHO to that of linoleoyl CGP (7.5/22.8=0.3), indicating the preferential formation of CGP-5CHO in AAPH-oxidized biomembranes. The sum of aldehydic CGP and CGP hydroperoxides, accounting for 25 nmol/mg/protein, corresponded closely to the loss of CGP due to peroxidation by AAPH (Fig. 1A). This result indicates that CGP was mainly converted into these two oxidized phospholipids in AAPH-peroxidized RBC membranes.

DISCUSSION

Hydroperoxides of phospholipids are the initial products of peroxidation of phospholipids in biological membranes. Significant amounts of hydroperoxides were produced in RBC membranes peroxidized with AAPH (Fig. 3). Hydroperoxides were predominantly formed in CGP, and small amounts of hydroperoxides were found in other classes of phospholipids. The accumulation of phospholipid hydroperoxides has been reported in a variety of pathological processes associated with oxidative stress, for example, in the carbon tetrachloride-treated liver (*17*), the ischemic liver (*18*), and the aging brain (*19*).

In contrast to AAPH peroxidation, XO/XOD/Fe^{3+} peroxidation failed to generate CGP hydroperoxides. This result indicates that CGP hydroperoxides are rapidly degraded to secondary products in the XO/XOD/Fe^{3+} peroxidation system. The instability of phospholipid hydroperoxides generated by the XO/XOD/Fe^{3+} system is probably due to their iron-catalyzed decomposition, a rapid cleavage that produces products of lipid peroxidation with high yield (*20*).

It is also of interest to examine the reactive aldehydes that remain in the parent phospholipid molecules during the β-sicission of lipid hydroperoxides. Recently, Kamido *et al.* isolated aldehydic phospholipids generated during the copper-catalyzed peroxidation of low- and high-density lipoproteins from human plasma after derivatization with dinitrophenylhydrazine (DNPH), although molecular spe-

cies of aldehydic phospholipids could not be separated (*21*). Aldehydic CGP was produced in AAPH-treated RBC membranes, while no consistent production of these oxidized phospholipids was evident with the XO/XOD/Fe^{3+} system (Fig. 4). The amount of CGP-5CHO generated by peroxidation with AAPH was significantly higher than that of CGP-9CHO, even though the amount of arachidonic acid (4.5%) in CGP was much lower than that of linoleic acid (22.8%) in RBC membranes (Fig. 5). The ratio of the amount of CGP-5CHO to that of arachidonyl CGP was 9 times higher than the ratio of the amount of CGP-9CHO to that of linoleoyl CGP. These results indicate that aldehydic CGP was preferentially produced from arachidonoyl rather than linoleoyl species of CGP. In a previous study, we demonstrated that the phospholipid hydroperoxides in AAPH-peroxidized RBC membranes originated from linoleoyl CGP, with little production of hydroperoxides derived from arachidonoyl CGP (*12*). Arachidonoyl hydroperoxides of CGP should be less stable than linoleoyl hydroperoxides. The lability of the arachidonoyl hydroperoxides of CGP seems to lead to the preferential formation of CGP-5CHO rather than CGP-9CHO in AAPH-peroxidized RBC membranes. By contrast, the stability of linoleoyl hydroperoxides indicates that the amount of hydroperoxides of CGP in biomembranes is determined by the relative level of linoleic acid in CGP. The production of CGP hydroperoxides in linoleic acid-rich rabbit RBC membranes was much greater than that in rat RBC membranes, which contain relatively low levels of linoleic acid (*12*).

Several reports have appeared concerning the oxidized products of chemically pure phospholipids. Itabe *et al.* detected CGP containing an *sn*-2-azelaoyl residue as a oxidized lipid produced by the peroxidation of linoleoyl species of CGP with oxyhaemoglobin (*22*). Tanaka *et al.* demonstrated that various kinds of products of CGP having an *sn*-2-butyrate, oxovalerate, *O*-methylglutarate, and 4-hydroxybutyrate residue were detected by GC-MS after peroxidation of 1-hexadecyl-2-arachidonyl or docosahexanoyl species of CGP with $FeSO_4$/ascorbate/EDTA (*23*). The profile of oxidation products of membrane phospholipids seems to be more simple than those of chemically pure phospholipid. In the present study, CGP in RBC membranes was predominantly converted into two kinds of oxidized lipids, hydroperoxide and aldehydic phospholipids, since the sum of these types of oxidized CGP corresponded closely to the loss of CGP by the peroxidation with AAPH. Hydroperoxide CGP was exclusively from linoleoyl species and aldehydic CGP was predominantly from the arachidonyl species.

The formation of aldehydic phospholipids during peroxidation of RBC membranes *in vitro* indicates that aldehydic CGP might also be formed *in vivo*. Recently, we demonstrated that a specific monoclonal antibody that recognizes human atherosclerotic lesions also reacted with aldehydic CGP, suggesting that aldehydic CGP is produced in human atherosclerotic lesions (*24*). Aldehydic phospholipids prepared from arachidonoyl CGP have PAF-like activity, being able to activate the adhesive response of leukocytes (*10*). CGPs with short-chain dicarboxylate residues, detected in brain tissues, induced the aggregation of platelets to the same extent as PAF (*25-27*). Oxidized low density lipoprotein (LDL) has a variety of biological effects on monocytes and endothelial cells (*28-30*). Lipids extracted from oxidized LDL can inhibit the migration of endothelial cells (*28*) and the phospholipid fraction of oxidized LDL also stimulates the binding of monocytes to endothelial cells (*5*). These observations suggest that extensive oxidation of biomembranes produces oxidized phospholipids similar to PAF by the oxidative cleavage of polyunsaturated fatty acids in phospholipids. These proinflammatory phospholipids could contribute to the initiation and progression of atherosclerosis.

The oxidation of individual phospholipids and the products of the oxidation of lipids depend greatly on the structures of lipids and phospholipids, as well as on the peroxidation conditions. The individual products of lipid peroxidation probably have different damaging effects on biomembranes.

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