Conformational Changes in Oxidized Phospholipids and Their Preferential Hydrolysis by Phospholipase A_2: A Monolayer Study†

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ABSTRACT: Cleavage of oxidized fatty acids by phospholipase A_2 has been implicated as the first step in the repair mechanism for oxidative damage to membrane phospholipids. However, the mechanism by which this enzyme preferentially hydrolyzes oxidized fatty acyl chains is poorly understood. Using a lipid monolayer technique, we found that the molecular surface areas of 1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)-phosphatidylcholine (PLPC-OOH) and 1-palmitoyl-2-(9/13-hydroxylinoleoyl)phosphatidylcholine (PLPC-OH) were increased by as much as 50% relative to the parent nonoxidized 1-palmitoyl-2–linoleoylphosphatidylcholine (PLPC). These experimental data directly indicate a drastically changed molecular conformation of oxidized phospholipids in which the hydroperoxy or hydroxy group in the *sn*-2 fatty acid is close to the lipid–water interface. Phospholipases A_2 from porcine pancreas and from bee venom were shown to break down PLPC-OOH and PLPC-OH monolayers much faster than PLPC monolayers. In all cases, the presence of serum albumin in the subphase enhanced monolayer breakdown by extracting hydrolysis products from the monolayer, but monolayer breakdown was always much faster for oxidized than for nonoxidized PLPC. This did not appear to be due to change in the extent of monolayer penetration by phospholipase A_2, since enzyme–monolayer interaction studies revealed essentially identical penetration behavior of bee venom phospholipase A_2 with PLPC, PLPC-OOH, and PLPC-OH monolayers. We propose that the altered molecular conformation of oxidized phospholipids facilitates access to the *sn*-2 ester bond, thereby ensuring their preferential hydrolysis in the presence of a phospholipase A_2.

The susceptibility of unsaturated membrane lipids to oxidative damage and the deleterious consequences of lipid peroxidation to membrane structure and function have been widely investigated and well documented (Chiu et al., 1989; Girotti, 1985; Sevanian & Hochstein, 1985). Some biological membranes such as the erythrocyte membrane are under continuous oxidative stress. Although erythrocyte lipid peroxidation products can be detected and their levels appear elevated in some pathological conditions, very sensitive detection techniques have to be employed to identify them as the absolute concentrations are usually very low. Indeed, primary products of phospholipid peroxidation, phospholipid hydroperoxides and hydroxides, do not appear to accumulate in biological membranes under oxidative stress.

The structural and functional well-being of biological membranes depends on the molecular species composition of their lipid matrix. The ability of red blood cells to repair oxidative damage to proteins is limited, but an elaborate mechanism apparently exists for the repair of oxidatively damaged membrane phospholipids, the altered physical properties of which may constitute a threat to membrane integrity. A number of enzymes are involved in this putative lipid repair mechanism (Lubin & Kuypers, 1991; MacDonald & Sprecher, 1991; van den Berg & Kuypers, 1992), and cleavage of an oxidized fatty acyl chain by a phospholipase represents a necessary first step in this repair process. Although it has been observed that lipid peroxidation leads to the activation of a phospholipase A_2, and that this enzyme preferentially hydrolyzes the *sn*-2 ester bonds of phospholipids with an oxidized fatty acid (Salgo et al., 1992; Sevanian & Kim, 1985; van Kuijk et al., 1987; Yasuda & Fujita, 1977), the structural basis for this phospholipase activity remains poorly understood.

Monomolecular lipid films spread on a buffer subphase have been very useful in the study of lipid–lipid and lipid–protein interactions. An electronic microbalance is used to measure the surface pressure in the lipid film. Compressibility and packing of the monolayer lipids are measured by monitoring the surface pressure while varying the total surface area using a movable Teflon barrier. Monolayer systems have also been used to study the hydrolytic action of phospholipases on a variety of substrates, e.g., establishing pressure limits for the penetration and activity of different phospholipases A_2 (Alsina et al., 1983; Demel et al., 1975). In this study, a monolayer technique was used to describe some physicochemical characteristics of purified and well-characterized oxidized phospholipids [1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)phosphatidylcholine (PLPC-OOH)[1] and 1-palmitoyl-2-(9/13-hydroxylinoleoyl)phosphatidylcholine (PLPC-OH)] compared to the native lipid [1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC)]. The measurements of molecular surface areas presented here provide an exper-

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[1] Abbreviations: BSA, bovine serum albumin; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; PLPC-OH, 1-palmitoyl-2-(9/13-hydroxylinoleoyl)phosphatidylcholine (PLPC hydroxide); PLPC-OOH, 1-palmitoyl-2-(9/13-hydroperoxylinoleoyl)phosphatidylcholine (PLPC hydroperoxide); PLA_2, phospholipase A_2; HPLC, high-performance liquid chromatography.

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imental basis for the hypothesis that conformational changes occur in oxidized phospholipids. In addition, it is shown that phospholipase A_2 degrades monolayers of oxidized phospholipid much more rapidly than monolayers of nonoxidized phospholipid. We propose that the altered conformation of oxidized phospholipids results in a membrane packing defect, making the *sn*-2 ester bond more accessible to the phospholipase, and that the resulting preferential hydrolysis of oxidized phospholipids is the basis for the rapid repair of oxidized phospholipids in biological membranes.

EXPERIMENTAL PROCEDURES

Materials. Phospholipases A_2 from bee venom and from porcine pancreas, sodium borohydride, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). 1-Palmitoyl-2-linoleoylphosphatidylcholine (PLPC) was purchased from Avanti (Birmingham, AL) and purified by high-performance liquid chromatography (HPLC) as described below. All solvents used were of HPLC grade.

Preparation of Oxidized Phospholipids. A dichloromethane solution of PLPC in a round-bottom flask was dried down on a rotary evaporator. The dried lipid film was subsequently left exposed to room air. At daily intervals, the extent of oxidation was checked by dissolving the lipid in MeOH and determining conjugated diene formation (A_{230}) from the absorption spectrum (200–300 nm). To limit formation of secondary oxidation products such as epoxides and dihydro-(pero)xides, oxidation was stopped when the absorption ratio A_{230}/A_{208} reached a value of 1.2.

PLPC hydroperoxide (PLPC-OOH) was purified from the oxidized lipid mixture by semipreparative reversed-phase HPLC on an ODS column (25 cm × 1 cm i.d., 5-μm particles) using methanol as solvent at 4 mL/min. Under these conditions, retention times for PLPC and PLPC-OOH were 19.8 and 8.7 min, respectively. Some secondary products were observed to elute between 3 and 7 min. PLPC hydroxide (PLPC-OH) was prepared from PLPC-OOH by reduction with $NaBH_4$, and subsequently purified by HPLC as described above, eluting with a retention time of 8.7 min.

The identity and purity of the isolated oxidation products were checked by gas chromatography–mass spectrometry (GC–MS) after conversion of fatty acyl groups to fatty acid methyl esters using Methprep II (Pierce, Rockford, IL) and subsequent derivatization of hydroxyl groups to trimethylsilyl ethers using bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane (Pierce). Samples were analyzed on an HP 5790 A series gas chromatograph coupled to an HP 5970 A MSD quadrupole mass spectrometer (Hewlett Packard, Palo Alto, CA) operated in the electron-impact mode at 70 eV. The gas chromatograph was equipped with a 15-m DB-1 fused silica column (0.25-mm i.d., film thickness 0.25 μm; J&W Scientific, Folsom, CA), and sample analysis involved temperature programming from 50 to 310 °C. GC–MS results indicated that the peroxidation products obtained after reduction and HPLC purification were a mixture of the 9- and 13-hydroxides [retention time 16.0–17.0 min; characteristic peaks at m/z 382 (M^+), 225 (9-OTMS), 311 (13-OTMS]. The same peaks were observed with a sample from the purified PLPC-OOH after reduction to PLPC-OH. Without reduction by $NaBH_4$, only the palmitic acid methyl ester peak was observed in the total ion chromatogram, but virtually no signal arising from the oxidized *sn*-2 fatty acid. This confirmes that the PLPC oxidation product obtained before reduction was indeed PLPC-OOH without significant amounts of PLPC-OH, since hydroxides, but not hydroperoxides, can be directly derivatized to TMS ethers.

Lipids were stored in MeOH under argon at −20 °C and redissolved in dichloromethane before use. The phospholipid concentration was determined according to standard procedures (Rouser et al., 1970).

Lipid Monolayer Experiments. (*A*) *Pressure–Area Curves.* To obtain information on the packing behavior of oxidized as compared to nonoxidized PLPC, pressure–area isotherms were recorded essentially as described before (Demel et al., 1972). A known amount of phospholipid, dissolved in dichloromethane, was applied by dropwise addition onto a buffer subphase (130 mM NaCl, 10 mM Tris-HCl, and 2 mM $CaCl_2$, pH 8.0) in a Langmuir trough at a temperature of 23 °C. The lipid film was slowly compressed using a movable Teflon barrier, and phospholipid surface pressure was recorded as a function of the total monolayer surface area. The results of these experiments are plotted in a graph of surface pressure versus mean area per molecule.

(*B*) *Hydrolysis of Lipid Monolayers by Phospholipase A_2.* Hydrolysis of phospholipid monolayers by PLA_2 results in the formation of lysophospholipid and free fatty acid. As these products are more water-soluble than the native phospholipid, they can leave the monolayer, which results in a surface pressure decrease. Thus, phospholipase activity was monitored as changes in surface pressure at constant total area. After the base-line surface pressure was recorded for 10 min, enzyme was injected under the monolayer, and surface pressure changes were followed with time. For the pancreatic enzyme, 3.0 IU were used, giving a final concentration of 4.3 nM and an enzyme:substrate ratio of approximately 1:40. Experiments with the bee venom enzyme used 0.1 IU, giving a final concentration of 0.07 nM and an enzyme:substrate ratio of approximately 1:3200. In experiments where BSA was used, the indicated concentration of protein was injected under the monolayer, and the system was allowed to equilibrate for 10 min before addition of phospholipase. The hydrolysis experiments were carried out at 25 °C in a trough with a total surface area of 80 cm^2 and a volume of 80 mL with continuous magnetic stirring.

(*C*) *Penetration Studies.* Previous studies have shown that the activity of a phospholipase on a membrane or a lipid monolayer is dependent on its ability to penetrate into the membrane/monolayer and reach the ester bond to be cleaved (Verger et al., 1973, 1976). In the case of a lipid monolayer, penetration of PLA_2 leads to an increase in surface pressure. This surface pressure increase is dependent on the packing of the monolayer lipids; i.e., the higher the initial surface pressure in the absence of PLA_2, the less the surface pressure increase upon addition of PLA_2, as it becomes more difficult for the enzyme to penetrate the tightly packed monolayer. Penetration characteristics for a PLA_2 can be determined by measuring the penetration-related surface pressure increase as a function of the monolayer starting pressure. These experiments require large amounts of PLA_2, since the surface pressure increase resulting from a catalytic quantity of enzyme is too small to be measured. Penetration characteristics of bee venom PLA_2 were obtained using PLPC, PLPC-OOH, or PLPC-OH monolayers. At various starting pressures, a large amount (100 μg, 83 IU) of the phospholipase was injected into the subphase, and the subsequent increase in surface pressure due to penetration of the enzyme was recorded. To avoid very rapid breakdown of the monolayers by this large amount of PLA_2, these experiments were performed at 25 °C in a Ca^{2+}-free buffer (50 mM phosphate/100 mM NaCl) at pH 5.0, which is out of the optimal activity pH range. The

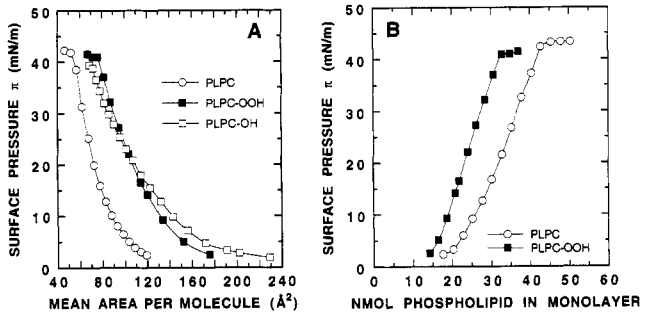

FIGURE 1: Monolayer packing behavior of oxidized and nonoxidized PLPC. (A) Pressure–area isotherms (T = 23 °C) for PLPC (○), PLPC-OOH (■), and PLPC-OH (□). A known amount of lipid was applied onto a buffer subphase, and the total surface area was subsequently reduced using a Teflon barrier. This increases the packing tightness of the lipids, which is expressed in increased monolayer surface pressure. Four experiments were done for each phospholipid; the curves shown here are representative. (B) Representative curves showing monolayer surface pressure as a function of the amount of phospholipid in the monolayer. These experiments were done by adding known quantities of PLPC (○) or PLPC-OOH (■) onto a buffer subphase at constant total area.

trough used was a small circular one with a surface area of 10 cm² and a volume of 10 mL.

RESULTS

Typical pressure–area curves obtained for PLPC, PLPC-OOH, and PLPC-OH are shown in Figure 1A. At any given surface pressure, the mean molecular areas of both types of oxidized PLPC are considerably larger compared to the molecular area of nonoxidized PLPC. Average values for mean molecular areas at a surface pressure of 10 mN/m as determined from several experiments are (areas in Å² ± SD, n = 4 in all cases) 92 ± 5 (PLPC), 136 ± 8 (PLPC-OOH), and 145 ± 7 (PLPC-OH). At 20 mN/m, these molecular areas are 74 ± 4 (PLPC), 108 ± 7 (PLPC-OOH), and 111 ± 7 (PLPC-OH).

Increasing the monolayer surface pressure by increasing the amount of phospholipid in the monolayer (by adding known quantities of lipid at a constant total surface area) instead of by reducing the total surface area (with the movable barrier) gives very similar results. Figure 1B shows that, on a given total surface area, significantly less oxidized phospholipid than nonoxidized phospholipid is needed to obtain comparable monolayer surface pressures.

PLA_2 from porcine pancreas can degrade pure PLPC monolayers up to a pressure of approximately 20 mN/m. In the experiments described here, phospholipase activity was monitored as a decrease in monolayer surface pressure in time, resulting from hydrolysis products (lysophospholipid, free fatty acid) leaving the monolayer. At low initial surface pressures (results not shown), all types of monolayers were broken down rapidly, although the surface pressure decrease was clearly more rapid for PLPC-OOH and PLPC-OH monolayers. For example, at a starting pressure of 16 mN/m, monolayers of PLPC-OOH or PLPC-OH showed a rapid pressure decrease immediately after addition of enzyme. Hydrolysis of the monolayers was complete within 20–25 min. In contrast, PLPC monolayers exhibited an initial pressure increase to approximately 18 mN/m over 6 min after addition of enzyme. Nonoxidized free fatty acid is less polar than oxidized free fatty acid; thus, this pressure increase probably reflects a buildup of hydrolysis products in the monolayer before they are expelled to the water phase. The pressure gradually decreased after this initial period as hydrolysis proceeded, and complete degradation of the PLPC monolayer took

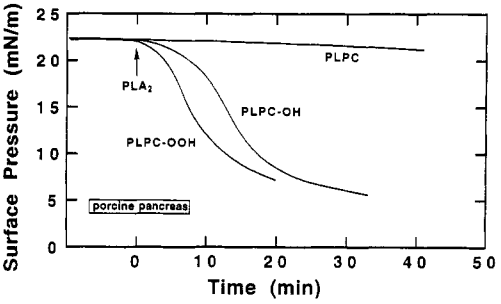

FIGURE 2: Breakdown of oxidized and nonoxidized PLPC monolayers by porcine pancreas phospholipase A_2. Enzyme activity was recorded as the decrease in monolayer surface pressure in time upon injection of 3 IU (4.3 nM, enzyme:substrate ratio approximately 1:40) of phospholipase (arrow) under monolayers consisting of pure PLPC, PLPC-OOH, or PLPC-OH (as indicated).

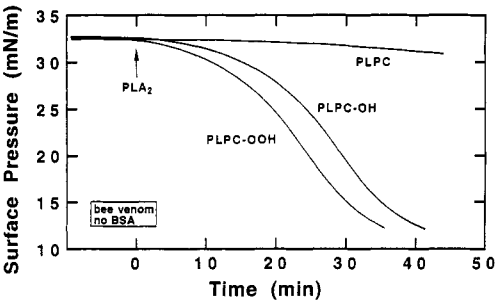

FIGURE 3: Breakdown of oxidized and nonoxidized PLPC monolayers by bee venom phospholipase A_2 in the absence of BSA. Monolayer surface pressure decrease was recorded in time upon injection of 0.1 IU of phospholipase (0.07 nM, enzyme:substrate ratio approximately 1:3200) under monolayers consisting of pure PLPC, PLPC-OOH, or PLPC-OH (as indicated).

approximately 60 min. Increasing the starting surface pressure resulted in a decreased rate of monolayer degradation in all cases. When the initial surface pressure was raised to 22 mN/m, hardly any decrease of surface pressure could be observed for PLPC monolayers over the time course of the assay (Figure 2). In contrast, both PLPC-OOH and PLPC-OH monolayers underwent rapid degradation, with the highest rate of pressure decrease observed for the PLPC-OOH monolayer.

PLA_2 from bee venom has a much higher pressure limit: it can degrade phospholipid monolayers at surface pressures of up to approximately 35 mN/m. At surface pressures well below that limit, hydrolysis of phospholipid monolayers was characterized by a rapid decrease in monolayer pressure for oxidized as well as nonoxidized PLPC (not shown). The order of observed pressure decrease rates was PLPC-OOH > PLPC-OH > PLPC. At initial surface pressures of 33 mN/m (Figure 3), only a very slow decrease of surface pressure was observed in the case of PLPC, whereas PLPC-OOH and PLPC-OH again exhibited rapid decreases, with the PLPC-OOH monolayer apparently being degraded faster than PLPC-OH.

The more polar nature of the oxidized free fatty acids that are produced as a result of PLA_2 activity might be expected to lead to a faster expulsion of these hydrolysis products to the water phase as compared to nonoxidized free fatty acid. This could lead to an underestimation of PLA_2 activity in the case of the nonoxidized PLPC monolayer, since with a comparable extent of hydrolysis the surface pressure would decrease less for nonoxidized PLPC than for oxidized PLPC due to more nonoxidized than oxidized free fatty acid remaining in the monolayer. Therefore, the above experiment was repeated with BSA added to the subphase in order to

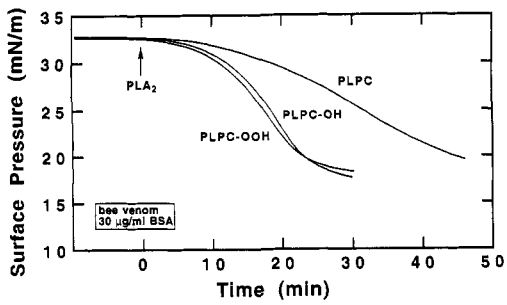

FIGURE 4: Preferential hydrolysis of oxidized versus nonoxidized PLPC by phospholipase A_2. Enzyme activity was recorded as the decrease in monolayer surface pressure upon injection of 0.1 IU of bee venom phospholipase A_2 under monolayers consisting of pure PLPC, PLPC-OOH, or PLPC-OH (as indicated). BSA (30 µg/mL) was present in the subphase buffer to facilitate removal of hydrolysis products from the monolayer.

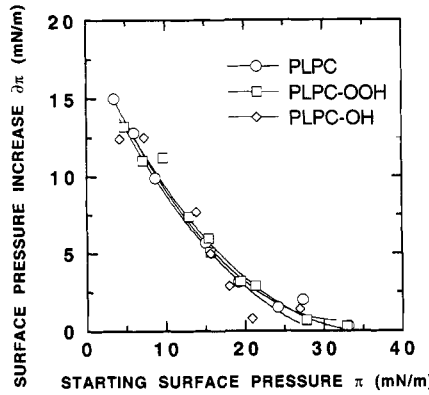

FIGURE 5: Interaction of phospholipase A_2 with oxidized and nonoxidized PLPC monolayers. Penetration of phospholipase into phospholipid monolayers was monitored as the monolayer surface pressure increase upon injection of 100 µg (83 IU) of bee venom phospholipase A_2 under monolayers consisting of pure PLPC (○), PLPC-OOH (□), or PLPC-OH (◇) at various starting pressures. In order to prevent enzyme activity, these experiments were performed in a Ca^{2+}-free buffer at pH 5.0.

extract free fatty acids from the monolayer upon their formation.

For each phospholipid, the rate of surface pressure decrease was higher in the presence of BSA and increased with increasing BSA concentration, indicating that BSA facilitates the removal of hydrolysis products from the monolayer. Using a BSA concentration that gave maximal pressure decrease rates (30 µg/mL), it was observed that degradation of the phospholipid monolayers by bee venom PLA_2 still proceeded much faster with monolayers consisting of PLPC-OOH and PLPC-OH than with PLPC (Figure 4). In the presence of BSA, PLPC-OOH and PLPC-OH exhibited essentially indentical monolayer degradation kinetics.

For all lipids tested, the enzyme penetration studies (Figure 5) show a decreasing ability of the phospholipase to penetrate the phospholipid monolayer as the initial surface pressure is increased. At starting pressure above 30 mN/m, only a marginal pressure increase could be observed after addition of the phospholipase. The curves obtained for PLPC, PLPC-OOH, and PLPC-OH are virtually identical, indicating similar penetration behavior of the PLA_2 in all cases.

DISCUSSION

The structural and functional integrity of a biological membrane is dependent on the condition of its lipid matrix. Studies on erythrocytes have shown that changes in the lipid molecular species composition of the membrane can have drastic consequences for cell shape and in vivo survival (Christiansson et al., 1985; Kuypers et al., 1984, 1985). Likewise, peroxidative damage to membrane lipids has been shown to affect membrane viscosity and barrier function (Deuticke et al., 1986; Richter, 1987; Sugihara et al., 1991; Wratten et al., 1993), and to ultimately lead to cell lysis (van den Berg et al., 1992). In the past, many research efforts have been devoted to the identification and characterization of enzymatic and nonenzymatic antioxidant defense mechanisms such as vitamins E and C, superoxide dismutase, catalase, and glutathione peroxidase (Clemens & Waller, 1987; Freeman & Crapo, 1982; Niki, 1987; Scott et al., 1991). Although these components are very efficient in the *prevention* of oxidative damage, they do not afford an absolute protection, and it is now widely accepted that an additional mechanism is in place to *repair* oxidatively damaged membrane lipids.

It has been known for a long time that even cells without the capacity for de novo synthesis of lipids, such as the erythrocyte, exhibit a continuous turnover of membrane phospholipids. This presumably reflects the need for a fine-tuned membrane lipid composition to ensure optimal membrane function. An important mechanism in this respect is the continuous hydrolysis and reacylation of membrane phospholipids. Early on, it was speculated that a specific role of phospholipase-mediated hydrolysis of membrane phospholipids might be found in the repair of oxidized lipids (van Deenen, 1965). In more recent years, experimental evidence has accumulated documenting the preferred hydrolysis of oxidized lipids by PLA_2 (Mead, 1980; Mead et al., 1982; Sevanian & Kim, 1985; Yasuda & Fujita, 1977). It was also observed that glutathione peroxidase could reduce fatty acid hydroperoxides, but prior release of the fatty acid from the parent phospholipid through the action of a PLA_2 was a requirement (van Kuijk et al., 1987). Recently, a selenium-dependent glutathione peroxidase has been identified that can directly reduce membrane phospholipid and cholesterol hydroperoxides (Thomas et al., 1990; Ursini et al., 1991). This enzyme detoxifies labile hydroperoxides, but the resulting hydroxy fatty acyl chains would still need to be hydrolyzed by PLA_2 in order to repair the phospholipid molecule. Complete repair of lipid oxidative damage subsequently entails activation of fresh fatty acids by acyl-CoA synthetase and reacylation of lysophospholipids by lysophospholipid–acyl-CoA transferases (Lubin & Kuypers, 1991; MacDonald & Sprecher, 1991; van den Berg & Kuypers, 1992). To date, characterization of the components of this important repair system for lipid oxidative damage and their interactions is incomplete. We attempt here to gain more insight into the mechanism by which PLA_2 could act on oxidized phospholipid substrates.

It has been proposed on theoretical grounds that the polar nature of a hydroperoxy or hydroxy group introduced into a phospholipid fatty acyl chain would tend to distort the normal conformation of that phospholipid molecule, pulling the oxidized fatty acyl chain toward the membrane surface (van Kuijk et al., 1987). The experimental data presented here regarding the enlarged molecular surface areas of oxidized PLPCs compared to native PLPC support this suggestion. The oxidized PLPC species exhibit an increase in molecular surface area compared to native PLPC of approximately 50% over the entire surface pressure range. This suggests a substantially altered conformation of the oxidized PLPC molecules, as is tentatively represented in Figure 6. Put differently, compressing oxidized phospholipids to the same

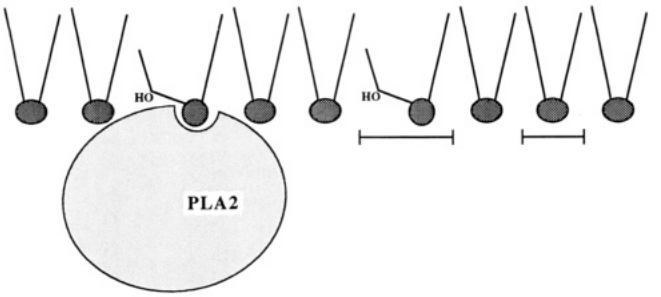

FIGURE 6: Conformational changes in oxidized phospholipids as a basis for their preferential hydrolysis by phospholipase A_2. This cartoon shows the conformational change in a phospholipid molecule in a monolayer or membrane resulting from the introduction of a hydroperoxy or hydroxy group in its *sn*-2 fatty acid. The polar group tilts the phospholipid molecule out of its normal position in the monolayer or bilayer by pulling the fatty acyl chain to the lipid–water interface. Resulting exposure of the *sn*-2 ester bond facilitates access for phospholipase A_2, which is proposed to be the basis for the preferential hydrolysis of oxidized phospholipids by this enzyme.

molecular surface area as nonoxidized phospholipid builds up a considerable packing stress in the monolayer as witnessed by the much larger monolayer surface pressure for oxidized phospholipids. Our data are in agreement with reports regarding the decreased electrical stability of oxidized membranes (Putvinsky et al., 1979) and the decrease in molecular order in planar phospholipid bilayers that contain peroxidation products (Wratten et al., 1993).

Comparing the pressure–quantity curves for PLPC and PLPC-OOH in Figure 1B, it is evident that less oxidized PLPC is needed to build a monolayer with identical surface pressure on a given total area. In the surface pressure range of 15–35 mN/m, the curves for oxidized and nonoxidized PLPC run virtually parallel, indicating comparable compression–expansion characteristics for these different molecular species at pressures where the molecules are forcibly packed very tightly. Although their molecular surface areas are different, the identical slopes of the curves for oxidized and nonoxidized PLPC indicate that removal of a nonoxidized or oxidized PLPC molecule from a monolayer with surface pressure in the 15–35 mN/m range will result in an identical drop in surface pressure. Therefore, although PLA_2 activity is surface pressure-dependent, the observed differences in degradation rates of oxidized and nonoxidized PLPC monolayers cannot be ascribed to different PLA_2 activities resulting from different surface pressure drops.

Additional evidence for changes in the molecular surface area and conformation of oxidized phospholipid was obtained in monolayer experiments where the effect of oxidants in the subphase on a PLPC monolayer was monitored with time (results not shown). For example, a combination of hydrogen peroxide and copper acted to increase the monolayer surface pressure at constant total area or to increase the total area at constant surface pressure. The kinetics of the pressure or surface area increase were dependent on the type and concentration of oxidant used.

Past investigations have established a correlation between the capability of a phospholipase A_2 to penetrate a membrane or monolayer and its hydrolytic activity. Each type of PLA_2 has a characteristic surface pressure limit above which the enzyme can no longer penetrate and hydrolyze the tightly packed phospholipid molecules (Demel et al., 1975; Verger et al., 1973, 1976). Thus, depending on the type of lipid used, PLA_2 from porcine pancreas has a surface pressure limit for activity of approximately 20 mN/m, while the pressure limit for PLA_2 from bee venom is much higher: approximately 35 mN/m. Our experiments with these two enzymes in the limiting pressure range for their activity (Figure 2 and 3) show hardly any monolayer pressure decrease for nonoxidized PLPC monolayers, whereas both PLPC-OOH and PLPC-OH monolayers are degraded rapidly.

The presence of BSA in the buffer subphase facilitates removal of hydrolysis products from the monolayer. Comparing Figures 3 and 4, it can be seen that in the absence of BSA the hydrolysis rate of the PLPC monolayer is underestimated. The more polar oxidized free fatty acids rapidly leave the monolayer upon formation, whereas nonoxidized free fatty acid does so much slower unless BSA is present. Nevertheless, even with BSA present the degradation of oxidized PLPC monolayers is still considerably faster. This indicates that the phospholipase has a preference for monolayers of oxidized versus nonoxidized phospholipid under otherwise identical conditions.

As the hydrolytic activity of PLA_2 has been linked to its penetrating capabilities, an explanation for the observed differences in hydrolysis rates of oxidized and nonoxidized PLPC monolayers could potentially be found in different penetration characteristics of the enzyme for these different types of monolayers. More specifically, enhanced breakdown of oxidized phospholipids might be explained by an increased extent of enzyme penetration, i.e., deeper into the lipid monolayer. Facilitated by the altered conformation of oxidized phospholipid molecules, this increased penetration could result in a more favorable positioning of the enzyme active center relative to the ester bond of the oxidized fatty acyl chain to be cleaved. A larger extent of enzyme penetration would be expected to be reflected in larger penetration-related surface pressure increases in the penetration experiments. However, the studies shown in Figure 5 indicate that the penetration behavior of the phospholipase is similar for all monolayers tested. Control experiments testing enzyme activity under the conditions used for the penetration studies (but in the presence of Ca^{2+}) revealed the same preference of the phospholipase for oxidized PLPC monolayers as described above. Therefore, increased penetration of PLA_2 into oxidized PLPC monolayers does not appear to offer a satisfactory explanation for the observed differences. However, one could speculate that enzyme penetration may be altered not with respect to the extent or depth of penetration but as a three-dimensional event in which oxidized phospholipid would allow a better positioning of the enzyme (active center) relative to the *sn*-2 ester bond.

It has been observed that PLA_2 preferentially hydrolyzes phospholipids at sites of membrane packing defects such as occur at the borders of coexisting solid and fluid lipid domains at the phase transition temperature (Grainger et al., 1990; Op den Kamp et al., 1974, 1975; Upreti & Jain, 1980). Thus, phosphatidylcholine liposomes were hydrolyzed by porcine pancreas PLA_2 only near the transition temperature, but above and below the transition temperature, hydrolysis was negligible. We propose a similar mechanism to explain the enhanced hydrolysis of oxidized PLPC monolayers observed here. As a result of the oxidation of its linoleoyl chain, the physical behavior of the PLPC molecule in a membrane or monolayer is altered. Averaged in time, the oxidized PLPC molecule will tend to adopt an orientation that can tentatively be depicted as in Figure 6. This altered molecular conformation results in facilitated access of phospholipase A_2 to the *sn*-2 ester bond. The PLA_2 thus preferentially hydrolyzes oxidized PLPC, with the altered three-dimensional configuration or "packing" of the phospholipid molecule being the

decisive factor rather than increased penetration of the enzyme.

It has been suggested that formation of oxidized phospholipid domains in a matrix of nonoxidized phospholipid, analogous to solid and fluid lipid domain formation at the phase transition temperature, could be the structural basis for phospholipase activation (Sevanian et al., 1988). Our experiments were not designed to study the role of the formation of oxidized phospholipid domains in phospholipase activation as we used either pure oxidized or pure nonoxidized lipid monolayers. However, without the possibility of domain formation in a mixture of oxidized and nonoxidized lipid, we still observed more rapid hydrolysis of oxidized phospholipid. Therefore, although domain formation may very well be an activating factor in the hydrolysis of oxidized phospholipids, it does not appear to be a requirement for phospholipase activity. Monomolecular lipid films are clearly distinct from biological membranes, but this technique allows us to address some very basic questions with regard to the behavior of individual lipid species. It is also clear that in a physiological situation no membrane will be made up exclusively of oxidized lipids. Therefore, subsequent experiments will focus on issues regarding enzyme specificity, i.e., the minimal concentration of oxidized phospholipid that is required for phospholipase activity (or the maximal concentration that is allowed without phospholipase being activated).

In conclusion, our results point toward a mechanism in which the drastically altered conformation of oxidized phospholipids, as expressed in their much larger molecular surface areas, predisposes them for preferential removal by a phospholipase A_2 enzyme because access to the *sn*-2 ester bond is facilitated. If this can be translated to a physiological situation, it would appear that the very same physical properties of oxidized phospholipids that underlie the mechanism by which they disrupt membrane organization render them susceptible to recognition and removal by endogenous phospholipases. Such an initial step in the lipid repair mechanism would allow for a "base-level" phospholipase activity for normal phospholipid and fatty acid turnover. Phospholipase activity is enhanced if oxidized phospholipids are present, returning to base-level activity after removal of the oxidized lipids.

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