Role of Lipid Structure in the Activation of Phospholipase A_2 by Peroxidized Phospholipids

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The time course of hydrolysis of a mixed phospholipid substrate containing bovine liver 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE) catalyzed by *Crotalus adamanteus* phospholipase A_2 was measured before and after peroxidation of the lipid substrate. The rate of hydrolysis was increased after peroxidation by an iron/adenosine diphosphate (ADP) system; the presence of iron/ADP in the assay had a minimal inhibitory effect. The rate of lipid hydrolysis was also increased after the substrate was peroxidized by heat and O_2. Similarly, peroxidation increased the rate of hydrolysis of soy PC liposomes that did not contain PE. In order to minimize interfacial factors that may result in an increase in rate, the lipids were solubilized in Triton X-100. In mixtures of Triton with soy PC in the absence of PE, peroxidation dramatically increased the rate of lipid hydrolysis. In addition, the rate of hydrolysis of the unoxidizable lipid 1-palmitoyl-2-[1-^{14}C]oleoyl PC incorporated into PC/PE liposomes was unaffected by peroxidation of the host lipid. These data are consistent with the notions that the increase in rate of hydrolysis of peroxidized PC substrates catalyzed by phospholipase A_2 is due largely to a preference for peroxidized phospholipid molecules as substrates and that peroxidation of host lipid does not significantly increase the rate of hydrolysis of nonoxidized lipids.
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The activity of phospholipase A_2 is increased in a number of tissues subjected to free-radical damage as a consequence of oxidative stress. Phospholipase inhibitors, such as chlorpromazine (1,2), inhibit the loss of phospholipid which is associated with peroxidative damage in cells. Extensive hydrolysis of cell membrane or lipoprotein phospholipids in the absence of adequate means for repair may lead to serious adverse consequences. For example, peroxidation of low density lipoproteins (LDL) and the associated hydrolysis of LDL phospholipids may play a role in the development of atherosclerosis (3). In addition, the structural reorganization of cell membranes consequent to lipid peroxidation and phospholipid hydrolysis may lead to edema (4), cell death and myocardial injury. Products of phospholipid hydrolysis, such as lysophospholipids (5), may also disrupt normal regulatory mechanisms that depend upon maintenance of the integrity of cell membrane structure. Adverse consequences of fatty acid and lysophospholipid accumulation have been demonstrated in brain and cardiac tissue in which the detergent effects of these molecules alter membrane function (5). In addition, release of arachidonic acid may produce eicosanoids that alter cardiovascular function, and phospholipid degradation has been observed in ischemia-reperfusion injury (6). However, control of lysolipid concentrations in membranes may be regulated by deacylation and reacylation of lipids. Accumulation of low concentrations of lysolipids has been shown to lead to increases in membrane permeability (7), activation of membrane-bound enzymes (8) and the activation of macrophages (9).

Preferential hydrolysis of peroxidized lipids has been observed in isolated brain capillaries (10), hepatic lysosomes (11), mitochondria (12) and microsomes (13) and in purified lipid mixtures (14,15). The rate of hydrolysis of these peroxidized lipids by phospholipase A_2 is some 2–3 times the rate of that of the unoxidized lipids in lipid mixtures that have been systematically examined (14). A repair mechanism based on this preference for peroxidized lipids has been proposed by van Kuijk *et al.* (16). The hydrolysis of peroxidized lipids results in excision of the peroxidized fatty acyl chains which are reduced, repaired and reesterified. This proposal is supported by several reports that demonstrate that phospholipase A_2 activity is required to release peroxidized fatty acids for subsequent detoxification by glutathione peroxidase (7,8, 17,18).

It is also conceivable that peroxidized lipids may promote the hydrolysis of neighboring unoxidized lipids, locally altering the membrane structure and possibly leading to cellular degeneration. Such a mechanism would suppose that peroxidation results in physical alterations in the substrate structure that lead to an increased rate of hydrolysis of the unoxidized lipids. A possible structural relationship between peroxidized lipids and increased activity of phospholipase A_2 was suggested by experiments of Sevanian *et al.* (19) who correlated an increase in microviscosity of diphenylhexatriene in liposomes following peroxidation with an increase in enzyme activity. In addition, an increase in the susceptibility of the lipids to fusion was observed following peroxidation suggesting a decrease in the stability of the liposomes.

In order to explore mechanisms that might explain the increase in rate of hydrolysis of peroxidized phospholipids by phospholipase A_2, the effect of lipid peroxidation on the rate of lipolysis in detergent-dispersed lipid and of a nonoxidizable radiolabeled substrate incorporated into liposomes comprising a host peroxidizable lipid were measured. The addition of detergent is expected to minimize differences in the surface structure of the substrates that may be due to lipid peroxidation. The host lipid experiments were designed to test whether the rate of hydrolysis of an unoxidized lipid increases when the host lipid is peroxidized.

MATERIALS AND METHODS

Lipid vesicles. To prepare lipid vesicles, bovine liver 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC; Type II-B, Sigma

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Abbreviations: ADP, adenosine diphosphate; LDL, low-density lipoproteins; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; TBARS, thiobarbituric acid reactive substances.

Chemical Company, St. Louis, MO) or soy phosphatidylcholine (Type III-S; Sigma) and bovine liver 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE; Type VII; Sigma) were mixed in chloroform at a 4:1 molar ratio. In some experiments, 5 mole% 1-palmitoyl-2-oleoyl PC (Avanti Polar Lipids, Birmingham, AL) or a trace quantity (0.1 μCi/mL of liposomes) of 1-palmitoyl-2-[1-^{14}C]oleoyl PC (58.0 mCi/mmol; New England Nuclear, Boston, MA) was included. The organic solvent was first dried with N_2, then under vacuum. The lipids were dispersed in 0.1 M KCl, 10 mM Tris-HCl, pH 7.6 at a concentration of 2.5 mM by incubating at 37°C with occasional vortexing for 1 h. The resulting liposomes were sonicated to clarity [15 min at setting 4 with a Branson (Branson Ultrasonic, Danbury, CT) 350 Sonifier operating on a 50% duty cycle] on ice under an argon atmosphere. These clear vesicles were stored at room temperature overnight to anneal (20). To prepare Triton/phospholipid mixtures (21), liposomes were prepared as described above without sonication at a concentration of 2.5 mM in 0.1 M KCl, 10 mM Tris-HCl, pH 7.6 containing 10 mM Triton X-100 (Ultragrade, LKB, Stockholm, Sweden). In some experiments additional Triton X-100 was added. The lipids were then suspended in the detergent-containing buffer by vortexing for 10 min.

The vesicles were peroxidized by one of three methods: (i) *Peroxidation by iron/adenosine diphosphate (ADP).* Vesicles (2.5 mM) were mixed with a fresh solution of 1.2 mM ADP and 0.2 mM $FeSO_4$ in water to attain a final concentration of either 20 μM $FeSO_4$ and 120 μM ADP or 10 μM $FeSO_4$ and 60 μM ADP. The vesicles were then incubated at 37°C for 30 min under air. (ii) *Peroxidation by iron (II)/iron (III).* A solution of 10 mM $FeCl_3$ and 11 mM ethylenediaminetetraacetic acid prepared in N_2-purged water was added to the vesicles to attain a concentration of 0.1 mM $FeCl_3$. Then a fresh solution of $FeSO_4$ (1 mg/mL water) was added to attain a concentration of 0.1 mM. (iii) *Peroxidation by heat and oxygen.* Prior to swelling the lipids in buffer, the tubes were flushed with oxygen, capped and heated at 60°C for up to 4 h. These peroxidized samples were stored at −20°C for up to 24 h. Then, buffer was added to the lipids and vesicles were prepared as described above. Prior to phospholipase measurements, aliquots were taken for measurement of thiobarbituric acid reactive substances (TBARS).

The rate of hydrolysis of lipids catalyzed by phospholipase was measured directly following peroxidation by the pH-stat technique or by measurements of released [^{14}C]oleic acid as described in detail by McLean *et al.* (21). The vesicles were diluted after peroxidation to 0.5 mM in 5 mL of 0.5 mM bicine, 0.1 M KCl, 10 mM $CaCl_2$, pH 8.0. The assay temperature was 37°C. Hydrolysis was initiated by addition of 3 μL of a 0.3 mg/mL solution of *Crotalus admanteus* phospholipase A_2 (Sigma) in standard buffer without calcium.

TBARS were measured by addition of 0.5 mL of sample to 0.1 mL of butylated hydroxytoluene (2%), followed by 1.5 mL each of 20% trichloroacetic acid and 0.67% thiobarbituric acid/0.05 N NaOH. Reaction proceeded for 30 min at 100°C. The tubes were then cooled, centrifuged for 15 min at 3000 rpm and transferred to plastic semimicro cuvettes. The difference in absorbance at 532 nm and 580 nm (to correct for light scattering) was measured in a Beckman DU-7 spectrophotometer (Fullerton, CA). The TBARS were calculated in units of malondialdehyde equivalents using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

RESULTS

Mixtures of PC and PE were chosen for evaluation of the effects of peroxidation on the rate of hydrolysis of phospholipids because of their abundance in biological membranes and the availability of previous data on similar mixtures (16). Unoxidized mixtures of bovine liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are hydrolyzed at a relatively slow rate by *Crotalus adamanteus* phospholipase A_2 at 37°C (Fig. 1). The rate is increased dramatically after peroxidation with iron and ADP. Because iron and ADP were not removed prior to assay, it is possible that the iron/ADP mixture directly affects the enzyme. Addition of iron and ADP to the assay mixture at the concentration at which it is present in the peroxidized samples inhibits the rate of hydrolysis slightly, so that the increased rate of hydrolysis observed in Figure 1 is not the result of a direct interaction of the iron and ADP with the enzyme. In a second set of experiments, dry lipids were peroxidized by heat and oxygen, to eliminate the presence of peroxidizing agents during the assay. This resulted in a slightly lower TBARS than with iron peroxidation, but peroxidation of the lipids by heat and oxygen increases the rate of lipid hydrolysis (Fig. 2), and the rate of hydrolysis increases progressively with time of incubation under oxygen at elevated temperature. The rate of hydrolysis of mixed PC/PE liposomes also depends upon the extent of lipid peroxidation and peaks after a time that depends on the particular substrate and experimental conditions under which it is examined (data not shown). With 10 μM $FeSO_4$ and 60 μM ADP at 37°C, the maximal rate occurs when the TBARS

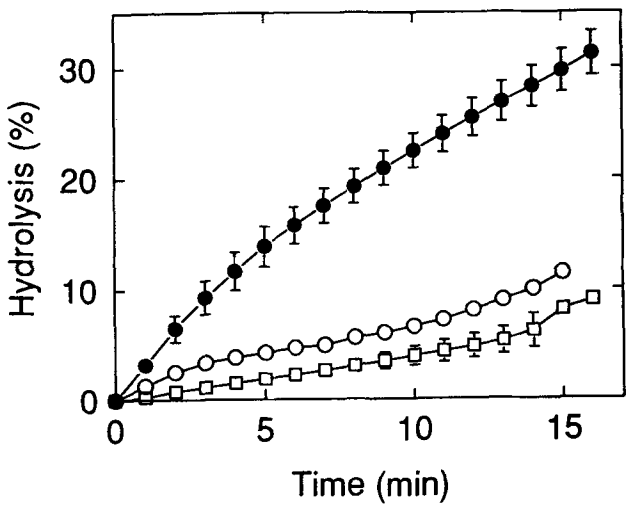

FIG. 1. Effect of peroxidation by iron adenosine diphosphate (ADP) on hydrolysis of 1,2-diacyl-*sn*-glycero-3-phosphocholine and phosphoethanolamine (PC/PE) (4:1) vesicles by phospholipase A_2. The rates of hydrolysis were measured by pH-stat (○) before incubation [thiobarbituric acid reactive substances (TBARS) of <0.2 nmol malondialdehyde (MDA)/μmole lipid]; (●) after incubation with 20 μM $FeSO_4$ and 120 μM ADP for 30 min at 37°C (TBARS of 7.9 ± 0.8 nmol MDA/μmole lipid); and (□) in the presence of 2 μM $FeSO_4$ and 12 μM ADP. Data are averages ± SEM of 3–4 experiments.

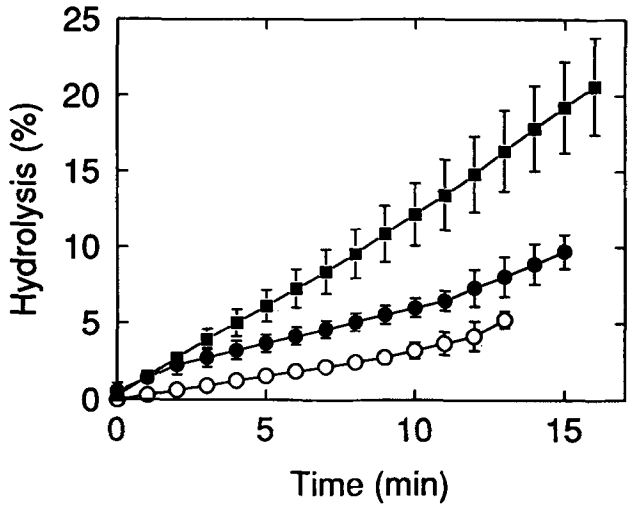

FIG. 2. Effect of incubation at elevated temperature under oxygen on the rate of hydrolysis of PC/PE vesicles by phospholipase A_2. Dry mixtures of PC/PE were incubated at 60°C for (○) 0 h (TBARS of <0.2 nmol MDA/μmole lipid), (●) 1 h (TBARS of 1.1 nmol MDA/μmole lipid) and (■) 4 h (TBARS of 2.1 nmol MDA/μmole lipid) prior to formation of vesicles and measurements of rates of hydrolysis. Abbreviations as in Figure 1.

reach ≈8 nmol/μmole lipid (Fig. 3). Further peroxidation of liposomal lipids as measured by TBARS formation results in slower rates of hydrolysis. Thus, the rate measured may depend critically on the extent of peroxidation. It is not possible to directly equate the TBARS results with the number of peroxidized lipid molecules.

One explanation for the increased rate of hydrolysis of peroxidized lipids in PC/PE mixtures is that the presence of the peroxidized lipids alters the structure of the vesicle in a manner that promotes interaction of the enzyme with substrate. One approach to test this hypothesis is to compare the rates of hydrolysis in the presence of a detergent which minimizes possible effects of substrate structure and physical form on the rates of hydrolysis. However, the presence of two types of phospholipids in the PC/PE mixture complicates interpretation of the data due to potential effects of the interface between the two lipids and the complications inherent in assessing experiments with more complex lipid mixtures. Thus, PC in the absence of PE was further investigated. Although more variability was observed in experiments with pure PC liposomes, the rate of hydrolysis of peroxidized PC liposomes exceeded that of the unoxidized liposomes (Fig. 4A) in a manner similar to that observed with the PC/PE mixtures. To minimize interfacial effects, samples were mixed with Triton X-100 at a molar ratio of 4:1 (Triton X-100 to lipid) and assayed. This addition of Triton to the peroxidized phospholipids resulted in a far more rapid rate of hydrolysis than with unoxidized PC mixtures. In the

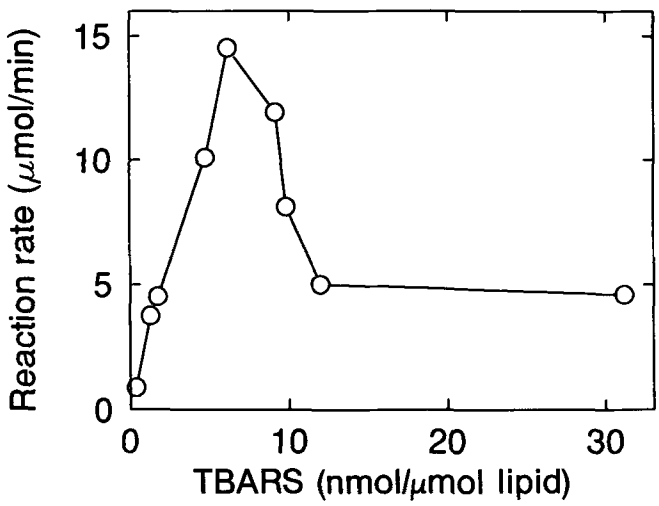

FIG. 3. Dependence of reaction rate on the extent of peroxidation as measured by TBARS. PC/PE (4:1) vesicles were peroxidized for various periods of time at 37°C with 10 μM $FeSO_4$ and 60 μM ADP. The rates were calculated from the initial linear portion of the kinetic course. Abbreviations as in Figure 1.

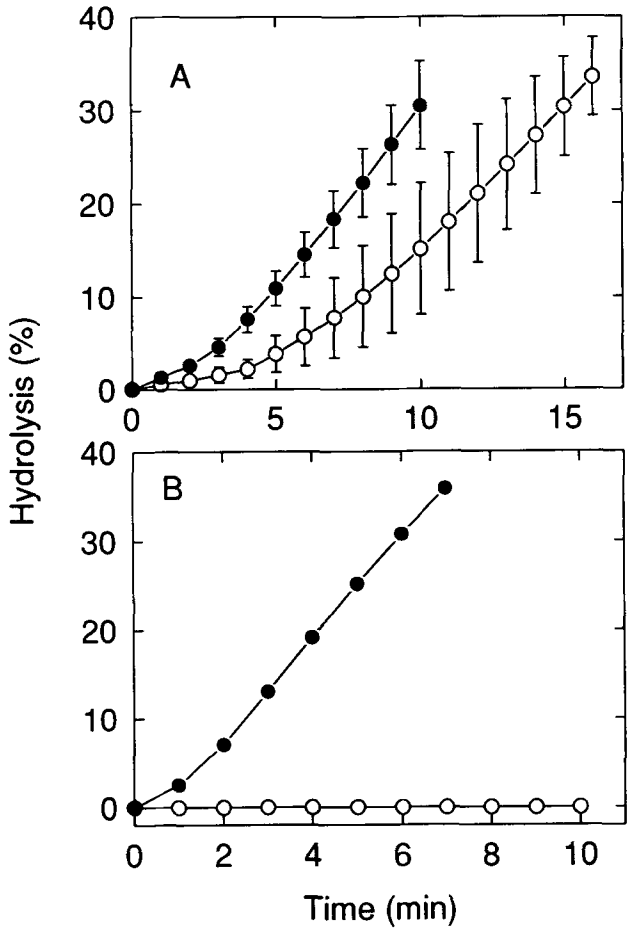

FIG. 4. Effect of peroxidation on hydrolysis of soy PC vesicles with and without Triton by phospholipase A_2. (A) Rates of hydrolysis of soy PC vesicles measured by pH-stat (○) before incubation (TBARS of 1.0 ± 0.1 nmol MDA/μmol lipid) and (●) after incubation with 100 μM Fe(II) and 100 μM Fe(III)/ethylenediaminetetraacetic acid for 30 min at 37°C (TBARS of 1.8 ± 0.2 nmol MDA/μmole lipid). Each curve is the average of two experiments. (B) Effect of peroxidation on rate of hydrolysis of soy PC in Triton X-100 micelles. Incubated (●) and unincubated (○) lipids from A were mixed with Triton X-100 (4:1 Triton/phospholipid mole/mole) and rates of hydrolysis were measured following addition of 3 μL (0.18 μg/mL) of enzyme. Abbreviations as in Figure 1.

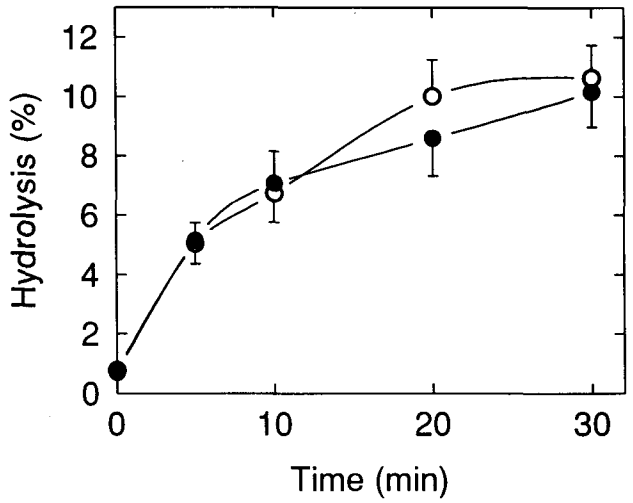

FIG. 5. Effect of peroxidation of host lipid on the rate of hydrolysis of 1-palmitoyl-2-[1-^{14}C]oleoyl PC by phospholipase A_2. PC/PE vesicles containing a trace amount of 1-palmitoyl-2-[1-^{14}C]oleoyl PC were peroxidized with iron/ADP as described in the legend to Figure 1. The rate of hydrolysis was measured by counting the released [^{14}C]oleic acid. (○) Unincubated vesicles (TBARS of 1.4 nmol MDA/μmol lipid), (●) incubated vesicles (TBARS of 8.7 nmol MDA/μmol lipid). Abbreviations as in Figure 1.

latter, no hydrolysis was measurable over a period of >60 min with a 4:1 ratio of Triton/PC (Fig. 4B). Higher ratios of Triton/PC were required to elicit measurable rates of hydrolysis in the unoxidized lipids, but peroxidized lipid/Triton mixtures were always more rapidly hydrolyzed (data not shown).

To test more directly whether peroxidation of the host lipid has any effect on the rate of hydrolysis of unoxidized lipids, the rate of hydrolysis of palmitoyl-[^{14}C]oleoyl PC in soy PC/soy PE vesicles before and after peroxidation was examined. Because palmitoyl-oleoyl PC is not readily peroxidized, it served as a marker for host lipid effects. The experiment described under Figure 1 was repeated with 1-palmitoyl-2-[1-^{14}C]oleoyl PC present in the liposomes, and the released fatty acids were assayed to determine the rate of hydrolysis of the radioactive palmitoyl-oleoyl PC tracer. In these experiments, peroxidation of the host lipid had no effect on the rate of hydrolysis of the palmitoyl-oleoyl PC (Fig. 5).

DISCUSSION

The present set of experiments demonstrates that peroxidation of mixed PC/PE vesicles by either iron/ADP or oxygen/heat results in a marked increase in the rate of phospholipase A_2-catalyzed hydrolysis that is not the result of a direct effect of peroxidizers on the enzyme. This supports the notion that phospholipase A_2 prefers peroxidized substrates (18,22). The dramatic increase in the overall rate of hydrolysis of PC/PE mixtures with peroxidation may be due to either a preference for phospholipid molecules with peroxidized acyl chains or a preference for structurally altered liposomes containing peroxidized lipids. It is difficult to distinguish between these two mechanisms in PC/PE mixtures because of potential differences in the rate of hydrolysis of the two components. The inability to make mechanistic conclusions with the mixed PC/PE mixture led us to study simple PC vesicles in more detail. To minimize interfacial interactions that may complicate interpretation of the effects of peroxidation of lipids on phospholipase activity, a detergent was included in some experiments. These experiments could not be interpreted at a molecular level with the PC/PE mixtures as addition of detergent may alter the substrate head-group preference of the enzyme (23,24).

In the simpler system containing PC in the absence of PE, the increase in rate of hydrolysis of lipids after peroxidation was relatively small. Addition of detergent increased the rate of hydrolysis of the lipids that had been incubated under peroxidizing conditions and reduced the rate of hydrolysis of unoxidized lipids. These data suggest that the peroxidized lipids are better substrates for the enzyme. An alternative explanation is that Triton enhances structural differences between the oxidized and peroxidized substrates. However, peroxidized lipids were still hydrolyzed more rapidly when the ratio of Triton to PC was increased. Consistent with this apparent substrate preference is the observation that the rate of hydrolysis of the nonoxidizable lipid, palmitoyl-[^{14}C]oleoyl PC, incorporated into a peroxidizable liposome was not influenced by peroxidation of host lipid.

These data suggest that in pure PC vesicles the primary effect of lipid peroxidation is to provide a phospholipid substrate with acyl chains that are preferentially hydrolyzed by the phospholipase. It does not appear that peroxidation of host lipid results in dramatic increases in the rate of hydrolysis of nonoxidized phospholipids. In terms of the proposed protective role of phospholipase A_2 in the repair of peroxidative membrane damage (16), the model provided by adding a trace of palmitoyl-oleoyl PC to peroxidizable lipids indicates that even in the case of transfer of nonoxidized lipids into regions of the membrane that have been damaged by peroxidation, the nonoxidized lipids may not be more rapidly hydrolyzed. However, the likelihood for peroxidation of lipid which diffuses into a region of peroxidized lipid is increased by its potential interaction with free radicals formed by peroxidative mechanisms at the site of injury. Such lipids would then be rapidly hydrolyzed in an attempt to minimize membrane damage.

No repair mechanism is available, to our knowledge, for the phospholipid hydrolysis associated with lipoproteins (25). We suggest that in cases of free-radical damage to lipoproteins or cells, in which the repair mechanism is unable to keep up with lipid hydrolysis, unchecked damage may occur that would lead to severe disruption of membrane or lipoprotein integrity and function. Part of this damage in LDL may be the result of transfer of liberated fatty acids from either the phospholipids or cholesteryl esters to amino groups on the surface of the lipoprotein (3). Recent experiments indicated that probucol prevents modification of the lipoprotein surface under conditions in which lipid peroxidation is observed, possibly by limiting transfer of fatty acids to the surface of the particle (26). These principles, when applied to the design of an antioxidant, suggest that one would need not only an effective free radical scavenger, but also a molecule that alters the structure of the membrane or lipoprotein surface in such a way that the transfer of nonoxidized lipids into the region of membrane peroxidative damage is limited. The possibility that other antioxidants may

exert their effects in a similar beneficial manner is currently under investigation.

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REFERENCES

1. Chien, K.R., Pfau, R.G., and Farber, J.L. (1979) *Am. J. Pathol. 97,* 505-530.
2. Jackson, M.J., Jones, D.A., and Harris, E.J. (1984) *Biosci. Rep. 4,* 581-587.
3. Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L., and Steinberg, D. (1984) *Proc. Natl. Acad. Sci. USA 83,* 3883-3887.
4. Chan, P.H., Yurko, M., and Fishman, R.A. (1982) *J. Neurochem. 38,* 525-531.
5. Katz, A.M., and Messineo, F.C. (1981) *Circ. Res. 48,* 1-16.
6. Otani, H., Prasad, M.R., Jones, R.M., and Das, D.K. (1989) *Am. J. Physiol. 257,* H252-H258.
7. Hochmann, Y. (1981) *J. Biol. Chem. 256,* 4783-4788.
8. Lawrence, A.J., Morrel, G.R., and Steele, J. (1974) *Eur. J. Biochem. 48,* 277-286.
9. Ngwenya, B.Z., and Yamamoto, N. (1985) *Biochim. Biophys. Acta 839,* 9-15.
10. Au, A.M., Chan, P.H., and Fishman, R.A. (1985) *J. Cell. Biochem. 27,* 449-453.
11. Weglicki, W.B., Dickens, B.F., and Mak, I.T. (1984) *Biochem. Biophys. Res. Commun. 124,* 229-235.
12. Yasuda, M., and Fujita, T. (1977) *Japan J. Pharmacol. 27,* 429-435.
13. Borowitz, S.M., and Montgomery, C. (1989) *Biochem. Biophys. Res. Commun. 158,* 1021-1028.
14. Sevanian, A., and Kim, E. (1985) *J. Free Radicals Biol. Med. 1,* 263-271.
15. Sevanian, A., and McLeod, L.L. (1987) *Lipids 22,* 627-636.
16. van Kuijk, F.J.G.M., Sevanian, A., Handelman, G.J., and Dratz, E.A. (1987) *Trends Biochem. Sci. 12,* 31-34.
17. van Kuijk, F.J., Handelman, G.J., and Dratz, E.A. (1985) *J. Free Radicals Biol. Med. 1,* 421-427.
18. Tan, K.H., Meyer, D.J., Belin, J., and Ketterer, B. (1984) *Biochem. J. 220,* 243-252.
19. Sevanian, A., Wratten, M.L., McLeod, L.L., and Kim, E. (1988) *Biochim. Biophys. Acta 961,* 316-327.
20. Apitz-Castro, R., Jain, M.K., and de Haas, G.H. (1982) *Biochim. Biophys. Acta 688,* 349-356.
21. McLean, L.R., Demel, R.A., Socorro, L., Shinomiya, M., and Jackson, R.L. (1986) *Meth. Enzymol. 129,* 738-763.
22. Roberts, M.F., Adamich, M., Robson, R.J., and Dennis, E.A. (1979) *Biochemistry 18,* 3301-3307.
23. Sevanian, A., Stein, R.A., and Mead, J.F. (1981) *Lipids 16,* 781-789.
24. Adamich, M., Roberts, M.F., and Dennis, E.A. (1979) *Biochemistry 18,* 3308-3314.
25. Sparrow, C.P., Parthasarathy, S., and Steinberg, D. (1988) *J. Lipid Res. 29,* 745-753.
26. McLean, L.R., and Hagaman, K.A. (1989) *Biochemistry 28,* 321-327.

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