Prothrombinase Acceleration by Oxidatively Damaged Phospholipids*

Thrombin production is controlled in vivo by a complex system of cascade and feedback mechanisms. By controlling thrombin production, these mechanisms regulate the various physiological activities of thrombin, including the maintenance of hemostasis, the signaling of smooth muscle and other cells, and the activation of platelets (1). Platelet plasma membranes are an especially important control point for thrombin production by prothrombinase. Oxidized arachidonoyl chains cause dose-dependent increases in prothrombinase activity up to 6-fold greater than control values. These increases were completely attenuated by the presence of α-tocopherol, γ-tocopherol, or ascorbate. Over the course of a 300-min oxidation, the ability of arachidonoyl lipids to accelerate prothrombinase peaked at 60 min and then declined to base-line levels. These results suggest that instead of being impeded by oxidative membrane damage, prothrombinase activity is enhanced by one or more products of nonenzymatic lipid oxidation.

EXPERIMENTAL PROCEDURES

MATERIALS—1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS), 1,2-dimyristoyl-sn-glycerol-3-phosphoholine (SAPC), 1-stearoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine (SAPC) were obtained from Avanti Polar Lipids (Alabaster, AL). All three lipid species were ordered specially packaged in 5–15-mg quantities, under argon, in sealed glass ampules and were stored at −20 °C until the day of use. Dansylarginine-N(3-ethyl-1,5-pentadecyl)amide, human prothrombin, and factors Xa delivery of substrate by confining these factors to the membrane surface and reducing three-dimensional diffusional processes to two-dimensional processes. However, PS head groups can also accelerate PTase activity by functioning as a regulatory cofactor (3).

There are compelling reasons to consider the possible effects of oxidative lipid damage on thrombin production. For instance, oxidation profoundly alters the architecture and chemical properties of phospholipid bilayers (4, 5), and the function of PTase is clearly sensitive to the physical state of the membrane (6). Many common cellular processes such as platelet activation release highly reactive oxygen species that can oxidize lipoproteins (7). Oxidation, in turn, dramatically increases the ability of lipoproteins to accelerate the activity of the PTase complex and produce more thrombin (8).

The principal sites of both enzymatic and non-enzymatic oxidation in a membrane are the olefinic groups of unsaturated fatty acyl chains. Although the fatty acyl chains of membrane lipids have long been regarded as having little or no effect on the catalytic activity of coagulation factor complexes (9), more recent studies have reported that unsaturated acyl chains do increase the intrinsic $k_{cat}$ for PTase, compared with saturated acyl chains (10). This difference was evident even after accounting for changes in substrate transport due to altered lipid “fluidity” and was confirmed using the soluble substrate, prothrombin-1. Other investigators (6, 11) have also used prothrombin-1 and found no such effect, but they nonetheless observed that PTase activity is accelerated by phosphatidylcholine vesicles if unsaturated acyl chains were present, especially at relatively low concentrations of phosphatidylserine.

Thus, the evidence suggests that the acyl chain composition of membrane lipids can influence PTase activity, at least under some circumstances. Taken together with the susceptibility of unsaturated acyl chains to oxidative damage, and the production of potent oxidizing agents by platelets and other cells capable of supporting PTase activity, these observations suggest that unsaturated and oxidized fatty acyl chains on PTase activity may have significant effects on thrombin production by PTase. To facilitate their detection and characterization of these effects, these investigations have been conducted in a chemically defined in vitro system consisting of synthetic lipid vesicles and purified human coagulation factors.

Edward A. Weinstein‡, Hongwei Li‡, John A. Lawson‡, Joshua Rokach§, Garret A. FitzGerald‡, and Paul H. Axelsen¶

From the 2Departments of Pharmacology and Medicine and the §Johnson Foundation for Molecular Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the ¶Claude Pepper Institute and Department of Chemistry, Florida Institute of Technology, Melbourne, Florida 32901

The optimally efficient production of thrombin by the prothrombinase complex relies on suitable positioning of its component factors and substrate on phosphatidylserine-containing lipid membranes. The presence of oxidatively damaged phospholipids in a membrane disrupts the normal architecture of a lipid bilayer and might therefore be expected to interfere with prothrombinase activity. To investigate this possibility, we prepared phosphatidylserine-containing lipid vesicles containing oxidized arachidonoyl lipids, and we examined their ability to accelerate thrombin production by prothrombinase. Oxidized arachidonoyl chains caused dose-dependent increases in prothrombinase activity up to 60 °C.

1 The abbreviations used are: PS, phosphatidylserine; PTase, prothrombinase; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPS, 1,2-dimyristoyl-sn-glycerol-3-phosphoholine; SAPC, 1-stearoyl-2-arachidonoyl-sn-glycerol-3-phosphocholine; BHT, butylated hydroxytoluene; PFB, pentafluorobenzyl.

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3 To whom correspondence should be addressed: Dept. of Pharmacology, University of Pennsylvania, 3620 Hamilton Walk, Philadelphia, PA 19104-6084. Tel.: 215-898-9238; Fax: 215-573-2236; E-mail: axe@pharm.med.upenn.edu.

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and Va were obtained from Hematologic Technologies, Inc. (Essex Junction, VT). Chromogenic substrates S-2238 and S-2765 were obtained from Diaphragma Group Inc. (Franklin, OH). Solvents for mass spectrometry were obtained from Burdick and Jackson (Muskegon, MI). All other reagents were obtained from Fisher or Sigma.

Vesicles—Phosphatidylcholine, dihydroxycholesterol, pyridine, and vitamin E were purchased from Avanti (Alabaster, AL). All organic and aqueous solvents were of reagent grade or higher purity. All vesicle lipids were dissolved in chloroform, mixed in the appropriate molar ratios, dried under a stream of nitrogen, and suspended in reaction buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM CaCl₂, 0.05% w/v PEG8000). The pentafluorobenzyl (PFB) ester was formed by adding 10 mol % diisopropylethylamine and 20 μl of 1M diisopropylethylamine to the stock solution of SAPC. When used, 400 μM vitamin C (Sigma) was added to the reaction mixture prior to the addition of oxidizing agents. When used, α-tocopherol (type V, Sigma, T-3634) or γ-tocopherol (Sigma) was added (0.1 mol/mol SAPC) to the stock solution of SAPC prior to any manipulations.

Prothrombinase Activity—Prothrombinase was assembled by incubation of lipids (vesicles) in “reaction” buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM CaCl₂, 0.05% w/v PEG8000) at 37 °C for 10 min. The solution was acidified with 100 μl of 0.1 M Tris buffer, pH 9.1. Samples of the oxidized lipid extract were dried under nitrogen and suspended in 1% acetic acid in water:methanol:chloroform (2:5:2). An aliquot of the sample solution was injected using a syringe pump at 20 μl/min into a VG Tracor 1000 gas chromatograph (Finnigan, San Jose, CA). The column temperature was held at 80 °C under argon until used. For control experiments, no Cu²⁺ or peroxide was added, but the samples were extracted after adding BHT and EDTA. Lipid extract efficiency (inferred from the fraction of phosphate recovered in the organic phase) approached 100% for unoxidized samples but fell as low as 40% for lipids that had been extensively oxidized. The extracted phosphoric acid was then mixed with chloroform solutions of DMPC and DMPS in a molar ratio of 10:70:20, dried under nitrogen, resuspended in reaction buffer, and extruded in the same manner as for vesicles not containing SAPC. When used, 400 μM vitamin C (Sigma) was added to the reaction mixture prior to the addition of oxidizing agents. When used, α-tocopherol (type V, Sigma, T-3634) or γ-tocopherol (Sigma) was added (0.1 mol/mol SAPC) to the stock solution of SAPC prior to any manipulations.

Prothrombinase Activity—Prothrombinase was assembled by incubation of lipids (vesicles) in “reaction” buffer (20 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM CaCl₂, 0.05% w/v PEG8000) at 37 °C for 5 min. The active concentration of a factor Xa stock solution was determined each day using the chromogenic substrate, S-2765 (Diaphragma, OH). Thrombin generation was initiated by the addition of prothrombin to the assembled PTase. The PTase activity increased with oxidation times up to 60 min incubation. The post-oxidation lipid extract was developed a broad band maximal at 242 nm (Fig. 3A). Overoxidation of SAPC was confirmed by the absorption spectrum of 8,12-iso-iPF₂-n-6 and oxo-iPF₂-n-6 at the concentrations used in the experiments (0.05) better than controls. The omission of any component of the PTase complex, namely Ca²⁺, liposomes, factors Xa, Va, or II, reduced PTase activity by at least 3 orders of magnitude (data not shown).

The presence of ascorbate, α-tocopherol, or γ-tocopherol in the oxidizing reaction dramatically attenuated the oxidation-induced increases in PTase activity. At times from 30 to 210 min, α-tocopherol was significantly (p < 0.05) better than ascorbate at attenuating this increase, although the effect of ascorbate was significant at all times examined (p < 0.05). The effects of α-tocopherol and γ-tocopherol were approximately equivalent at all times, bringing PTase activity to the level seen in unoxidized vesicles. In all of the experiments described to this point, SAPC was oxidized at atmospheric oxygen, the other lipids to facilitate the removal of oxidizing agents, simplify the analysis of oxidation products, and to make unambiguous the lipid species undergoing oxidation. Products from the oxidation reaction were characterized spectrophotometrically and by mass spectrometry. UV spectra of SAPC vesicles oxidized with Cu²⁺/peroxide oxidation initially developed a broad band maximal at 242 nm (Fig. 3A). Overoxidation of SAPC was confirmed by the absorption spectrum of 8,12-iso-iPF₂-n-6 and oxo-iPF₂-n-6 at the concentrations used in the experiments (0.05) better than controls. The omission of any component of the PTase complex, namely Ca²⁺, liposomes, factors Xa, Va, or II, reduced PTase activity by at least 3 orders of magnitude (data not shown).

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time, this maximum shifted toward 234 nm, providing evidence for the formation of conjugated dienes (14). Weak vibronic bands apparent at 274 and 285 nm between 60 and 120 min indicated the formation of conjugated trienes, but a single broad band maximal at 265 nm dominates this region of the spectrum after 300 min and may represent either ketone dienes or conjugated trienes (16). The shape and amplitudes of these spectra were reproducible to within 10%.

The oxidation-induced absorption maximum at 234 nm initially increased with time and then gradually decreased (Fig. 3B). This is evidence that conjugated dienes are formed and then decay in the course of this reaction (17). The data also demonstrate that oxidation proceeds at a significant rate even without the Cu$^{2+}$/peroxide oxidants. SAPC vesicles in Tris buffer that are merely kept in a cuvette at room temperature for 6 h without added oxidants or antioxidants exhibit an absorbance at 234 nm that was more than 12% of the absorbance at 2 h for a sample oxidized with Cu$^{2+}$/peroxide. By this measure, therefore, the chemical oxidation procedure used in these experiments was approximately 30-fold faster than air oxidation.

An extract of the SAPC oxidation product was assayed for the presence of 8,12-iso-iPF$_2$-$\omega$-VI, a major urinary F$_2$-isoprostane (18). F$_2$-isoprostanes are chemically stable products of free radical-catalyzed lipid peroxidation (19, 20). As shown in Fig. 4, 8,12-iso-iPF$_2$-$\omega$-VI was readily detected after 30 min of oxidation. These results show that in vitro oxidation of SAPC produces at least some of the same complex oxidation products produced in vivo.

Extracted SAPC oxidation products were also characterized by electrospray mass spectroscopy. As shown in Fig. 5, peaks at m/z 810 and 833 confirm the identity of SAPC and sodiated...
SAPC before oxidation. The absence of other peaks is evidence for the purity of the SAPC lipid used. Higher molecular weight species appear with progressively larger signals after 120, 210, and 300 min of oxidation. We attribute peaks at $m/z$ 842, 874, 906, and 938 after 300 min of oxidation to the presence of mono-, di-, tri-, and tetra-hydroperoxide derivatives of SAPC. The peaks at $m/z$ 864, 896, 928, and 960 are likely to be the corresponding sodiated derivatives. There are small peaks at $m/z$ 858, 890, and 922 suggesting the addition of 3:2, 5:2, and 7:2 molecules of O$_2$ to SAPC. Peaks appearing at $m/z$ 857 and 931 are not identified. Despite the multitude of oxidation products after 300 min, the parent peak at $m/z$ 810 remains the dominant feature of the spectrum. Although we have made no attempt to quantitate the absolute amount of SAPC in these samples, normalization of each spectrum according to the magnitude of $m/z$ 810 permits us to conclude that the relative concentration of these oxy addition products increases over the course of 300 min. Mass spectra below $m/z$ 800 at 60 min exhibit very few peaks down to $m/z$ 400, and at no point is there a peak in the 60-min spectrum that is more prominent than at all other times. At 300 min, the mass spectra exhibit numerous peaks below $m/z$ 800; identification is difficult, however, because the sample is a complex mixture of reaction products and not the fragmentation of a single species.

As expected, the presence of a-tocopherol (10 mol % relative to SAPC) suppresses the formation of all oxidation products (Fig. 5). Only a small amount of monohydroperoxide at $m/z$ 842 and a trace amount of dihydroperoxide at $m/z$ 874 are detected after 300 min of oxidation. The lack of a more substantial peak at $m/z$ 874 in the presence of a-tocopherol and the increasing magnitude of this peak with time in the absence of a-tocopherol weigh against the possibility that this peak is due to a cuprous ion, Cu(I), adduct. Although the concentration of conjugated
dienes appears to decay after 120 min (Figs. 3 and 4), we are unable to identify any peaks in the mass spectra at 120 min that subsequently decay (Fig. 5).

**DISCUSSION**

Our results indicate that the oxidation of a polyunsaturated phospholipid increases PTase activity in a dose-dependent manner and that the antioxidants ascorbate, \( \alpha \)-tocopherol, and \( \gamma \)-tocopherol are effective at preventing the chemical changes that cause this increased activity. Our findings corroborate an earlier report in which the phospholipid component of low density lipoprotein particles was shown to accelerate PTase when oxidized (8). The findings also add two important dimensions to the earlier results by reproducing the PTase acceleration in a system where the substrate undergoing oxidation (SAPC) is chemically defined and by showing that the effect is not related in a simple way to the concentration of the principal oxidation products.

Esterified arachidonoyl chains comprise the vast majority of the olefinic groups in a membrane. Arachidonate and oleate are the two main unsaturated fatty acyl chains in the phospholipids of a platelet plasma membrane and are present in roughly equal portions (21, 22). However, there are four times as many olefinic groups in arachidonate as in oleate. Hence, the vast majority of sites in a platelet membrane that are susceptible to nonenzymatic oxidation are found in SAPC. Dimyristoyl lipids were chosen as a “background” lipid because they are saturated and in liquid crystalline phase at 37 °C.

The mechanism by which lipid peroxidation increases PTase activity may be either physical or chemical. Physical explanations are plausible because the introduction of SAPC into DMPC/DMPS perturbs membrane structure with longer acyl chains and olefinic groups. The olefinic groups are relatively hydrophilic in this context (i.e. compared with completely saturated chains), and they become much more so when oxidized. Therefore, both the introduction of longer chains and the oxidation of those chains will tend to disorder the bilayer membrane. Thus, one possible explanation for our results is that increased PTase activity is due to increased membrane disorder, at least to a point. This would be consistent with reports that olate chains accelerate PTase activity (10, 11), and it is compatible with the suggestion that PTase activity is sensitive to the precise way in which its components are juxtaposed on a membrane (6). Even if this is not the sole explanation for accelerated PTase, the relatively short acyl chains of DMPC/DMPS may amplify the effects of the direct cause. However, a physical explanation as the primary cause of this effect is not supported by our finding that PTase activity is unchanged from base-line values in the presence of 10 mol % SAPC and lipophilic antioxidants.

Chemical explanations for the acceleration of PTase activity by oxidized SAPC include the generation of specific accelerators of PTase via oxidation or phase separation of lipids. Our finding that the effect of SAPC oxidation diminishes after 60 min (Fig. 2) is consistent with the transient creation of par-
tially oxidized compounds with PTase-accelerating properties. Lipid peroxidation is a complex process resulting in heterogeneous mixtures of derivatives that accumulate at different rates (23). Isoprostanes arise in vivo via nonenzymatic oxidation of arachidonic acid and are considered surrogate markers for oxidative stress in human and animal studies (19, 24). Their detection in this system indicates that our procedure for the in vitro oxidation of SAPC produces at least some of the same complex oxidation products produced in vivo.

However, mass spectrometry results weigh against acceleration due specifically to the presence of isoprostanes or to the oxidized derivatives of SAPC seen in mass spectra, because the accelerator effect wanes despite an increase in the concentration of these compounds after 90 min (Figs. 4 and 5). Acceleration due to conjugated dienes also seems unlikely, since the peak absorbance at 234 nm occurs at 120 min oxidation, whereas peak PTase activity occurs around 60 min. Of course, extensive oxidation may produce PTase inhibitors or phase separations that reverse the accelerator effect of one of these compounds. However, it would be remarkably coincidental to create an inhibitor that exactly canceled the effect of the accelerator and left PTase activity at its base-line level after 300 min of oxidation. It seems more likely that SAPC oxidation transiently produces a specific accelerator of PTase that is simply not detected by mass spectrometry.

The pathological significance of increased endovascular thrombin production cannot be ascertained in this type of in vitro system. However, a substantial body of evidence suggests that oxidative chemical processes are involved in the pathogenesis of atherosclerosis (25), whereas another largely independent body of evidence suggests that thrombin may have an important role by virtue of its ability to modulate cell behavior (26). Observations suggesting a link between these processes include large quantities of oxidized phospholipid in regions of atherosclerotic plaques that are especially thrombogenic (27), high levels of thrombin receptor (PAR-1) found in association with atherosclerotic changes (28), and the ability of thrombin to stimulate the proliferation of vascular smooth muscle that is characteristic of atherosclerotic lesions (29). Small increases in thrombin production resulting from oxidized lipids may have significant local effects on endothelial cells, and these effects may be integrated over many years.

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