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EFFECT OF OZONATION PRODUCTS ON PHOSPHOLIPASE A_2 HYDROLYTIC ACTIVITY: USE OF BIS(1-HYDROXYHEPTYL)PEROXIDE AS A PRECURSOR OF THE OZONATION PRODUCT 1-HYDROXY-1-HYDROPEROXYHEPTANE

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Bis(1-hydroxyheptyl)peroxide (BisC7) upon spontaneous hydrolysis affords the difficult-to-isolate ozonation product 1-hydroxy-1-hydroperoxyheptane along with heptanal. PLA_2 hydrolytic activity is enhanced when the BisC7 hydrolysis products are incorporated in the membrane of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine liposomes, suggesting they cause a pronounced alteration in the bilayer packing order. Conversely, inhibition is observed when PLA_2 is incubated with BisC7 hydrolytic products prior to incubation with the liposomes, suggesting that these products are capable of reacting with and modifying the enzyme when present in solution.

The activity of phospholipase A_2 (PLA_2) is increased in tissues subjected to free radical damage as a consequence of oxidative stress. Preferential hydrolysis of peroxidized lipids has been observed in *in vivo* and *in vitro* systems (1-6) and it has also been shown that peroxidized lipids promote the hydrolysis of neighboring unoxidized lipids (7). Recently, we have shown that PLA_2 also can recognize ozone-induced damage (8).

Unsaturated fatty acids in phospholipids are important targets for reaction with ozone (9-11). The study of the effect of lipid ozonation products on PLA_2 hydrolytic activity is important since these molecules may be responsible for triggering an ozone-induced inflammatory response by activating the lipases PLA_2

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Abbreviations: BisC7, bis(1-hydroxyheptyl)peroxide; PLA_2, phospholipase A_2; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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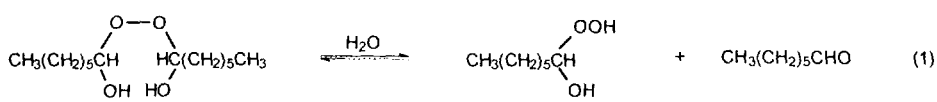
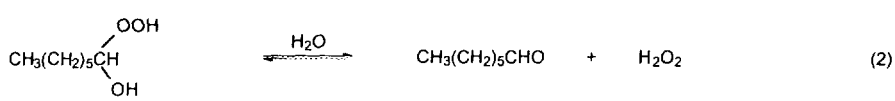
and PLC. The ozonation of unsaturated fatty acids (UFA) yields primarily 1-hydroxy-1-hydroperoxyalkanes, aldehydes and Criegee ozonides (12-14). We have previously studied the effects of phospholipid Criegee ozonides and aldehydes on the hydrolytic activity of PLA_2 and found that they display remarkably different activities. While the Criegee ozonide of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine is an excellent substrate for PLA_2, its hydrolysis product, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine, activates PLA_2 toward intact phospholipids within the same bilayer (8).

1-Hydroxy-1-hydroperoxyalkanes are difficult to isolate in pure form. However, they can be conveniently generated *in situ* from the spontaneous hydrolysis of the corresponding bis(1-hydroxyalkyl)peroxides which can be obtained as stable crystalline compounds. We recently succeeded in synthesizing bis(1-hydroxyheptyl)peroxide (BisC7). (The synthesis and characterization of BisC7, along with those of other ozonation products, will be published elsewhere; see equation 1 for the structure of BisC7.) Upon partial hydrolysis, BisC7 affords 1-hydroxy-1-hydroperoxyheptane (see equations 1-2), a major ozonation product from phospholipids containing palmitoleic acid. Here we report the change in PLA_2 hydrolytic activity caused by the hydrolysis products of BisC7, as reflected by oleic acid release from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) liposomes.



Bis(1-hydroxyheptyl)peroxide (BisC7)

1-Hydroxy-1-hydroperoxyheptane



MATERIALS AND METHODS

The following materials were used as received: L-α-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine from Avanti Polar Lipids (Alabaster, AL); methyl sulfoxide from Aldrich (Milwaukee, WI); phospholipase A_2 (Crotalus Adamanteus; EC 3.1.1.4) from Sigma (St.Louis, MO); L-α-1-palmitoyl-2-oleoyl-[oleoyl-1-^{14}C]-*sn*-glycero-3-phosphocholine sp.act. 58 mCi/mmol) from New England Nuclear Research Products (Boston, MA). The EcoLite liquid scintillation cocktail was purchased from ICN Biomedicals (Irvine, CA).

Liposome preparation. Liposomes were prepared by extrusion, using POPC as phospholipid core, following a procedure described previously (8). Briefly,

unlabeled POPC was mixed with the corresponding labeled [^{14}C]-POPC (0.0015 μCi ^{14}C-POPC /mg POPC), vortexed and dried under a nitrogen stream and resuspended in 10 mM Tris/150 mM KCl buffer (pH 7.4), to a final concentration of 10 mg/mL. The phospholipid suspension was then extruded ten times through an extruder (Lipex Biomembranes, Vancouver, BC) using two 0.1μm pore polycarbonate membrane, under an argon pressure of 2000 KPa.

PLA_2 mediated hydrolysis. The time course of PLA_2 hydrolysis of intact POPC liposomes shows linear rates of hydrolysis over a 15 min period. After this period, the hydrolytic activity plateaus, with 5.5% of the total POPC having undergone hydrolysis, presumably because the unilamellar liposomes have been destroyed. Thus, we chose to allow PLA_2 to act upon the liposomes for a period of 20 min for all experiments.

Sample aliquots of 2 mg POPC/0.003 μCi [^{14}C]-POPC in 10 mM Tris/150 mM KCl buffer (pH 7.4) containing 15 mM $CaCl_2$, were incubated in the presence of BisC7, which was added in 20 μL DMSO to give a final concentration of 10 μM, in a final volume of 1 mL, for 15, 30, 60, 90, 120 minutes at 37°C in a shaking bath. After each incubation period, 0.5 U of PLA_2 was added to each sample and incubated for an additional 20 min. Control experiments indicated that DMSO alone has no effect on PLA_2 hydrolytic activity.

In order to study the direct effect of the hydrolytic products of BisC7 on PLA_2, sample aliquots containing 0.5 U PLA_2 were incubated with 10 μM BisC7, for 15, 30, 60, 90, 120 min at 37°C in a shaking bath. After each incubation, 2 mg POPC/0.003 μCi [^{14}C]-POPC, liposome aliquots, were added and incubated for an additional 20 min. A third group of samples, containing liposomes aliquots and BisC7 were incubated under identical conditions but in the absence of enzyme, to estimate the degree of nonenzymatic (spontaneous) hydrolysis.

The percentage of POPC hydrolysis catalyzed by PLA_2, which was used as a standard activity, was measured following the release of [^{14}C]-oleic acid in a time-course assay, incubating liposomes (2 mg POPC/ 0.003 μCi [^{14}C]-POPC) for 0, 5, 10, 15, 20 min at 37 °C, under standard conditions.

The reactions were stopped by adding 5 mL of $CHCl_3$/MeOH (2:1 v/v) followed by centrifugation and recovery of the organic phase. A second extraction was made using 3 mL of $CHCl_3$. The organic phases were pooled and evaporated under a stream of nitrogen, the residue was dissolved in 3 mL of $CHCl_3$ and applied to a diol solid phase extraction column (Bond-Elute, Analychem) (25 mg, 3 mL). The chloroform fraction containing the free fatty acids, was collected into scintillation vials for measurements of radioactivity. The columns were then eluted with 3 mL of MeOH and the eluent collected in a separate set of vials, representing the phospholipid fraction. All samples were evaporated and redissolved in 10 mL of EcoLite liquid scintillation cocktail for measurement of radioactivity.

RESULTS AND DISCUSSION

The inhibitory effects of the hydrolysis products of BisC7 on PLA_2 were evaluated by incubating PLA_2 with BisC7 for up to 120 min prior to the addition of liposomes (Table 1). The amount of oleic acid released decreases with the length of time in which BisC7 was incubated with PLA_2, suggesting a direct inactivation of PLA_2 by hydrolysis products of BisC7.

Table 1. Percentage hydrolysis of oleic acid from POPC liposomes by phospholipase A_2 preincubated with bis(1-hydroxyheptyl)peroxide

Preincubation[a] (min)	% Hydrolysis[c]
0[b]	5.5 ± 0.25
15	4 ± 2
30	3 ± 2
60	1.8 ± 1
90	1.1 ± 0.4
120	0.46 ± 0.04

a) Aliquots of liposomes containing 2 mg POPC/0.003 μCi [^{14}C]-POPC in 10 mM Tris/150 mM KCl buffer (pH 7.4) were preincubated for 15, 30, 60, 90, 120 min, in the presence of BisC7 (10 μM), in a final volume of 1 mL at 37 °C in a shaking bath.
b) Liposomes were not preincubated with BisC7.
c) PLA_2 hydrolytic activity is expressed as percentage of [^{14}C]-oleic acid released from POPC liposomes over a 20 min incubation period with PLA_2. (See Materials and Methods for experimental details.)

Strikingly contrasting results were obtained as a result of a change in the order of addition. Liposomes were incubated with BisC7 (Figure 1) for up to 120 min, in the absence of PLA_2, to allow BisC7 to partially hydrolyze (according to

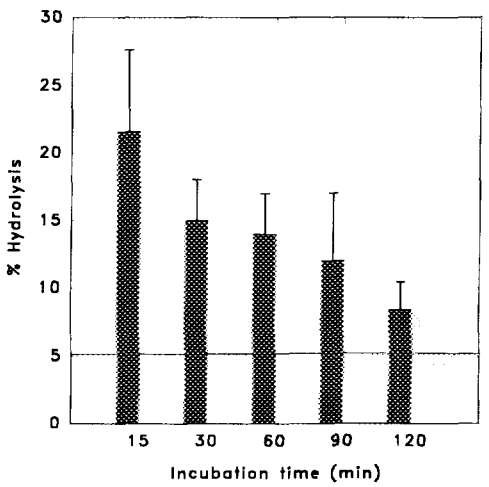


Figure 1. Phospholipase A_2 hydrolysis of oleic acid from POPC liposomes preincubated with bis(1-hydroxyheptyl)peroxide. The horizontal line at 5.5% hydrolysis represents the enzymatic activity in the absence of bis(1-hydroxyheptyl)peroxide.

equations 1 and 2) and the products to incorporate into the liposome bilayers. Then, 0.5 U PLA_2 was added and the mixture incubated for an additional 20 min. Under these conditions, PLA_2-mediated hydrolysis of oleic acid from POPC corresponded to 21 to 7.5% of POPC, a 1.4- to 3.8-fold **increase** in hydrolytic activity. This contrasts sharply with the **decrease** in hydrolytic activity that was observed when PLA_2 was incubated with BisC7 and then the liposomes added. Therefore, PLA_2 is **activated** when the hydrolysis products of BisC7 are incorporated into the bilayer. The hydrophobic hydrolysis products of BisC7 partition into the bilayer, generating heterogeneous microdomains that affect lipid organization and increase the area of the phospholipid-water interface, making the membrane more susceptible to PLA_2 attack (15). We previously observed a similar effect when another lipid ozonation product, 1-palmitoyl-2-(9-oxononanoyl)-*sn*-glycero-3-phosphocholine, was incorporated in POPC liposomes (8).

The **decrease** in hydrolytic activity, observed when liposomes are added last, occurs because the hydrolysis products of BisC7 react directly with PLA_2 **in solution** causing a decrease in oleic acid release (Table 1), suggesting the possibility of Schiff base formation at the lysine residue in the active site of PLA_2 (16) and/or noncovalent binding of 1-hydroxy-1-hydroperoxyheptane to the active site (17) and/or oxidations caused by 1-hydroxy-1-hydroperoxyheptane. When BisC7 is preincubated with the liposomes, the hydrophobic hydrolysis products preferentially partition into the bilayer, sharply lowering their effective concentrations in the aqueous phase where the PLA_2 resides, and thus, preventing direct reaction of the hydrolysis with the enzyme.

A **decrease** in PLA_2 hydrolytic activity was observed with longer times of BisC7 incubation with liposomes (Figure 1). This effect presumably occurs also as a result of the destabilizing effect that the incorporation of the hydrolysis products of BisC7 have on the liposomes bilayer. In the presence of the hydrolysis products of BisC7, and independently of PLA_2, the unilamellar liposomes appear to have a faster intrinsic decay constant.

Thus, PLA_2 hydrolytic activity is influenced in two different ways by the hydrolysis products of bis(1-hydroxyheptyl)peroxide, one when the products are present in solution, and a second if they are embedded in the membrane. The hydrolysis products, namely 1-hydroxy-1-hydroperoxyheptane and heptanal, **activate** PLA_2 when embedded in the membrane, but **inactivate** PLA_2 when they are present in solution.

We propose long-chain 1-hydroxy-1-hydroperoxyalkanes will reside preferentially in the membrane and will **activate** PLA_2, whereas the more water-soluble short-chain compounds will **inactivate** PLA_2. The toxic effects of 1-hydroxy-1-hydroperoxyalkanes produced by the ozonation of unsaturated phospholipids during inhalation of ozone-containing air can be due to alteration of membrane packing order with consequent PLA_2 hydrolytic activation, and/or direct modification of the enzyme in solution, inhibiting its activity. Studies of the thermodynamics and kinetics of these processes will help understand the markedly different effects that have been observed for several lipid ozonation products on eicosanoid metabolism (18) and phospholipases A_2 and C (19) in airway epithelial cells.

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