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Amphotericin B as an Intracellular Antioxidant

PROTECTION AGAINST 2,2′-AZOBIS(2,4-DIMETHYLVALERONITRILE)-INDUCED PEROXIDATION OF MEMBRANE PHOSPHOLIPIDS IN RAT AORTIC SMOOTH MUSCLE CELLS

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ABSTRACT. The antifungal activity of amphotericin B (AmB) and its side-effects (e.g. nephrotoxicity and hemolytic action) are suggested to be associated with its prooxidant effects in target cells. To test this hypothesis, we have undertaken studies to examine the role of AmB in oxidative stress in cultured rat aortic smooth muscle cells (SMC) incubated in the absence or in the presence of a lipid-soluble azo-initiator of peroxyl radicals, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN). No changes in the pattern of membrane phospholipids could be detected by two-dimensional high performance thin-layer chromatography (HPTLC) after oxidative stress induced by AMVN in which the cells remained viable, as judged by trypan blue exclusion. To improve the sensitivity of detection of oxidative stress in the cells, *cis*-parinaric acid (PnA) was incorporated biosynthetically into the membrane phospholipids [using PnA–human serum albumin (hSA) complex]. Incubation of the cells under aerobic conditions in the presence of up to 10 μM AmB showed no significant change in the pattern of PnA-labeled phospholipids, suggesting that AmB was not affecting the oxidative state of the cells. In contrast, treatment with AMVN (0.5 mM, incubation in the dark for 2 hr at 37°—conditions in which the viability of the cells was maintained) caused a significant reduction of all fluorescently labeled phospholipid fractions separated by HPLC. When PnA-labeled cells were subjected to oxidative stress by incubation with 0.5 mM AMVN in the presence of AmB, the loss of fluorescent phospholipids was reduced in a concentration-dependent manner over a concentration range of 0.25 to 10 μM. Thus, AmB does not produce any prooxidant effect but rather acts as an intracellular antioxidant. BIOCHEM PHARMACOL **54**;8:937–945, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. amphotericin B; oxidative stress; antioxidant; phospholipid peroxidation; parinarioyl-labeled phospholipids; smooth muscle cells

AmB†† is a heptaene antibiotic drug (Fig. 1) commonly used in the therapy of systemic fungal infections. Despite the subsequent development of newer drugs to combat such infections, AmB remains the drug of choice in the most severe cases of systemic infections. There are, however, known side-effects of the drug, which include cytotoxicity in a number of mammalian cells resulting, for example, in nephrotoxicity [1, 2] and hemolysis [3].

The antifungal action of AmB is believed to be due to two, possibly interrelated, effects on the cell membranes of target cells. First, AmB forms well characterized complexes with cholesterol and the formation of such complexes in the plasma membrane, which is relatively rich in cholesterol, may cause irreversible changes in the permeability properties of the membrane [4]. Second, AmB is said to act as a prooxidant in membranes, causing oxidative stress [5] possibly associated with the formation of free radical intermediates. The fact that antioxidants appear to protect erythrocytes from AmB-induced lysis [3] has been used to support the idea that antioxidants and/or antioxidant enzymes might be effective in counteracting the harmful effects of the drug.

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†† *Abbreviations:* AmB, amphotericin B; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); BHT, butylated hydroxytoluene; DMEM, Dulbecco's Modified Eagle's Medium; DMEM/F12, Dulbecco's Modified Eagle's Medium/F12Ham Medium; DPG, diphosphatidylglycerol; FBS, fetal bovine serum; HBSS, Hank's Balanced Salt Solution; HPTLC, high performance thin-layer chromatography; hSA, fatty acid free human serum albumin; LPC, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PEA, phosphatidylethanolamine; PI, phosphatidylinositol; PnA, *cis*-parinaric acid; PS, phosphatidylserine; SMC, smooth muscle cell(s); and SPH, sphingomyelin.

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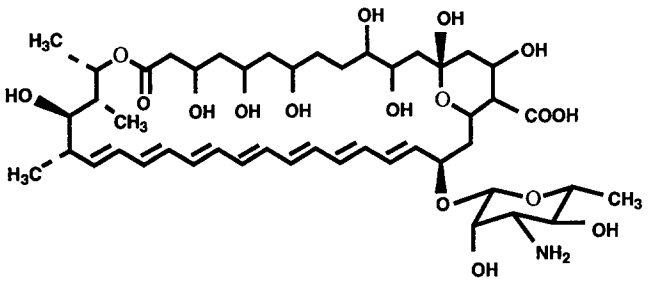

FIG. 1. Structural formula of AmB.

It should be emphasized that AmB possesses seven conjugated double bonds, which also make it likely that the drug itself would be susceptible to autoxidation resulting in an antioxidant effect in line with the carotenoids and retinoids. A more precise knowledge of the mechanism of action of AmB is therefore necessary in order to pave the way for the development of more effective fungicides with potentially reduced side-effects.

In an attempt to resolve this question, we have undertaken studies to examine the role of AmB in oxidative stress. We have chosen an *in vitro* system of SMC as a model for these studies and induced oxidative stress by generating peroxyl radicals from AMVN. These radicals are the major type of free radicals expected to be involved in membrane damage [6].

Because the level of oxidation of polyunsaturated membrane lipids required to detect changes by conventional analytical methods results in death of the cells, we have exploited a fluorescent method to monitor levels of lipid oxidation that do not perturb cell viability. The method consists of incorporating PnA biosynthetically into the constituent membrane phospholipids of SMC *in vitro*. The fatty acid has four conjugated double bonds that render it slightly more susceptible to free radical attack compared with other polyunsaturated fatty acyl residues [7, 8]. We have undertaken preliminary experiments using PnA to study the antioxidant/prooxidant activities of lipophilic compounds like α-tocopherol, β-carotene, and ubiquinol in model membrane systems [9], and, more recently, we showed that AmB inhibits oxidation of PnA both in aqueous systems and in model membranes but the effect is blocked by the formation of complexes with cholesterol [10]. The present work was designed to investigate whether such effects could be demonstrated in a whole cell system. Using our newly developed assay of site-specific peroxidation of major classes of intracellular membrane phospholipids, we attempted to determine whether AmB acts as a prooxidant or an antioxidant in live cells.

MATERIALS AND METHODS

Reagents

PnA (Z-9, E-11, E-13, Z-15-octadecatetraenoic acid) was purchased from Molecular Probes, Inc. (Eugene, OR). The purity of each lot of PnA purchased was determined by UV spectrophotometry (Shimadzu UV 160U spectrophotometer) using the molar extinction $\varepsilon_{304\ nm}$ in ethanol, 80,000 $M^{-1}cm^{-1}$. AMVN, used as a lipid-soluble radical initiator, was purchased from Polysciences, Inc. (Warrington, PA). HEPES, magnesium chloride, sodium phosphate, hSA, phospholipids, DMSO, sodium molybdate, malachite green base, BHT, Tween 20, and EDTA were purchased from the Sigma Chemical Co. (St. Louis, MO). Silica G plates (5 × 5 cm; Whatman, Clifton, NJ), NaCl, and HCl were purchased from Fisher Scientific (Pittsburgh, PA). Methanol, chloroform, hexane (HPLC grade), 2-propanol (HPLC grade), and water (HPLC grade) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). Phenol red-free DMEM, DMEM/F12, HBSS, penicillin, streptomycin, 0.25% trypsin-EDTA solution, and all tissue culture ware were purchased from Gibco Laboratories Inc. (Grand Island, NY). FBS was obtained from HyClone Laboratories Inc. (Logan, UT). All other chemicals used were of tissue culture or best grade available.

Aortic Smooth Muscle Cell Culture

Sprague–Dawley male rats (Charles River, Wilmington, MA) weighing 150–200 g were fed standard rat chow and tap water *ad lib*. SMC were cultured as explants from the ascending thoracic aortas, obtained from ether-anesthetized rats, after a midline abdominal incision including the diaphragm as described previously [11]. Briefly, the medial layer of the aorta was removed surgically under the microscope, and minced sections of this layer were suspended in primary cell culture medium (DMEM/F12 supplemented with penicillin [100 U/mL], streptomycin [100 μg/mL], $NaHCO_3$ [13 mmol/L], and HEPES [25 mmol/L]) containing 10% FBS plated in tissue-culture flasks (75 cm^2), and incubated under standard tissue-culture conditions (37°, 5% CO_2/95% air, and 98% humidity). The SMC grew as explants from the medial tissue and were confluent in 12–14 days. Confluent monolayers of SMC were dislodged by treatment with 0.25% trypsin-EDTA solution and passaged further. SMC purity was characterized by immunofluorescence staining with smooth muscle-specific anti-smooth muscle α-actin monoclonal antibodies and by morphological criteria specific for smooth muscle, as described in detail previously [12]. SMC between the second and third passages were used for cellular lipid peroxidation studies. Cell viability was assayed by the trypan blue dye exclusion test.

Incorporation of PnA into Smooth Muscle Cell Phospholipids

PnA was incorporated into SMC by incubation of cell suspensions in the presence of PnA in the form of a complex with hSA (PnA–hSA). The complex was prepared by adding PnA (500 μg, 1.8 μmol) in 25 μL of DMSO to hSA (50 mg, 760 nmol) in 1 mL of PBS containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4

and 8 mM Na_2HPO_4 (pH 7.4). Cells in log phase of growth were rinsed twice with L1210 medium containing 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM NaH_2PO_4, 10 mM glucose, and 25 mM HEPES (pH 7.4) and resuspended finally in this medium to give a cell density of 2×10^6 cells/mL. PnA–hSA complex was added to the cell suspension to give a final concentration of 5 μg/mL PnA, and the cells were incubated for 2 hr at 37° in the dark under aerobic conditions to allow incorporation of PnA into phospholipid. At the end of the incubation period, the cells were washed twice with L1210 medium with or without medium containing hSA (0.5 mg/mL) to remove unincorporated PnA. Aliquots were taken to assess cell viability using the trypan blue dye exclusion test.

AMVN-Induced Lipid Peroxidation

SMC (2×10^6 cells) preloaded with PnA and suspended in L1210 medium were incubated for 2 hr in the presence or absence of AMVN at 37° under aerobic conditions (pO_2 150 mm Hg) in the dark. In experiments to study the effects of AmB, the different concentrations of AmB (0.25 to 10 μM) were added to the cell suspension 10 min before the addition of AMVN. After incubation, aliquots of cell suspension were taken for determination of both cell viability and lipid analysis.

Extraction of Cell Lipids

Total lipids were extracted from SMC (10^6 cells in 1 mL) using a slightly modified Folch procedure [13]. Briefly, methanol (2 mL) containing BHT (0.1 mg) was added to the cell suspension and mixed. Then chloroform (4 mL) was added, and the mixture was stored for 1 hr at 0° under a nitrogen atmosphere in the dark to ensure complete extraction. After the addition of 0.1 M NaCl (1 mL) and vortex mixing (still in a nitrogen atmosphere), the chloroform layer was separated by centrifugation (1500 g, 5 min). The lower chloroform layer was evaporated with a stream of nitrogen, and the lipid extract was dissolved in 0.2 mL of 2-propanol:hexane:water (4:3:0.16, by vol.). Control experiments confirmed that more than 95% of cell phospholipids were extracted by this procedure.

HPTLC Analysis of Cell Lipids

The phospholipid classes in the extracts were separated by two-dimensional HPTLC on silica G plates (5 × 5 cm, Whatman). The plates were first developed with a solvent system consisting of chloroform:methanol:28% ammonium hydroxide (65:25:5, by vol.). After drying the plate with a forced air blower to remove the solvent, the plates were developed in the second dimension with a solvent system consisting of chloroform:acetone:methanol:glacial acetic acid:water (50:20:10:10:5, by vol.). The phospholipids were visualized by exposure to iodine vapor and identified by comparison with migration of authentic phospholipid standards. The spots identified by iodine staining were scraped off the plates and transferred to tubes. Lipid phosphorus was determined as described in Ref. 14. The identity of each phospholipid was established by comparison with the R_f values measured for authentic standards.

HPLC Analysis of Cell Lipids

Lipid extracts were separated by HPLC using an ammonium acetate gradient essentially as described by Geurts van Kessel *et al.* [15] and modified by our laboratory [16]. The lipid extracts were applied to a 5-mm Supelcosil LC-Si column (4.6 × 250 mm) equilibrated with a mixture of 1 part solvent A [2-propanol:hexane:water (57:43:1, by vol.)] and 9 parts solvent B [2-propanol:hexane:40 mM aqueous ammonium acetate (57:43:10), pH 6.7]. The column was eluted during the first 3 min with a linear gradient from 10% solvent B to 37% solvent B, then 3–15 min isocratic at 37% solvent B, 15–23 min linear gradient to 100% solvent B, 23–45 min isocratic at 100% solvent B; the solvent flow rate was maintained at 1 mL/min. The separations were performed using a Shimadzu HPLC (model LC-600, Kyoto, Japan) equipped with an in-line configuration of fluorescence (model RF-551) and UV-VIS (model SPD-10AV) detectors. The effluent was monitored by absorbance at 205 nm to detect lipids and fluorescence of PnA by emission at 420 nm after excitation at 324 nm. UV and fluorescence data were processed and stored in digital form with Shimadzu EZChom software. The identity of phospholipids in the chromatogram was established by collecting each of the peak fractions and subjecting them to HPTLC analysis as described above.

Determination of Lipid Phosphorus in Lipid Extracts

Lipid phosphorus in lipid extracts was determined using a modification of the method described by Chalvardjian and Rubnicki [17]. Aliquots of lipid extract were pipetted into test tubes, and the solvent was evaporated to dryness under a stream of oxygen-free dry nitrogen. Then 50 μL of 70% perchloric acid was added to samples, and mixtures were incubated for 20 min at 170–180° in heating elements. After the tubes were cooled, 0.4 mL of distilled water was added to each tube followed by 2 mL of sodium molybdate malachite green reagent [4.2% sodium molybdate in 5.0 N HCl:0.2% malachite green (1:3)]. Without delay, 80 μL of 1.5% Tween 20 was added, and the tubes were shaken immediately to stabilize the developed color. The color was measured at 660 nm using a Shimadzu UV 160U spectrophotometer. Triplicate determinations were made on each sample.

Statistical Evaluation

Data are expressed as means ± SEM. Changes in variables for different incubations were analyzed by one-way analysis of variance and covariance (ANOVA). If changes between

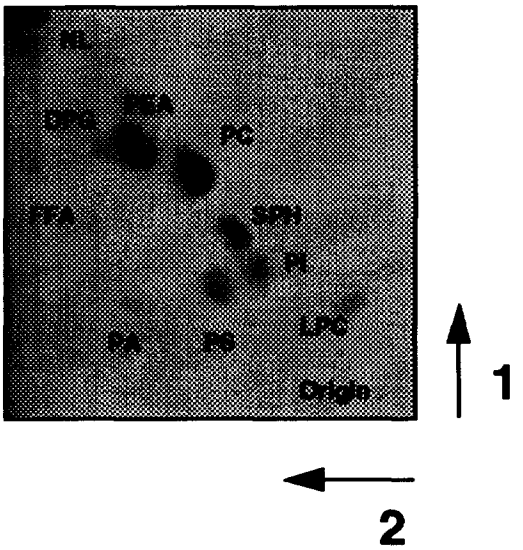

FIG. 2. A typical HPTLC two-dimensional chromatogram of a total lipid extract from SMC stained by exposure to iodine vapor. The identities of the lipids are: FFA, free fatty acids; NL, neutral lipids; DPG, diphosphatidylglycerol; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin, PA, phosphatidic acid; and LPC, lysophosphatidylcholine.

groups were substantial, unpaired Student's *t*-tests were performed. Differences among means were considered significant when the value was $P < 0.05$.

RESULTS

Effect of AMVN on the Phospholipid Composition in Smooth Muscle Cells

To determine the role of AmB in the oxidant status of SMC, it was necessary to develop a sensitive assay of oxidative stress that did not result in loss of viability of the cells yet was sufficiently sensitive to detect oxidative damage. Since the target site of AmB is believed to be confined to the cell membranes, an index of oxidative stress in membranes was required. Initial studies were undertaken to attempt to use the loss of polyunsaturated membrane lipids as a measure of an oxidative stress that was lower than that observed to kill the cells. Figure 2 shows a typical HPTLC separation of a total polar lipid extract of SMC. PC represents about half of the total phospholipids, with PEA the next most prominent phospholipid.

Incubation of SMC with AmB (in the concentration range 0.25 to 10 μM, 2 hr at 37°) did not cause any changes in the phospholipid composition of cell membranes as detected by HPTLC (data not shown). The patterns of distribution of polar lipids detected in control cells and in cells incubated in the presence of AMVN (under conditions where full viability of the cells was preserved according to vital staining methods) were similar (Table 1). There was a 2-fold increase in content of LPC. However, this is a relatively minor phospholipid in AMVN-treated cells. Since lipid peroxidation is known to activate phospholipase

TABLE 1. Effect of AMVN on the phospholipid composition of smooth muscle cells

	% of Total Phospholipids	
Phospholipid	Control	AMVN
PC	44.1 ± 2.5	43.9 ± 2.8
PEA	27.5 ± 1.6	27.1 ± 1.5
PI	8.7 ± 0.6	8.9 ± 0.5
PS	9.7 ± 0.7	8.4 ± 0.8
SPH	7.3 ± 0.5	8.5 ± 0.8
DPG	1.7 ± 0.2	1.3 ± 0.3
LPC	0.5 ± 0.2	1.1 ± 0.3
PA	0.5 ± 0.1	0.8 ± 0.2

All values are mean percent of total phospholipids ± SEM (N = 5). Cells were incubated with 0.5 mM AMVN for 2 hr at 37° in L1210 medium.

A_2-catalyzed phospholipid hydrolysis [18, 19], AMVN-induced accumulation of LPC in cells could be caused by this mechanism.

Incorporation of PnA into Smooth Muscle Cells

A more sensitive method to detect oxidative stress in SMC was developed, which consisted of metabolically incorporating PnA into the constituent phospholipids and monitoring oxidative processes by fluorescence techniques [16]. SMC were incubated in the presence of PnA–hSA for up to 2 hr to incorporate PnA into cellular phospholipids. Preliminary study showed that the maximal incorporation of PnA into all detected phospholipid classes was reached within 2 hr of incubation. The incorporation of PnA in the various phospholipids was differential, and the amount of PnA incorporated was in the following order: PC > PEA > PS > SPH > PI. The constituent phospholipids were separated from the total lipid extract by HPLC. A typical fluorescence emission profile in the column eluate is shown in Fig. 3. Major fluorescence peaks were identified using authentic phospholipid standards including PI, PEA, PS and PC; trace amounts of SPH could also be detected. Control incubations of cells with hSA alone showed no fluorescent HPLC components, under the excitation and emission limits used.

Susceptibility of PnA-Labeled Phospholipids to AmB

To determine whether AmB caused any change in fluorescence of PnA biosynthetically incorporated into phospholipids of SMC membranes, washed cell suspensions were incubated aerobically in the dark for 2 hr at 37° in the absence and presence of 0.25 to 10 μM AmB, and changes in fluorescence emission intensity of individual phospholipid classes at the end of the incubation were determined by HPLC. The results are presented in Table 2 and show that there were no significant changes in PnA fluorescence, indicating that AmB does not by itself cause lipid peroxidation in viable cells. Cell viability was also shown to be unaltered, as evidenced by trypan blue exclusion during

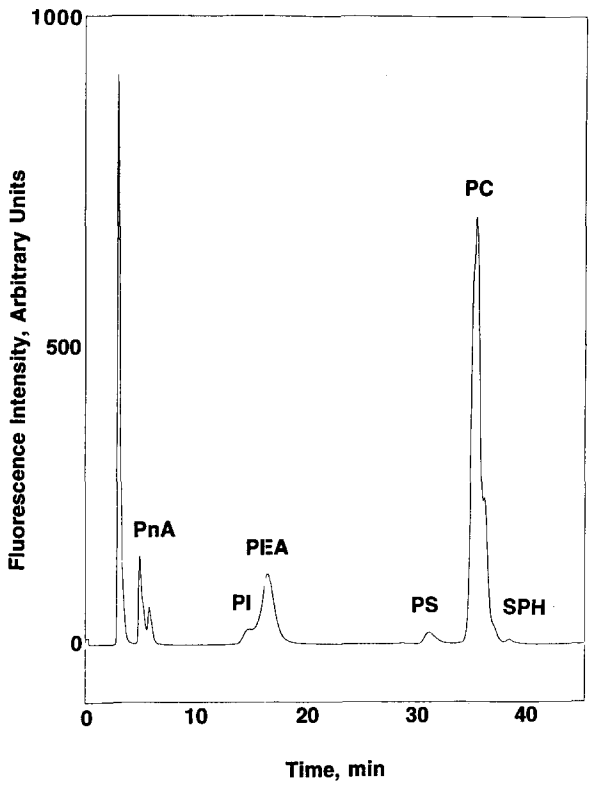

FIG. 3. A typical HPLC tracing of total lipids extracted from SMC: Fluorescence emission intensity profile, excitation at 324 nm, emission at 420 nm. Cells were incubated with the PnA–hSA complex (5 μg of PnA/0.5 mg hSA/mL of L1210 medium) in the dark for 2 hr at 37° and then were washed twice with hSA (0.5 mg/mL of L1210 medium) and L1210 medium. Lipids were extracted and resolved by HPLC as described in Materials and Methods. Abbreviations: PnA, *cis*-parinaric acid; PI, phosphatidylinositol; PEA, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; and SPH, sphingomyelin.

incubation of the cells in the presence of up to 10 μM AmB.

Protective Effect of AmB against AMVN-Induced Peroxidation

To determine the effects of AmB on cells subjected to oxidative stress, SMC suspensions labeled with PnA were incubated with or without AmB (0.25 to 10 μM) in the dark at 37° in the presence of 0.5 mM AMVN. Based on the known rate constant of AMVN decomposition under the experimental conditions employed [6], at 0.5 mM it can be estimated that 2.4 nmol of peroxyl radicals are generated per hr at 37° in each incubation containing 10^6 cells. Incubation of SMC with AMVN for 2 hr induced significant oxidation of all the detected phospholipids (Fig. 4). Trypan blue exclusion from cells after incubation showed that AmB at concentrations from 0.25 to 10 μM did not result in significant loss of cell viability, while at higher concentrations (50 μM) it was cytotoxic (Fig. 5). The effect of AmB on the oxidation of PnA-labeled phospholipids provoked by AMVN is presented in Fig. 4. It is clear that the presence of 1–10 μM AmB significantly reduced the loss of all PnA-labeled phospholipid from oxidation ($P < 0.05$ or 0.01), except for SPH. Furthermore, the protective effect was concentration dependent. However, in no instance was there complete protection from AMVN-induced oxidation. Based on our results on phospholipid fractional composition in SMC (Table 1), the specific rates of PnA peroxidation in phospholipid classes can be estimated (Table 3). In SMC, the order of AMVN-induced oxidation effectiveness was the same for both specific rates and relative rates: PC > PEA > PS > PI > SPH. A decrease in both relative and specific oxidation rates by AmB was observed for all phospholipid classes.

DISCUSSION

In the present study, we attempted to evaluate the pro-/antioxidant effects of AmB using a sensitive assay of oxidative stress in a cell culture system. The major finding of this study is that not only did AmB not enhance oxidation of PnA but it also caused an inhibitory effect when a lipophilic azo-initiator of radicals, AMVN, was used as an inducer of lipid peroxidation. Since AMVN did not cause changes in SMC viability, the observed protection of PnA-labeled phospholipids against peroxidation was characteristic of live cells. Hence, the protective effect of AmB against AMVN-induced phospholipid peroxidation implies that AmB acts as an intracellular membrane antioxidant.

A number of studies have implicated oxidative processes in the metabolism of therapeutic drugs to induce toxic

TABLE 2. Incorporation of PnA into membrane phospholipid of smooth muscle cells

Phospholipid	Relative concentration (ng PnA/μg of total P_i)		Specific concentration (ng PnA/μg P_i in phospholipid fraction)	
	Control	AmB (1 μM)	Control	AmB (1 μM)
PI	2.71 ± 0.30	2.13 ± 0.50	31.6 ± 3.6	24.8 ± 5.8
PEA	18.0 ± 1.41	18.1 ± 0.8	64.9 ± 4.5	65.3 ± 14.8
PS	6.51 ± 0.53	5.02 ± 1.8	67.4 ± 5.0	52.0 ± 18.6
PC	114.6 ± 16.1	105.5 ± 19.0	261.1 ± 36.7	240.4 ± 18.0
SPH	0.7 ± 0.1	0.7 ± 0.2	9.6 ± 1.2	9.5 ± 2.7

All values are means ± SEM (N = 5). The cells were incubated with PnA–hSA complex for 2 hr at 37° in L1210 medium.

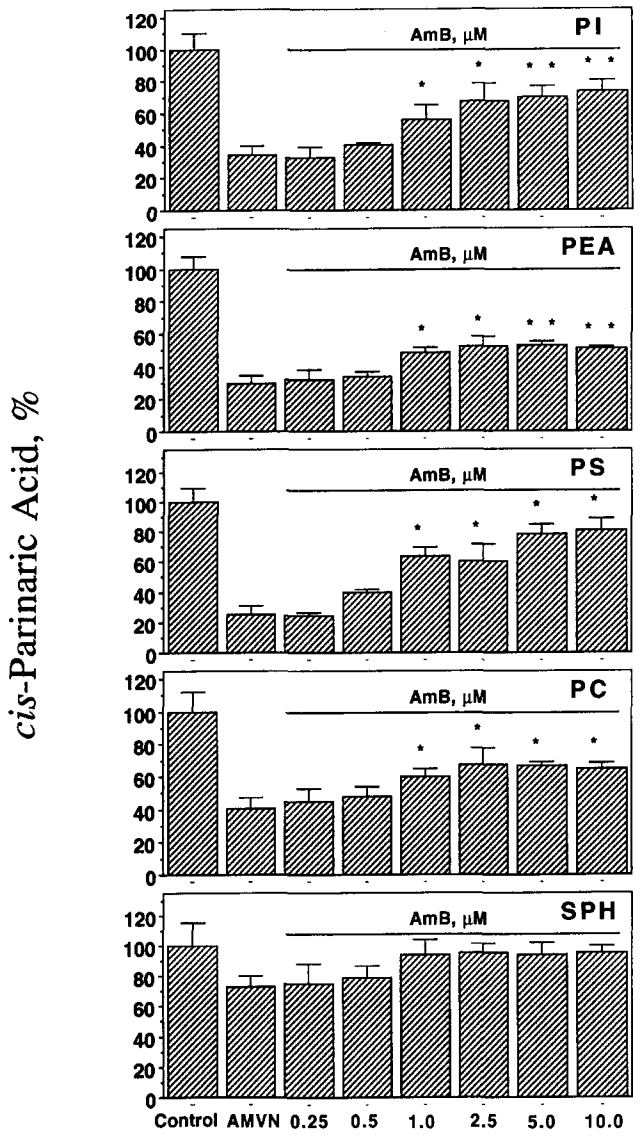

FIG. 4. Effect of AmB on fluorescently labeled phospholipids of smooth muscle cells. Cells prelabeled with PnA were incubated under aerobic conditions in the dark for 2 hr at 37° in the presence of 0, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 μM AmB. Individual components of total polar lipid extracts were separated by HPLC, and fluorescence intensity of each of the major phospholipid peaks is plotted as a function of AmB concentration. Abbreviations are defined in the legend of Fig. 2. The protective effect of AmB against AMVN-induced phospholipid peroxidation was significant at the following levels: * $P < 0.05$, or ** $P < 0.01$. All values are means ± SEM (N = 5).

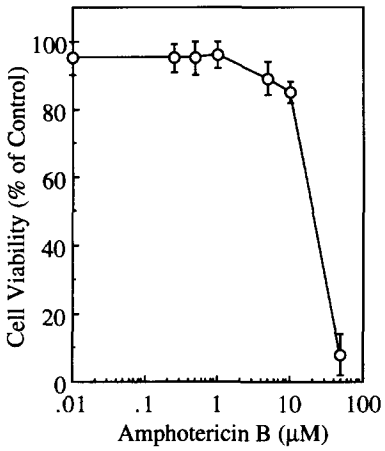

FIG. 5. Effect of AmB on viability of smooth muscle cells. Cells (1×10^6/mL) were incubated under aerobic conditions in the dark for 2 hr at 37° in the presence of 0, 0.25, 0.5, 1.0, 5.0, 10, and 50 μM AmB. All values are means ± SEM (N = 5).

side-effects including autoxidation of AmB [3, 20]. In the case of AmB, indirect evidence elicited to support the idea that oxidative processes are responsible for the observed cytotoxicity of the drug is that the effects can be counteracted by antioxidant enzymes or antioxidant compounds. Nevertheless, despite reports that oxidative damage is said to be associated with AmB-induced hemolysis of erythrocytes [3], other workers found no evidence of any oxidative stress associated with AmB-induced injury to endothelial cells [21].

The present results are more consistent with the latter findings. In our system a lipid-soluble source of peroxyl radicals was produced by the azo-initiator AMVN. This reagent generates peroxyl radicals at a constant rate at a given temperature independently of intracellular metabolism. Because of its hydrophobic character, AMVN partitions into the acyl lipid region of membranes and generates radicals initially within this domain [6]. Radical species generated in this way do not escape from the hydrophobic lipid environment [22]. In a recent study of the AmB/phospholipid (dipalmitoylphosphatidylcholine) complex structure, it has been demonstrated that the conjugated heptaene stretch of AmB interacts with the methylene groups of lipid acyl chains, while the sugar moiety interacts with the phosphate head group by the formation of a hydrogen bond [23]. This topography of AmB in lipid bilayer is reminiscent of that of vitamin E (α-tocopherol) whose phenolic hydroxyl group is located at the lipid/water interface where it interacts with the polar region of phospholipid molecules (likely hydrogen-bonded to carbonyl or phosphate groups) and its hydrophobic side chain interacts with phospholipid acyl chains [24]. Such orientation of α-tocopherol is critical for its function as a major lipid-soluble antioxidant of membranes [25]. Vitamin E is known to effectively scavenge AMVN-derived peroxyl radicals and protect membranes against AMVN-induced lipid peroxidation [6]. Since our goal was to determine antioxidant effects of AmB in live cells, we chose to use AMVN as a source of peroxyl radicals capable of generating peroxyl radicals and inducing measurable peroxidation of PnA-labeled phospholipids in membranes without causing significant cell death. Peroxyl radicals derived from water-soluble azo-initiators [e.g. 2,2' - azobis(2 - aminodinopropane)dihydrochloride (AAPH)] would, most likely, attack the cell plasma membrane from extracellular environments, resulting in cell death and the oxidation of the membrane phospholipids by completely unphysiological conditions. Another feature of oxidation of

TABLE 3. Rates of AMVN-induced PnA oxidation in membrane phospholipids of smooth muscle cells

Phospholipid	Amphotericin B (μM)						
	0	0.25	0.5	1.0	2.5	5.0	10.0
	Relative rate (ng PnA/μg of total P_i/hr)						
PC	33.9 ± 3.8	31.5 ± 4.4	29.8 ± 3.3	22.7 ± 2.7	18.7 ± 0.2	19.2 ± 1.6	20.7 ± 2.2
PEA	6.3 ± 0.5	6.1 ± 0.6	5.9 ± 0.3	4.7 ± 0.3	4.3 ± 0.6	4.3 ± 0.2	4.4 ± 0.1
PS	2.4 ± 0.2	2.1 ± 0.2	1.9 ± 0.1	1.2 ± 0.2	1.3 ± 0.6	0.9 ± 0.5	0.6 ± 0.5
PI	0.89 ± 0.07	0.92 ± 0.01	0.80 ± 0.02	0.60 ± 0.13	0.43 ± 0.15	0.42 ± 0.09	0.36 ± 0.09
SPH	0.09 ± 0.02	0.07 ± 0.03	0.07 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.04 ± 0.02	0.03 ± 0.02
	Specific rate (ng PnA/μg P_i in phospholipid fraction/hr)						
PC	78.3 ± 7.8	72.0 ± 9.8	67.8 ± 7.7	52.2 ± 5.7	39.6 ± 11.2	43.8 ± 3.4	47.8 ± 5.0
PEA	22.3 ± 2.4	22.0 ± 2.0	21.5 ± 1.2	16.6 ± 1.0	15.3 ± 2.0	15.2 ± 0.9	15.9 ± 0.5
PS	25.2 ± 2.2	25.4 ± 0.6	19.6 ± 0.3	12.6 ± 1.7	13.0 ± 7.2	8.9 ± 4.8	6.7 ± 4.9
PI	10.4 ± 1.0	10.7 ± 1.2	9.4 ± 0.3	7.0 ± 1.2	5.1 ± 1.6	5.7 ± 0.5	4.2 ± 1.2
SPH	1.3 ± 0.4	1.0 ± 0.7	0.9 ± 0.4	0.5 ± 0.3	0.3 ± 0.2	0.4 ± 0.3	0.5 ± 0.4

All values are means ± SEM (N = 5).

phospholipids initiated by AMVN is that the reactions tend to be nonselective, so it is, in principle, possible to determine whether particular phospholipid classes are intrinsically more susceptible to oxidation than others.

ESR is the only direct method to detect, identify, and characterize free radicals; ESR-based assays are utilized routinely for measurements of different radicals in model systems. Unfortunately, relatively low sensitivity of the method makes its use in live cells very difficult, if not impossible. Since our major goal in this study was to establish pro-/antioxidant effects of AmB in live cells, we chose to use a newly developed and very sensitive methodology utilizing fluorescent labeling of phospholipids with oxidation-sensitive PnA.

Our initial experiments using conventional methods of separation and analysis of phospholipids from cells incubated in the presence of AMVN failed to reveal any significant differences in membrane phospholipid composition resulting from oxidative stress. It was of concern to ensure that the cells remained viable during exposure to the stress, and it is clear that preservation of membrane phospholipids from oxidative damage is integral to cell survival. So as to be able to detect oxidative processes with the required degree of sensitivity necessary to detect oxidative damage, a technique that has been developed to incorporate PnA into membrane phospholipids was adapted to label the SMC [16]. The method creates membrane phospholipid molecular species labeled with PnA that are markedly more susceptible to oxidation than are molecular species normally found as constituents of the membranes of these cells [7, 8]. Moreover, the oxidation of these PnA-labeled phospholipids can be monitored with a high degree of precision by measuring changes in fluorescence.

In a model system consisting of PnA interpolated into phospholipid liposomes, AmB acted as an antioxidant by scavenging peroxyl radicals generated by AMVN [10]. This mode of action is entirely consistent with its action in SMC and relies on preferential reaction of the molecule with the peroxyl radicals in competition with the parinaryl-labeled phospholipids. In our model experiments with liposomes, we found that cholesterol decreased the antioxidant effect of AmB against AMVN-induced peroxidation of PnA at high cholesterol:AmB ratios (by 30–40% at 10:1) [10]. Cholesterol concentrations in SMC may be as high as 15–20 μg/mg protein [26, 27], providing for cholesterol concentrations up to 15 μM in our incubation systems. Since AmB concentrations used were in the range of 0.25 to 10 μM, its interactions with cholesterol might diminish its antioxidant effects, at least at low AmB concentrations. Indeed, AmB did not yield antioxidant protection at 0.25 μM.

The antioxidant mechanism of AmB's contrasts with the mechanism of action of the lipid-soluble phenolic chain-breaking antioxidants like ubiquinol and probucol [9] or α-tocopherol [28]. β-Carotene is believed to act by a mechanism similar to that proposed for the antioxidant action of AmB [28, 29], and these molecules share extensive conjugated double bond structures [30]. Thus, when β-carotene reacts with phenoxyl radicals, it competes with the allylic hydrogens of polyunsaturated membrane lipids to form a carotenyl radical [31, 32]. The antioxidant action of β-carotene radical, in turn, depends on the concentration of dissolved oxygen [33]. Under hypoxic conditions the carotenyl radical acts as a chain terminator, but in high oxygen concentrations it reacts with oxygen to form a chain-propagating species, a β-carotene peroxyl radical that initiates further oxidations [34, 35]. The concentration of oxygen in the SMC suspension during the oxidative stress presumably is low because the cells are oxidatively metabolizing substrates so that the action of AmB is likely to be as a chain-breaking antioxidant.

It is noteworthy that high concentrations of AmB are reported to undergo autoxidation and to generate free radicals detectable by spin traps [36] despite earlier studies that had failed to detect such radicals [37]. The concentra-

tion-dependent protective effect of AmB against oxidative stress observed in the SMC system suggests that if AmB radicals do form, they are not effective in propagating PnA-labeled membrane lipid peroxidation. In conclusion, the results of this study extend our previous data on antioxidant effects of AmB in simple model systems in that they directly demonstrate that AmB acts as an intracellular antioxidant in live cells.

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