Abstract — The goal of this study was to test the hypothesis that antioxidant estrogens, by a mechanism independent of the estrogen receptor, protect phospholipids residing in the plasma membrane of vascular smooth muscle cells from peroxidation and peroxidation-induced cell growth and migration. Peroxidation of membrane phospholipids was assessed by HPLC analysis of phospholipids extracted from rat aortic vascular smooth muscle cells prelabeled with cis-parinaric acid (a fatty acid that is susceptible to peroxidation, which quenches its fluorescent properties). Incubation of cells for 2 hours with the peroxyl radical donor 2,2'-azobis-2,4-dimethylvaleronitrile (AMVN) caused peroxidation of all measured membrane phospholipids. This effect was attenuated by pretreating cells for 15 minutes with 50 to 5000 ng/mL of 2-hydroxyestradiol (strong antioxidant but weak estrogen-receptor ligand) or 4-hydroxytamoxifen (strong antioxidant and potent estrogen-receptor ligand), but not by estrone or droloxifene (both weak antioxidants but potent estrogen-receptor ligands). Moreover, pretreatment of cells for 20 hours with physiological concentrations (0.3 ng/mL) of 2-hydroxyestradiol or pharmacologically relevant concentrations of 4-hydroxytamoxifen (40 ng/mL) also decreased AMVN-induced phospholipid peroxidation. Both 2-hydroxyestradiol and 4-hydroxytamoxifen were as effective as 2,2,5,7,8-pentamethyl-6-hydrochromane (an antioxidant homolog of vitamin E) in attenuating AMVN-induced peroxidation of membrane phospholipids. Also, physiological concentrations of 2-hydroxyestradiol, but not estrone, and pharmacologically relevant concentrations of 4-hydroxytamoxifen attenuated AMVN-induced DNA synthesis, cell proliferation, and cell migration. These studies demonstrate in vascular smooth muscle cells that antioxidant estrogens via a non-estrogen receptor–dependent mechanism attenuate peroxidation of membrane phospholipids and peroxidation-induced cell growth and migration. (Circ Res. 1999;84:229-239.)

Key Words: estrogen ■ metabolism ■ antioxidant ■ cardiovascular disease ■ free radical
ling cell migration and proliferation.\textsuperscript{20,21} processes critical to vascular neointimal formation. Moreover, because estrogens are lipophilic, high local concentrations of these compounds would exist in the biophase of the lipid bilayer during hormone replacement therapy. Thus, the hypothesis that estrogens may attenuate oxidation of membrane components has a strong a priori rationale. Accordingly, the main objective of the present study was to test the hypothesis that antioxidant estrogens, by a mechanism independent of the estrogen receptor, protect phospholipids residing in the plasma membrane of vascular smooth muscle cells from free radical–induced peroxidation and peroxidation-induced cell growth and migration.

Materials and Methods

\textit{cis}-Parinaric acid (Z-9,E-11,E-13,Z-15-octadecatetraenoic acid) was purchased from Molecular Probes, Inc. The purity of each batch of \textit{cis}-parinaric acid used was determined by UV spectrophotometry (Shimadzu UV 160U spectrophotometer) using a molar extinction at 304 nm in ethanol of $8 \times 10^4$ (mol/L)$^{-1}$ cm$^{-1}$. 2,2'-Azobis (2,4-dimethylvaleronitrile) (AMVN) was procured from Polysciences, Inc. 2,2'-Azobis (2-aminodipropionate) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. Fatty acid-free human serum albumin (hSA), phospholipids, 5,5'-dithiobis (2-nitrobenzoic acid), estrone, glutathione, glutathione-peroxidase, cumene hydroperoxide, 2-mercaptoethanol, and 2-mercaptoethylamine (cysteamine) hydrochloride. Tween 20, sodium molybdate, malachite green base, nitrobenzoic acid, estrone, glutathione, glutathione-peroxidase, cumene hydroperoxide, malachite green base, butylated hydroxytoluene, and 3-[4,5-dimethylthiozol-2-yl]-2,5-di-phenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. Methanol, chloroform, hexane, 2-propanol, and water were purchased from Aldrich Chemical Co. Deferoxamine mesylate was purchased from Ciba. 2,2,5,7,8-Pentamethyl-6-hydrochromane (PMC) was a generous gift from Eisai Co. 4-Hydroxytamoxifen and droloxifene were purchased from Ciba. 2,2,5,7,8-Pentamethyl-6-hydrochromane (PMC) or cysteamine were added to the incubation medium 15 minutes after a midline abdominal incision including the diaphragm and as described previously.\textsuperscript{24} Smooth muscle cell purity was characterized by immunofluorescence staining with specific anti–smooth muscle \textalpha-actin monoclonal antibodies and by morphological criteria specific for smooth muscle as described in detail previously.\textsuperscript{25} Smooth muscle cells between the second and third passages were used for the cellular lipid peroxidation and thiol (GSH) oxidation studies.

General Experimental Approach

To test the hypothesis that antioxidant estrogens inhibit oxidation of membrane structures, it was necessary to measure free radical–induced oxidation of membrane lipids in intact vascular smooth muscle cells. In this regard, we used the novel strategy of monitoring fluorescence intensity of membrane-incorporated \textit{cis}-parinaric acid, a naturally occurring 18-carbon fatty acid that is highly susceptible to free radical–induced peroxidation, which markedly quenches its fluorescence.\textsuperscript{22} Because \textit{cis}-parinaric acid fluorescence is lost permanently on oxidation of the conjugated double-bond system, repair mechanisms, which are normally present in live cells, cannot mask the oxidative events. This is true even if \textit{cis}-parinaric acid is oxidized and replaced with unoxidized fatty acids, since the evidence for oxidation remains as loss of \textit{cis}-parinaric acid fluorescence. This characteristic is not found in assays currently used in cultured cells and animal models. Furthermore, use of \textit{cis}-parinaric acid also enables the localization of the effects of oxidative stress on different classes of phospholipids. Peroxyl radicals were generated by the addition of AMVN,\textsuperscript{23} and fluorescence intensity of the reporter molecule, \textit{cis}-parinaric acid, was monitored in several membrane phospholipid components in the absence and presence of 2-hydroxyestradiol, 4-hydroxytamoxifen, estrone, and droloxifene. These particular estrogens were selected because 2-hydroxyestradiol is a strong antioxidant but different in their potency as estrogen-receptor ligands (4-hydroxytamoxifen $>$ 2-hydroxyestradiol), whereas estrone and droloxifene are weak antioxidants but potent estrogen-receptor ligands.\textsuperscript{6,8,11} We anticipated that using a diverse set of estrogens with different antioxidant and estrogen-receptor ligand affinities would allow inferences regarding whether a given effect was dependent or independent of estrogen receptors. Moreover, we used PMC, a potent antioxidant, as a reference benchmark for evaluating the antioxidant potency of the estrogens tested in this study. We also investigated the selected estrogens with regard to their ability to attenuate peroxyl radical–induced changes in endogenous thiols (reduced glutathione [GSH] and protein sulhydryls) in vascular smooth muscle cells, using in this case AAPH\textsuperscript{20} as the oxidant stress. The addition of these experiments allowed us to contrast the antioxidant effects of estrogens at 2 important targets for oxidative changes. Finally, to evaluate the physiological relevance of the antioxidant effects of estrogens, we conducted cell proliferation, \textsuperscript{[3]}H]thymidine incorporation, and cell migration studies to determine whether peroxyl radicals induce smooth muscle cell growth and migration and whether the selected estrogens inhibit free radical–induced DNA synthesis, cell proliferation, and cell migration.

Aortic Smooth Muscle Cell Culture

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 150 to 200 g were kept in standard rat chow with tap water ad libitum. Aortic smooth muscle cells were cultured as explants from the lower abdominal aortas that were obtained from ether-anesthetized rats after a midline abdominal incision including the diaphragm and as described previously.\textsuperscript{24} Smooth muscle cell purity was characterized by immunofluorescence staining with specific anti–smooth muscle \textalpha-actin monoclonal antibodies and by morphological criteria specific for smooth muscle as described in detail previously.\textsuperscript{25} Smooth muscle cells between the second and third passages were used for the cellular lipid peroxidation and thiol (GSH) oxidation studies.

Incorporation of \textit{cis}-Parinaric Acid Into Smooth Muscle Cell Phospholipids

\textit{cis}-Parinaric acid was incorporated into smooth muscle cells by addition of \textit{cis}-parinaric acid complexed with hSA (\textit{cis}-parinaric acid (500 $\mu$g, 1.8 $\mu$mol) in 25 $\mu$L of DMSO and hSA (50 mg, 760 nmol) in 1 mL of PBS (in mmol/L: 137 NaCl, 2.7 KCl, 1.5 KH$_2$PO$_4$, and 8 NaHPO$_4$, pH 7.4)) to the cell suspensions. Briefly, confluent monolayers of smooth muscle cell were dislodged by trypsinization, washed once with DMEM containing 10% FCS, and washed subsequently with DMEM alone. Before the experiments, the smooth muscle cells were rinsed twice with L1210 buffer containing (in mmol/L) 115 NaCl, 5 KCl, 1 MgCl$_2$, 5 NaHPO$_4$, 10 glucose, and 25 HEPES (pH 7.4). Cells were diluted to a density of 1.0 $\times$ 10$^6$ cells/mL and then incubated with \textit{cis}-parinaric acid-hSA complex (final concentration of 5 $\mu$g/mL \textit{cis}-parinaric acid) in L1210 buffer at 37$^\circ$C in the dark and under air. The smooth muscle cells were incubated for different time periods (30, 60, 120, and 180 minutes), and the time course of \textit{cis}-parinaric acid incorporation into cellular phospholipids was evaluated after washing the cells twice with L1210 buffer with and without hSA (0.5 mg/mL). Trypan blue exclusion tests for cell viability were conducted in parallel by taking aliquots of smooth muscle cells treated for different time periods.

AMVN-Induced Peroxidation of Membrane Lipids

AMVN, a lipid-soluble azo- compound that is a thermally activated initiator of peroxyl radicals, was used to induce lipid peroxidation. Because of its hydrophobic character, AMVN partitions into the acyl lipid region of membranes and generates radicals\textsuperscript{29} that do not escape from the hydrophobic lipid environment.\textsuperscript{23} Moreover, oxidation of phospholipids by AMVN tends to be nonselective, so in principle it is possible to determine whether particular phospholipid classes are intrinsically more susceptible to oxidation than others. Smooth muscle cells were incubated for 2 hours with \textit{cis}-parinaric acid, and \textit{cis}-parinaric acid–labeled cells were subsequently incubated in the presence of AMVN (500 $\mu$mol/L) in L1210 buffer at 37$^\circ$C in the dark in air for 2 hours. Appropriate amounts of phenolic compounds (4-hydroxytamoxifen, droloxifene, estrone, 2-hydroxyestradiol, or PMC) or cysteamine were added to the incubation medium 15
minutes before addition of AMVN. After incubation for 2 hours, aliquots of cell suspension were taken for both determination of cell viability and lipid analysis.

To evaluate whether long-term treatment of cells with 4-hydroxymuconic, estrone, 2-hydroxystilbestradiol, and PMC prevents AMVN-induced peroxidation of smooth muscle cells, trypsinized cells were suspended in DMEM containing 0.4% albumin and treated for 20 hours with or without 0.3 to 30 ng/mL of the above agents. Following incubation the smooth muscle cells were loaded with cis-parinaric acid (as described above) and subsequently incubated with AMVN (250 μmol/L) for 3 hours. After incubation, aliquots of cell suspension were taken for both determination of cell viability and lipid analysis.

**Extraction of Cell Lipids**

Total lipids were extracted by using a procedure adapted from that of Folch et al. Briefly, methanol (2 mL) containing butyralized hydroxytoluene (0.1 mg) was added to the cell suspension (1×10^6 cells). The suspension was mixed with chloroform (4 mL) and kept for 1 hour under a nitrogen atmosphere on ice in the dark to ensure complete extraction. After addition of 0.1 mol/L NaCl (1 mL) and vortex mixing (still under a nitrogen atmosphere), the chloroform layer was separated by centrifugation (1500g, 5 minutes). The chloroform was evaporated with a stream of nitrogen, and the lipid extract was dissolved in 4:3:0.16 (vol/vol) 2-propanol–hexane–water (0.2 mL). Control experiments demonstrated that the procedure recovered >95% of cell phospholipids.

**HPLC Analysis of Cell Lipids**

Lipid extracts were separated with an ammonium acetate gradient using normal-phase HPLC by slight modification of the method of Geerts van Kessel et al. Briefly, a 5-μm Supelcosil LC-Si column (4.6×250 mm) was used with the following mobile phase flowing at 1 mL/min: solvent A (2-propanol–hexane–water 57:43:1 vol/vol), solvent B (2-propanol–hexane–40 mmol/L aqueous ammonium acetate, pH 6.7), 0- to 3-minute linear gradient from 10% solvent B to 37% solvent B, 3- to 15-minute isocratic elution at 37% solvent B, 15- to 23-minute linear gradient to 100% solvent B, and 23- to 45-minute isocratic elution at 100% solvent B. A Shimadzu high-performance liquid chromatograph (model LC-600) equipped with an in-line configuration of fluorescence (model RF-551) and absorbance detectors was used. The effluent was monitored at 205 nm to gauge separation of lipids. Fluorescence emitted by cis-parinaric acid in eluates was monitored fluorometrically at 420-nm emission and 324-nm excitation, and the data were processed with Shimadzu EZChrom software.

**Determination of Lipid Phosphorus in Lipid Extracts**

Lipid phosphorus in lipid extracts was estimated spectrophotometrically at 660 nm as described by Chalvardjian and Rubnicky. Aliquots of lipid extract were pipetted into test tubes, and the solvent was evaporated to dryness under a stream of nitrogen. Perchloric acid (50 μL) was then added to samples, and the mixtures were incubated for 20 minutes at 170°C to 180°C. 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 5.0N hydrochloric acid/0.2% malachite green, 1:3 vol/vol). Without delay, 80 μL of 1.5% Tween 20 was added, mixed immediately to stabilize the color, and read spectrophotometrically at 660 nm.

**High-Performance Thin-Layer Chromatograph Analysis of Cell Lipids**

Different classes of phospholipid extracted from smooth muscle cells were separated by 2-dimensional high-performance thin-layer chromatography 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, vol/vol). After drying, the plates were developed in the second dimension with a mixture of chloroform, acetone, methanol, glacial acetic acid, and water (5:20:10:10.5, vol/vol). The phospholipids were then visualized by exposing the plates to iodine vapor and the spots identified by comparison with migration of authentic phospholipid standards. The spots identified by iodine staining were scraped off the plates, and the silicic acid was transferred to tubes. Lipid phosphorus in individual phospholipid spots of the HPLC plates were extracted and quantified spectrophotometrically with the method of Aruini et al. The identity of each phospholipid was established by comparison with the RI values measured for authentic standards.

**AAPH-Induced Oxidation of Intracellular Thiols and Glutathione**

AMVN-derived peroxyl radicals do not escape the lipid environment of the membrane; hence, AMVN is not a good agent with which to study the effects of peroxyl radicals on intracellular glutathione oxidation. Therefore, we used AAPH, an analog of AMVN that is a water-soluble azo-initiator. Smooth muscle cells were incubated with AAPH (20 mmol/L in L1210 buffer, pH 7.4) for 2 hours at 37°C in the absence or in the presence of different concentrations of the phenolic compounds (4-hydroxymuconic, droloxifene, estrone, 2-hydroxystilbestradiol, or PMC). The reaction was terminated by cooling the samples to 4°C. In parallel to the above vascular smooth muscle cell experiments, we also investigated the direct effects of the phenolic compounds on the oxidation of pure GSH. Briefly, glutathione (50 μmol/L) was incubated for 10 minutes in phosphate buffer (50 mmol/L), pH 7.4, both in the absence and in the presence of phenolic compound, and the levels of glutathione were assayed.

**Determination of Glutathione Concentration in Smooth Muscle Cells**

Glutathione concentration in smooth muscle cells was determined by estimating the difference in 5,5′-dithiobis(2-nitrobenzoic acid)-titratable thiols in the presence and in the absence of glutathione peroxidase and cumene hydroperoxide. Smooth muscle cells (5×10^5 cells) were sonicated and then incubated with glutathione peroxidase (1.94 U/μL), cumene hydroperoxide (333 μmol/L), and deferoxamine mesylate (100 μmol/L) for 30 minutes at 25°C. An aliquot of cell lysate was then added to 200 μmol/L 5,5′-dithiobis(2-nitrobenzoic acid), and the precipitated protein was separated by centrifugation for 5 minutes, 10 000g at 4°C. The absorbance of supernatant was determined spectrophotometrically at 412 nm.

**Growth Studies**

[3H]Thymidine incorporation, cell number, and cell migration studies were conducted to investigate the effects of agents on AMVN-induced DNA synthesis, cell proliferation, and cell migration. Smooth muscle cells were plated at a density of 4×10^4 cells/well in 24-well tissue-culture dishes and allowed to grow to subconfluence in DMEM/F12 (phenol red–free) medium containing 10% FCS (steroid free and delipidated) under standard tissue culture conditions. The cells were then growth arrested by feeding with DMEM (phenol red free) containing 0.4% albumin for 48 hours. For DNA synthesis, growth was initiated by treating growth-arrested cells for 20 hours with DMEM containing 0.5% FCS and supplemented with AMVN (1 to 100 μmol/L) and containing or lacking estrone, 2-hydroxystilbestradiol, 4-hydroxymuconic, or droloxifene. After 2 hours of incubation, the treatments were repeated with freshly prepared solutions but supplemented with [3H]thymidine (1 μCi/mL; ICN Biomedicals) for an additional 4 hours. The experiments were terminated by washing the cells twice with Dulbecco’s PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 500 μL of 0.3N NaOH and 0.1% SDS after incubation at 30°C for 2 hours. Aliquots from 4 wells for each treatment with 10 mL of scintillation fluid were counted in a liquid scintillation counter. For cell number experiments, smooth muscle cells were allowed to attach overnight, were growth arrested for 48 hours, were treated every 24 hours for 4 days, and on day 5 were dislodged and counted on a Coulter counter.
Cell Migration Studies

Modified Boyden chambers (Neuro Probe Inc) were used to evaluate the effects of AMVN-derived peroxyl radicals on smooth muscle cell migration as previously described.24 Briefly, confluent monolayers of smooth muscle cells were growth arrested by feeding with DMEM supplemented with 0.4% albumin for 48 hours. Growth-arrested smooth muscle cells were trypsinized, washed, and suspended at a concentration of \(1 \times 10^6\) cells/mL in fresh DMEM containing 0.4% albumin and supplemented with AMVN (50 \(\mu\)mol/L) and containing or lacking 0.3 to 30 ng/mL estrone, 2-hydroxyestradiol, 4-hydroxytamoxifen, or PMC. Following incubation, the smooth muscle cells (50,000 cells/50 \(\mu\)L; 6000 cells/mm²) were layered on the top chamber, and DMEM containing the respective treatments plus 0.5% FCS was added to the lower chamber. After 6 hours of incubation, the membranes were removed, the nonmigrated cells on the top surface wiped, and the migrated cells fixed and stained in Dif Quick stain (Baxter Scientific Corp). The migrated cells on the lower surface of the membrane were counted manually at 6 different areas of the membrane.

Cell Viability Assays

Trypan blue exclusion, MTT assay, and cell adhesion studies (for cells treated in suspension) were conducted to confirm cell viability after various treatments. For the trypan blue exclusion assay, cells were stained with trypan blue, and the cells taking up the dye were counted microscopically. For the MTT assay, we used a modified colorimetric assay based on the selective ability of living cells to reduce the yellow dye, MTT, to intracellular formazan via mitochondrial enzymes, as described before.32 Briefly, aliquots of cells treated for different times in suspension with the various experimental agents were plated in multwell plates, allowed to attach, and subsequently treated with MTT (5 mg/mL) under standard tissue-culture conditions. In monolayers treated with different agents, MTT was added directly to the wells after treatment. Following incubation for 4 hours, the supernatant was aspirated without disruption of the formazan precipitate, and the intracellular formazan crystals were dissolved by adding 150 \(\mu\)L of DMSO to each well. The absorbance was measured at 570 nm using a microplate spectrophotometer. For cell adhesion viability assays, aliquots of cells treated in suspension were plated in multwell plates, and the dead floating cells remaining after 5 hours of incubation were counted under the microscope. In all experiments, cells grown in medium and without any treatment served as controls.

Statistics

All experiments were conducted in triplicate or quadruplicate with 3 to 5 separate cultures. Data are presented as mean±SEM, and statistical analysis was performed using ANOVA, paired Student’s \(t\) test or Fisher’s least significant difference test as appropriate. A value of \(P<0.05\) was considered statistically significant.

Results

Incorporation of cis-Parinaric Acid Into Smooth Muscle Cell Lipids

Figure 1A shows a typical high-performance liquid chromatogram of fluorescence detected in phospholipids extracted from smooth muscle cells incubated in the presence of hSA-cis-parinaric acid complex. Four phospholipid peaks were well resolved and identified as phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine, and sphingomyelin. Under the same excitation and emission limits used above, fluorescent compounds were not detected by HPLC in extracts from cells incubated with hSA in the absence of cis-parinaric acid (data not shown).

Cis-Parinaric acid incorporation into different membrane phospholipids in smooth muscle cells was dependent on the incubation time of these cells with albumin-cis-parinaric acid complex (Figure 2A). The maximal incorporation of cis-parinaric acid into all detected phospholipid classes was reached within 1 to 2 hours of incubation (Figure 2A). cis-Parinaric acid was differentially incorporated into the various phospholipids (phosphatidylethanolamine>phosphatidylserine>phosphatidylinositol>phosphatidylglycerol).

Effect of AMVN on Smooth Muscle Cell Membrane Lipids

Figure 1B depicts a chromatogram of fluorescence detected in cis-parinaric acid–acylated lipids extracted from cis-parinaric acid–labeled smooth muscle cells incubated in the presence of AMVN. Incubation of smooth muscle cells with AMVN (500 \(\mu\)mol/L) induced significant oxidation of cis-parinaric acid in all the detected phospholipids (Figure 2B) as indicated by the reduction in fluorescence. In smooth muscle cells treated with 50 to 100 \(\mu\)mol/L of AMVN, no oxidation of cis-parinaric acid–labeled phospholipids could be observed (Figure 2B). Incubation of cis-parinaric acid–labeled smooth muscle cells with 250 \(\mu\)mol/L AMVN resulted in a decrease of cis-parinaric acid fluorescence of phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine by 39%, 22%, and 16%, respectively (Figure 2B). The extent of oxidation of cis-parinaric acid that was incorporated into the smooth muscle cell phospholipids was dependent on the duration of incubation of these cells with AMVN (data not shown). No loss in cell viability was observed in cells treated for various times and concentrations of AMVN and as

Figure 1. Representative HPLC chromatogram showing the fluorescent detection of various phospholipids eluted from total lipids extracted from \(1 \times 10^6\) smooth muscle cells incubated with cis-parinaric acid or cis-parinaric acid plus AMVN (500 \(\mu\)mol/L). A, Vascular smooth muscle cells labeled with cis-parinaric acid; control. B, Vascular smooth muscle cells treated with cis-parinaric acid plus AMVN. Various phospholipids (phosphatidylethanolamine, PEA; phosphatidylserine, PS; phosphatidylinositol, PC; sphingomyelin, Sph; and free cis-parinaric acid, PnA) were detected fluorometrically at 324-nm excitation and 420-nm emission.
Effect of Various Estrogens on the AMVN-Induced Oxidation of cis-Parinaric Acid–Acylated Phospholipid in Smooth Muscle Cells

2-Hydroxyestradiol, 4-hydroxytamoxifen, and PMC protected cis-parinaric acid–acylated phospholipids against AMVN-induced oxidation in a concentration-dependent manner, whereas droloxifene and estrone did not (Figures 3 and 4). The potency of PMC in protecting the various phospholipids was in the following order: phosphatidylethanolamine > phosphatidylserine > phosphatidylcholine = sphingomyelin. Concentrations of 2-hydroxyestradiol and 4-hydroxytamoxifen higher than 250 ng/mL were as effective as PMC in preventing AMVN-induced oxidations of phosphatidylethanolamine and more effective than PMC in preventing phosphatidylserine, phosphatidylcholine, and sphingomyelin oxidation. However, at lower concentrations (<250 ng/mL), PMC was more effective than 2-hydroxyestradiol and 4-hydroxytamoxifen in protecting all the phospholipids (Figures 3 and 4). AMVN-induced oxidation of the various phospholipids was completely blocked (100%) by 2.5 to 5 µg/L per 10^6 cells of PMC, 2-hydroxyestradiol, and 4-hydroxytamoxifen. The overall efficacy of the various estrogens and phenolic compounds in protecting against lipid peroxidation was PMC > 2-hydroxyestradiol > 4-hydroxytamoxifen > droloxifene = estrone (Figures 3 and 4).

In smooth muscle cells pretreated for 20 hours, physiological concentrations (0.3 ng/mL) of 2-hydroxyestradiol selectively decreased AMVN-induced peroxidation of phosphatidylinositol and phosphatidylserine by 34% and 25%, respectively (Figure 5). In contrast to 2-hydroxyestradiol, estrone even at high concentrations (30 ng/mL) was a weak inhibitor of AMVN-induced peroxidation of phosphatidylserine and phosphatidylinositol (Figure 5). Similarly to 2-hydroxyestradiol, 4-hydroxytamoxifen (40 ng/mL) and PMC (20 ng/mL) selectively protected against AMVN-induced peroxidation of phosphatidylinositol and phosphatidylserine (Figure 5), but not phosphatidylethanolamine and phosphatidylcholine (data not shown). No loss in cell viability was observed in cells treated for various times and...
concentrations of the experimental agents and AMVN as assessed by trypan blue exclusion test, MTT assay, and cell adhesion assay (data not shown).

Effect of AMVN on the Phospholipid Composition in Smooth Muscle Cells

High-performance thin-layer chromatographic analysis of total polar lipids is shown in the Table. Phosphatidylcholine and phosphatidylethanolamine represented the major fraction of the total phospholipids in smooth muscle cells and was ≈50% and ≈27% of the total phospholipid content, respectively. Additionally, the other prominent phospholipids in order of their abundance were as follows: phosphatidyserine > phosphatidylglycerol > sphingomyelin > diphosphatidylglycerol > lysophosphatidylcholine > phosphatidic acid. No significant differences in phospholipid distribution or content were detected in smooth muscle cells following oxidative stress imposed by incubation of the cells in the presence of AMVN (Table), with the exception of a slight increase in lysophosphatidylcholine, a relatively minor phospholipid.

Effect of Various Estrogens on the AAPH-Induced Oxidation of Intracellular Thiols and Glutathione

The incubation of smooth muscle cells with AMVN (500 μmol/L) for 2 hours at 37°C resulted in a mild oxidation (15% to 17%) of intracellular thiols (glutathione

**Table.** Phospholipid composition of smooth muscle cells after incubation with AMVN.

<table>
<thead>
<tr>
<th>Phospholipids</th>
<th>Percentage of Total Lipids</th>
<th>AMVN 500 μmol/L</th>
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<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>44.2 ± 2.6</td>
<td>44.1 ± 2.7</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>27.3 ± 1.5</td>
<td>27.5 ± 1.6</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>8.7 ± 0.5</td>
<td>8.4 ± 0.5</td>
</tr>
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<td>Phosphatidyserine</td>
<td>9.1 ± 0.7</td>
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<tr>
<td>Sphingomyelin</td>
<td>8.1 ± 0.5</td>
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<td>Diphosphatidylglycerol</td>
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<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>0.5 ± 0.2</td>
<td>0.9 ± 0.3*</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>0.5 ± 0.1</td>
<td>0.6 ± 0.3</td>
</tr>
</tbody>
</table>

Cells were incubated with or without AMVN (500 μmol/L) for 2 hours at 37°C and various phospholipids separated from the total extracts of phospholipids by high-performance thin-layer chromatography and quantified. Each value represents percentage of total phospholipids and is mean ± SEM from 5 separate experiments.

*P < 0.05 as compared with untreated controls.
plus intracellular thiols; data not shown). The GSH level, measured as thiol compound after pretreatment of cells with glutathione-peroxidase and cumene hydroperoxide in the presence of deferoxamine mesylate, was 23.81 ± 6.34 nmol/10^6 cells. The intracellular glutathione content in smooth muscle cells was ~75% to 85% of total thiol content in smooth muscle cells. The incubation of smooth muscle cells with AAPH (20 mmol/L) for 2 hours at 37°C resulted in a significant oxidation of the intracellular thiols and glutathione (Figure 6, top panels). In the presence of PMC, AAPH-induced oxidation of intracellular thiols was inhibited in a concentration-dependent manner, suggesting that PMC protects glutathione against oxidation. In contrast to PMC, 4-hydroxytamoxifen, droloxifene, estrone, and 2-hydroxyestradiol increased the AAPH-induced oxidation rate of thiols in smooth muscle cells. However, in the absence of AAPH, these phenolic compounds did not affect the levels of intracellular thiols and glutathione in the smooth muscle cells (data not shown). Additionally, when pure glutathione was incubated in phosphate buffer containing 100 μmol/L deferoxamine mesylate, auto-oxidation of glutathione was not observed. However, when AAPH (20 mmol/L) was added, the peroxyl radicals produced by AAPH oxidized glutathione at a rate of 1.45 nmol/min. Moreover, PMC protected glutathione from AAPH-induced oxidation in a concentration-dependent manner (Figure 7B). In contrast to the protective effects of PMC, the estrogens (4-hydroxytamoxifen, droloxifene, estrone, and 2-hydroxyestradiol) did not inhibit AAPH-induced oxidation of glutathione.

**Effects of Various Estrogens on AMVN-Induced DNA Synthesis, Cell Number, and Cell Migration**

Treatment with AMVN stimulated DNA synthesis in a concentration-dependent manner (P < 0.001 versus 0.5% FCS; Figure 7A). 2-Hydroxyestradiol and 4-hydroxytamoxifen inhibited AMVN-induced DNA synthesis in a concentration-dependent manner. As compared with 2-hydroxyestradiol, estrone was a very weak inhibitor of AMVN-induced DNA synthesis (Figure 7B). The lowest concentrations of estrone, 2-hydroxyestradiol, and 4-hydroxytamoxifen that inhibited AMVN-induced DNA synthesis were 0.3 ng/mL for 2-hydroxyestradiol, 30 ng/mL for estrone, and 0.4 ng/mL for 4-hydroxytamoxifen (Figure 7B). A 50% decrease in AMVN-induced DNA synthesis in smooth muscle cells was observed at ~3 ng/mL 2-hydroxyestradiol and 40 ng/mL 4-hydroxytamoxifen. In contrast to 2-hydroxyestradiol, estrone at a concentration of 300 ng/mL inhibited AMVN-induced DNA synthesis by only 10%. Similarly to the effects on DNA synthesis (Figure 7B), the AMVN- and 2-hydroxyestradiol- induced peroxyl radicals on [3H]thymidine incorporation. Each data point represents mean ± SEM from 3 experiments conducted in quadruplicate. **P < 0.05 vs control (C; vascular smooth muscle cells treated with 0.5% FCS). B. Line graph showing the concentration-response relationship for the inhibitory effects of 2-hydroxyestradiol, estrone, and 4-hydroxytamoxifen on 50 μmol/L AMVN-induced DNA synthesis. Results are expressed as percentage of control, with 100% defined as thymidine incorporation in the presence of 50 μmol/L AMVN. Each data point represents mean ± SEM from 3 experiments, each conducted in quadruplicate. **P < 0.05 vs AMVN.
The present study demonstrates that aortic smooth muscle cells labeled with cis-parinaric acid provide a sensitive and quantitative method to assess the effects of drugs on lipid peroxidation. Using this technique, this study establishes that AMVN-induced peroxidation of various smooth muscle cell membrane phospholipids is not prevented by estrone, an estrogen-receptor ligand, but is completely blocked by 2-hydroxyestradiol, a major metabolite of 17β-estradiol that does not have a high affinity for the estrogen receptor. Moreover, 4-hydroxytamoxifen (a metabolite of tamoxifen with high affinity for estrogen receptors) effectively blocks peroxidation of smooth muscle cell phospholipids, while droloxifene, a tamoxifen analog known to be an estrogen-receptor ligand, does not prevent peroxidation. Both 2-hydroxyestradiol and 4-hydroxytamoxifen are as effective as PMC in protecting membrane phospholipids against peroxidation. Moreover, 2-hydroxyestradiol and 4-hydroxytamoxifen inhibit peroxyl radical–induced proliferation and migration of smooth muscle cells. In contrast to the effects on lipid peroxidation and cell growth, 2-hydroxyestradiol and 4-hydroxytamoxifen do not prevent AAPH-induced oxidation of cellular thiols/glutathione in smooth muscle cells. These studies provide the first evidence that the cardioprotective effects of estrogen and tamoxifen may in part be due to the direct antioxidant effects of their major metabolites on membrane phospholipids of smooth muscle cells.

Because our aim was to evaluate oxidative damage in cells membranes, we required a sensitive method for detecting oxidation of phospholipids. Thus, we selected the cis-parinaric acid/AMVN paradigm. In this regard, our results indicate that cis-parinaric acid covalently labels various phospholipids in smooth muscle cells in a differential and time-dependent fashion and that cis-parinaric acid fluorescence in the various phospholipids is uniformly reduced in smooth muscle cells treated with AMVN. These results indicate that smooth muscle cells can be labeled with cis-parinaric acid and that AMVN-derived peroxy radicals cause peroxidation of a broad array of lipid components in the cell membrane. Even though cis-parinaric acid–labeled phospholipids constitute <1% of the total lipid content, we can detect AMVN-induced peroxidation in cis-parinaric acid–labeled smooth muscle cells, as well as protection against AMVN-induced peroxidation by PMC, a potent antioxidant. The actual content of various membrane phospholipids analyzed using conventional methods did not vary in control and AMVN-treated smooth muscle cells, indicating that decreases in unoxidized cis-parinaric acid in labeled smooth muscle cells were not due to loss of cis-parinaric acid–labeled phospholipids, but rather to the change in the oxidation state of incorporated cis-parinaric acid. Moreover, the finding that cell viability was not reduced in smooth muscle cells treated with AMVN suggests that the effects of AMVN on lipid peroxidation were not due to cell death. Together, these findings strongly suggest that cis-parinaric acid–labeled smooth muscle cells provide an excellent in vitro system to evaluate the effects of both oxidative stress-induced lipid peroxidation and the antioxidant capabilities of chemicals and new therapeutic drugs. In the present study, we found that AMVN-induced peroxidation of various membrane phospholipids and AMVN-induced cell growth are not prevented by estrone, but are completely blocked by 2-hydroxyestradiol.

Discussion

The present study demonstrates that aortic smooth muscle cells labeled with cis-parinaric acid provide a sensitive and quantitative method to assess the effects of drugs on lipid peroxidation. Using this technique, this study establishes that AMVN-induced peroxidation of various smooth muscle cell membrane phospholipids is not prevented by estrone, an estrogen-receptor ligand, but is completely blocked by 2-hydroxyestradiol, a major metabolite of 17β-estradiol that does not have a high affinity for the estrogen receptor. Moreover, 4-hydroxytamoxifen (a metabolite of tamoxifen with high affinity for estrogen receptors) effectively blocks peroxidation of smooth muscle cell phospholipids, while droloxifene, a tamoxifen analog known to be an estrogen-receptor ligand, does not prevent peroxidation. Both 2-hydroxyestradiol and 4-hydroxytamoxifen are as effective as PMC in protecting membrane phospholipids against peroxidation. Moreover, 2-hydroxyestradiol and 4-hydroxytamoxifen inhibit peroxyl radical–induced proliferation and migration of smooth muscle cells. In contrast to the effects on lipid peroxidation and cell growth, 2-hydroxyestradiol and 4-hydroxytamoxifen do not prevent AAPH-induced oxidation of cellular thiols/glutathione in smooth muscle cells. These studies provide the first evidence that the cardioprotective effects of estrogen and tamoxifen may in part be due to the direct antioxidant effects of their major metabolites on membrane phospholipids of smooth muscle cells.

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This finding suggests that the cardioprotective effects of 17β-estradiol may, in part, be due to the direct antioxidant effects of its metabolite on smooth muscle cells. Differences in the antioxidant capabilities of estrone and 2-hydroxyestradiol may largely be due to the chemical nature of the molecule. Indeed, 2-hydroxyestradiol is a catechol estrogen and is a more potent antioxidant than 17β-estradiol. It is also possible that, similar to 17β-estradiol, the metabolism of estrone to 2-hydroxyestrone is necessary to induce its antioxidant effects. Indeed, a recent study has shown that 2-hydroxyestrone is several times more potent than estrone in protecting against lipid peroxidation. Moreover, as compared with 17β-estradiol, 2-hydroxyestradiol is several times more potent in inhibiting DNA synthesis in smooth muscle cells and proliferation as well as extracellular matrix synthesis in cardiac fibroblast.

Whether the cardioprotective effects of estrogens are receptor dependent is under intense debate. Recent studies suggest that the cardioprotective effects of estrogen(s) are non–receptor mediated; however, the mechanisms involved remain unclear. Our finding that 4-hydroxytamoxifen, a phenolic metabolite of tamoxifen and an estrogen-receptor ligand, is as potent as 2-hydroxyestradiol in protecting phospholipids against AMVN-induced peroxidation and our finding that estrone and droloxifene, which bind to estrogen receptors with high affinity, are ineffective in preventing peroxidation, suggest that the non–receptor-mediated cardioprotective effects of estrogens may be due to direct antioxidant effects of their metabolites on smooth muscle cells. This notion is further supported by our observation that, similarly to 2-hydroxyestradiol, 4-hydroxytamoxifen inhibited AMVN-induced growth of smooth muscle cells. Nonetheless, further experiments will be required to determine whether the observed protective effects of estrogen and tamoxifen metabolites are greater in females and whether the effects are blocked by specific estrogen receptor antagonists.

To evaluate the antioxidant potency of various estrogens, we compared their effects with PMC, a homolog of the natural membrane antioxidant, and growth inhibitor vitamin E, a very potent antioxidant. An important observation in the present study is that 2-hydroxyestradiol and 4-hydroxytamoxifen are as effective as PMC in preventing lipid peroxidation. It is well documented that 4-hydroxytamoxifen is severalfold more potent than tamoxifen in preventing LDL oxidation, iron-induced microsomal peroxidation, and cardiovascular disease. Moreover, it has been suggested that the cardioprotective effects of tamoxifen are largely mediated via generation of 4-hydroxytamoxifen. This is in agreement with our observation that low concentrations (1 nmol/L) of 4-hydroxytamoxifen inhibited AMVN-induced growth of smooth muscle cells. With regard to 2-hydroxyestradiol, no studies have been conducted to compare its potency with that of 17β-estradiol in inducing cardioprotective effects in vivo. However, numerous studies suggest that 2-hydroxyestradiol is the major metabolite of 17β-estradiol and that some biological effects of estrogens are mediated in part through their metabolites. As compared with 17β-estradiol and estrone, their 2-hydroxymetabolites (2-hydroxyestradiol and 2-hydroxyestrone) are severalfold more effective in preventing lipid peroxidation in liposomes. Hence, similarly to tamoxifen, estrogens may largely induce their cardioprotective effects via generation of the potent antioxidant metabolites. This hypothesis is further supported by our observation that low concentrations of 2-hydroxyestradiol, but not estrone, inhibited free radical–induced DNA synthesis in smooth muscle cells.

The cardioprotective effects of several drugs with antioxidant effects are thought to be mediated via modulation of the endogenous free radical scavenging system, including the glutathione reductase system. Hence, the possibility that estrone, 2-hydroxyestradiol, droloxifene, and 4-hydroxytamoxifen may also induce their cardioprotective effects by preventing peroxyl radical–induced oxidation of intracellular thiols in smooth muscle cells was also investigated. In smooth muscle cells treated with AAPH, the intracellular thiol/GSH is oxidized rapidly. In contrast to the effects on lipid peroxidation, 4-hydroxytamoxifen and 2-hydroxyestradiol did not prevent AAPH-induced oxidation of smooth muscle cell thiol/glutathione. Thus, the antioxidant effects of 2-hydroxyestradiol and 4-hydroxytamoxifen are exerted within the cell membrane, rather than in the cytoplasm.

Do estrogens prevent lipid peroxidation and free radical–induced growth in vivo? The observation that physiological concentrations (0.3 ng/mL) of 2-hydroxyestradiol inhibited free radical–induced growth and migration of smooth muscle cells suggests that 2-hydroxyestradiol may importantly contribute to the cardioprotective effects of estrogen in vivo. Moreover, the finding that pretreatment of cells for 20 hours with physiological concentrations of 2-hydroxyestradiol, but not estrone, selectively protects phosphatidylinositol and phosphatidylserine against AMVN-induced peroxidation suggests that estradiol metabolites may protect against peroxyl radical–induced growth via mechanisms linked to these phospholipids. In this regard, it is important to note that cell viability, as assessed by trypan blue exclusion, MTT assay, and a cell adhesion assay, was not reduced by prolonged treatment with AMVN, 2-hydroxyestradiol, 4-hydroxytamoxifen, or estrone. Therefore, cell death cannot account for the reduced AMVN-induced peroxidation by 2-hydroxyestradiol. Finally, it should be mentioned that plasma levels of 17β-estradiol do not reflect the true physiological levels of antioxidant estrogens in the cell membrane, since in addition to 17β-estradiol, several other estrogens and their metabolites are present in vivo and estrogen metabolites concentrate in cell membranes.

Regarding the mechanisms mediating oxidation-induced cell growth, peroxidation of phospholipids has been shown to activate the extracellular signal–regulated protein kinases (ERK1 and ERK2), induce the expression of c-fos and c-jun oncogene proteins, increase activator protein-1 DNA binding activity, and induce nuclear factor-κB. Moreover, the acidic membrane phospholipids phosphatidylinositol and phosphatidylserine are known to selectively activate protein kinase C activity, whereas phos-
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phatidylethanolamine and phosphatidylcholine do not.\(^{45}\) Moreover, oxidized metabolites of phosphatidylinositol have been shown to induced cell migration. Hence, it is possible that 2-hydroxyestradiol and 4-hydroxytamoxifen prevent smooth muscle cell growth by regulating protein kinase C activity by selectively protecting peroxidation of phosphatidylinositol and phosphatidylserine. However, further detailed studies will be required to clarify the mechanisms by which peroxidation of phospholipids enhances cell growth.

In conclusion, our findings suggest that 2-hydroxyestradiol and 4-hydroxytamoxifen may be more potent and clinically important than their parent compounds in preventing lipid peroxidation, growth of smooth muscle cells, and vaso-occlusive disorders.

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References


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