Preparation and Characterization of 8a-(Phosphatidylcholine-dioxy)-
α-tocopherones and their Formation during the Peroxidation
of Phosphatidylcholine in Liposomes

Ryo Yamauchi, Hiromi Mizuno, and Koji Kato

Department of Food Science, Faculty of Agriculture, Gifu University, Gifu 501-1193, Japan

Received December 8, 1997

α-Tocopherol was reacted with the phosphatidylcholines (PCs), 1-palmitoyl-2-linoleoyl-3-sn-PC (PLPC), 1-palmitoyl-2-linolenoyl-3-sn-PC, 1-palmitoyl-2-arachidonoyl-3-sn-PC (PAPC) and 1-stearyl-2-arachidonoyl-3-sn-PC, in the presence of the free radical initiator, 2,2'-azobis(2,4-dimethylvaleronitrile), at 37°C. The addition products of α-tocopherol with the PC peroxyl radicals were isolated and identified as 8a-(PC-dioxy)-α-tocopherones, in which the peroxyl radicals derived from each PC molecule attack the 8a-position of the α-tocopherol radical. The antioxidative efficiency of α-tocopherol against the peroxidation of PLPC and PAPC in liposomes was assessed by the formation of the reaction products of α-tocopherol. When α-tocopherol was oxidized in the presence of the water-soluble free radical initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride, epoxy-α-tocopherolquinones were mainly produced together with 8a-(PC-dioxy)-α-tocopherones and α-tocopherolquinone. The yield of α-tocopherolquinone was increased by treating each sample with dilute acid which indicates the presence of tocopherone precursors other than the 8a-(PC-dioxy)-α-tocopherones. The same products were also detected from iron-dependent peroxidation, although the yields were very low.

Key words: α-tocopherol; antioxidant; lipid peroxidation; liposomes; phosphatidylcholines

Peroxidation of lipid membranes is implicated in a variety of damaging pathological events.1-3) α-Tocopherol protects biological membranes from lipid peroxidation. The antioxidative activity of α-tocopherol is related to scavenging of peroxyl radicals of the unsaturated fatty acyl chains of lipids in the membranes.4) α-Tocopherol efficiently transfers a hydrogen atom to a peroxyl radical, giving a hydroperoxide and an α-tocopherol radical.5-7) This α-tocopherol radical, once formed, reacts with a second peroxyl radical to form a non-radical product. Thus, each α-tocopherol molecule traps two peroxyl radicals. The reaction products of α-tocopherol with peroxyl radicals have been extensively studied to explain the mechanism for the inhibition of autoxidation by α-tocopherol.8-18)

Liposomes are generally accepted to be a suitable model for studying the membrane structure and properties, given that they are surrounded by a lipid bilayer that is structurally similar to the lipidic matrix of a cell membrane. Therefore, phospholipid liposomal systems have been employed to investigate the peroxyl radical scavenging reactions of α-tocopherol in biological membranes.13,14,16,17,19-21) We have previously reported the isolation and characterization of 8a-(phospholipid-dioxy)-α-tocopherones as the primary reaction products of the α-tocopheroyl radical with phospholipid peroxyl radicals during the peroxidation of 1,2-dilinoleoyl-3-sn-phosphatidylcholine (D LPC) in liposomes.10) In addition to the 8a-substituted α-tocopherones, α-tocopherylquinone and epoxy-α-tocopherophylnones have been detected as the products of α-tocopherol.17) DLPC is relatively rare in biological membranes; animal tissue contains phospholipids of molecular species defined by a saturated esterified fatty acid, usually 16 or 18 carbons in length, at the sn-1 position of glycerol and an unsaturated esterified fatty acid that is usually substituted at the sn-2 position.20) Therefore, it is important to elucidate the reaction of α-tocopherol with peroxyl radicals derived from such molecular species of phosphatidylcholine (PC) in liposomes.

This paper reports the preparation and characterization of 8a-(PC-dioxy)-α-tocopherones from the reaction between α-tocopherol and the peroxyl radicals of 1-palmitoyl-2-linoleoyl-3-sn-PC (PLPC), 1-palmitoyl-2-linolenoyl-3-sn-PC (PPLPC), 1-palmitoyl-2-arachidonoyl-3-sn-PC (PAPC), and 1-stearyl-2-arachidonoyl-3-sn-PC (SAPC). The antioxidative efficiency of α-tocopherol against the peroxidation of PLPC and PAPC in large unilamellar liposomes is assessed by the formation of PC hydroperoxides (PC-OH) and the reaction products of α-tocopherol. Peroxidation of the liposomes was initiated by peroxyl radicals generated in the aqueous phase by the thermal decomposition of the azo-initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), or by an iron-dependent system.

1 Corresponding author (Fax: +81-58-293-2930; E-mail: yamautir@cc.gifu-u.ac.jp)

Abbreviations: AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile); DLPC, 1,2-dilinoleoyl-3-sn-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-sn-phosphatidylcholine; EDTA, ethylenediaminetetraacetate; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; PAPC, 1-palmitoyl-2-arachidonoyl-3-sn-phosphatidylcholine; PC, phosphatidylcholine; PC-OH, phosphatidylcholine monohydroperoxides; PLPC, 1-palmitoyl-2-linoleoyl-3-sn-phosphatidylcholine; PPLPC, 1-palmitoyl-2-linolenoyl-3-sn-phosphatidylcholine; SAPC, 1-stearyl-2-arachidonoyl-3-sn-phosphatidylcholine; UV, ultraviolet.
Materials and Methods

Materials. **RRR-α-Tocopherol** was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and purified by reversed-phase high-performance liquid chromatography (HPLC).** RRR-γ-Tocopherol,** which was used as an internal standard, was prepared from mixed isomers of tocopherol (Hohnen Oil Co.; Tokyo, Japan). Authentic α-tocopherylquinone, 2,3-epoxy- and 5,6-epoxy-α-tocopherylquinones, and the α-tocopherol spirolide dimer were prepared as described previously.** PLPC, PLnPC, PAPC and SPC were synthesized by 2-acylation of 1-palmitoyl- or 1-stearoyl-3-sn-glycerophosphocholine with fatty acid anhydrides according to the method of Gupta et al.** 1-Palmitoyl- and 1-stearoyl-3-sn-glycerophosphocholines were respectively prepared from 1,2-dipalmitoyl- and 1,2-distearyl-3-sn-PCs (Wako Pure Chemical Co.; Osaka, Japan) by a phospholipase A₂ treatment.** Linoleic, linolenic and arachidonic anhydrides were synthesized from the reaction of the corresponding fatty acid (99%, Sigma Chemical Co.) with dicyclohexylcarbodiimide.** Synthesized PC was purified by reversed-phase HPLC, using an Inertsil Prep-ODS column (2.0×25 mm, GL Sciences; Tokyo, Japan) with methanol to remove the contaminating hydroperoxides. 1,2-Dimyristoyl-3-sn-PC (DMPC) was purchased from Sigma Chemical Co. Authentic PC monohydrated PC (PC-OH) were prepared by the oxidation of PLPC or PAPC liposomes in the presence of 1 mol% α-tocopherol that was initiated by 2,2′-azobisisobutyronitrile (AMVN)** and purified by HPLC under the same conditions as those already described. Free-radical initiators AAPH and AMVN were obtained from Wako Pure Chemical Co., and ferric monosodium ethylenediaminetetraacetate (Fe(III)-EDTA) was from Dojindo Laboratories (Kumamoto, Japan).

Apparatus. HPLC was carried out with a Waters model 600E system (Waters; Milford, MA, U.S.A.) connected to a Waters model 486 UV/VIS detector or a Jasco model 820-FP spectrofluorometer (Japan Spectroscopic Co.; Tokyo, Japan). The ultraviolet (UV) spectrophotometer was measured with a Jasco Ubest-30 UV/VIS spectrophotometer, the ε value being calculated from the phosphorus content.** Fast atom bombardment mass spectrometry (FAB-MS) was done with a 9020-DF instrument (Shimadzu Co.; Kyoto, Japan), using a xenon fast atom bombardment gun. Samples were placed in the 5.5 kV xenon atom beam on a probe with triethanolamine as the matrix. The proton nuclear magnetic resonance (1H-NMR) spectrum was recorded at 270.17 MHz with a GX-270 instrument (JEOL; Tokyo, Japan), using CDCl₃ as the solvent and tetramethylsilane as the internal standard. The 1H-1H chemical shift-correlated NMR technique was employed to assign 1H shifts and couplings.

Preparation and characterization of 8a-(PC-dioxy)-α-tocopherones. The 8a-(PC-dioxy)-α-tocopherones were prepared by reacting α-tocopherol with PC initiated by AMVN.** Multilamellar liposomes of PLPC, PLnPC, PAPC or SPC (1 mmol) containing α-tocopherol (5 μmol) and AMVN (0.1 mmol) in 100 ml of a 50 mM sodium phosphate buffer (pH 7.4, containing 50 mM NaCl and 0.1 mM Na₂-EDTA) were incubated at 37°C for 15 h (PAPC and SPC) and for 20 h (PLPC and PLnPC) under air with mechanical shaking. The reaction mixture was extracted with 200 ml of chloroform-methanol (1:1, v/v). The chloroform layer was evaporated in vacuo and dissolved in 1 ml of ethanol. The ethanol solution for HPLC was injected into a reversed-phase Inertisil C8 column (4.6×150 mm or 7.5×250 mm, GL Sciences) with a solvent system of methanol-water (99:1, v/v) at a flow rate of 1.0 or 4.0 ml/min and at 240 nm. The following 8a-(PC-dioxy)-α-tocopherones were obtained as reaction products:

A mixture of 1-palmitoyl-2-[8a-(dioxy-α-tocopherone)-octadecadienoyl]- and 1-palmitoyl-2-[8a-(dioxy-α-tocopherone)-octadecadienoyl]-3-sn-PCs (1). UV λmax (ethanol) nm (ε): 233 (35,400), FAB-MS m/z: 1219 (MH⁺), 790 (MH─429+), 757 (MH─462+), 702, 430 ([α-tocopherone]+), 403, 290, 224 ([CH=CH─O-phosphocholine]+), 198, 184, 165 ([phosphocholine]+), and 104 ([choline]+). NMR δH (CDCl₃): 0.80–0.90 (18H, m), 1.07 and 1.25 (3H, s), 1.15 (3H, m), 1.20–1.40 (56H, m, CH₂), 1.40–1.80 (6H, m), 1.86 (6H, s), 1.95 (3H, s), 2.0–2.2 (4H, m), 2.28 and 2.30 (4H, t, J=7 Hz), 2.60 (2H, m), 3.35 (9H, s), 3.77 (2H, m), 3.97 (2H, m), 4.10 (1H, m), 4.16 (2H, m), 4.36 (2H, m), 4.42 (1H, m), 5.20 (1H, m), 5.3–5.6 (2H, m), 5.9–6.0 (1H, m), 6.3–6.4 (1H, m). The isomeric compositions were determined by normal-phase HPLC after NaBH₄ reduction and transmethylation.** The 10E,12Z-9- (45%), 10E,12E-9- (12%), 9Z,11E-13- (32%), and 9E,11E-13-isomers (11%) of methyl hydroxyoctadecadienatoxides were detected on the chromatogram.

A mixture of four positional isomers of 1-palmitoyl-2-[8a-(dioxy-α-tocopherone)-octadecatrienoyl]-3-sn-PC (2). UV λmax (ethanol) nm (ε): 234 (32,300), FAB-MS m/z: 1217 (MH⁺), 788 (MH─429+), 755 (MH─462+), 702, 496, 430 ([α-tocopherone]+), 403, 290, 224 ([CH=CH─CH₂─O-phosphocholine]+), 198, 184, 165 ([phosphocholine]+), and 104 ([choline]+). NMR δH (CDCl₃): 0.80–0.91 (18H, m), 1.07 and 1.25 (3H, s), 1.15 (3H, m), 1.2–1.4 (50H, m), 1.4–1.6 (8H, m), 1.86 (6H, s), 1.95 (3H, s), 2.0–2.2 (4H, m), 2.28 and 2.30 (4H, t, J=7 Hz), 2.61 (2H, m), 2.91 (2H, m), 3.35 (9H, s), 3.79 (2H, m), 3.99 (2H, m), 4.10 (1H, m), 4.15 (1H, m), 4.38 (2H, m), 4.42 (1H, m), 5.22 (1H, m), 5.3–5.6 (4H, m), 5.9–6.0 (1H, m), 6.3–6.5 (1H, m). The isomeric compositions were determined to involve the 10E,12Z,15Z-9- (30%), 10E,12E,15Z-9- (2%), 9Z,11E,15Z-12- (10%), 9Z,11E,15Z-13- (13%), E,E,E-12- and (E,E,E)-13- (3%), 9Z,12Z,14E-16- (40%), and (E,E,E)-16-isomers (2%).** A mixture of six positional isomers of 1-palmitoyl-2-[8a-(dioxy-α-tocopherone)-eicosatetraenoyl]-3-sn-PC (3). UV λmax (ethanol) nm (ε): 235 (32,200), FAB-MS m/z: 1243 (MH⁺), 814 (MH─429+), 796, 781 (MH─462+), 578, 496, 478, 430 ([α-tocopherone]+), 403, 290, 224 ([CH=CH─CH₂─O-phosphocholine]+),
Oxidation of Vitamin E in Liposomes

198, 184, 165 ([phosphocholine]+), 104 ([choline]+).
NMR $^{1}H$ (CDCl$_3$): 0.80-0.91 (18H, m), 1.07 and 1.25 (3H, s), 1.15 (3H, m), 1.2-1.35 (52H, m), 1.4-1.8 (4H, m), 1.85 
(6H, s), 1.95 (3H, s), 2.05 (4H, m), 2.28 and 2.30 (4H, t, 
J=7 Hz), 2.60 (2H, m), 2.81 (4H, m), 3.36 (9H, s), 3.77 
(2H, m), 4.00 (2H, m), 4.13 (1H, m), 4.21 (1H, m), 4.37 
(2H, m), 4.43 (1H, m), 5.22 (1H, m), 5.37 (4H, m), 5.4-5.5 
(2H, m), 5.9-6.1 H, m), 6.3-6.5 (1H, m). The isomeric compositions were determined to involve the 
(6E,8Z,11Z,14Z)5- (11%), (5Z,9E,11Z,14Z)8-hydroxy- 
(14%), (5Z,7E,11Z,14Z)-9-hydroxy- (16%), 
(5Z,8Z,12E,14Z)-11- (16%), (5Z,8Z,10E,14Z)-12- (17%), and 
(5Z,8Z,11Z,13E)-15-isomers (26%).
Decomposition occurred during the NaNBH$_4$ treatment, so the accurate isomeric compositions were uncertain.

A mixture of six positional isomers of 1-stearoyl-
2-[(8a-dioxy-α-tocopherol)-eicosatetraenoyl]-3-sn-PC (4).
UV $^{\lambda}_{max}$ (ethanol nm) (ε): 235 nm (32,300). FAB- 
MS m/z: 1271 (MH$^+$), 842 ([MH - 429]$^+$), 824, 809 
([MH - 462]$^+$), 578, 506, 430 ([α-tocopherol]$^+$), 290, 
224 ([CH=CH-CH$_2$O-phosphocholine]$^+$), 184, 165 
([phosphocholine]+), 104 ([choline]+). NMR $^{1}H$ 
(CDCl$_3$): 0.80-0.92 (18H, m), 1.07 and 1.25 (3H, s), 
1.15 (3H, m), 1.2-1.35 (52H, m), 1.4-1.8 (4H, t, 
J=7 Hz), 2.60 (2H, m), 2.81 (4H, m), 3.35 (9H, s), 3.77 
(2H, m), 4.00 (2H, m), 4.13 (1H, m), 4.21 (1H, m), 4.37 
(2H, m), 4.43 (1H, m), 5.22 (1H, m), 5.37 (4H, m), 5.4-5.5 
(2H, m), 5.9-6.1 H, m), 6.3-6.5 (1H, m). The isomeric compositions were determined to involve the 
(6E,8Z,11Z,14Z)5- (11%), (5Z,9E,11Z,14Z)8-hydroxy- 
(14%), (5Z,7E,11Z,14Z)-9-hydroxy- (16%), 
(5Z,8Z,12E,14Z)-11- (16%), (5Z,8Z,10E,14Z)-12- (17%), and 
(5Z,8Z,11Z,13E)-15-isomers (26%).
Decomposition occurred during the NaNBH$_4$ treatment, so the accurate isomeric compositions were uncertain.

Peroxidation of liposomes. Large unilamellar liposomes containing α-tocopherol were prepared by the ex- 
trusion method. Mixtures of PC and α-tocopherol (0.1 mol%), based on PC) were suspended in a 50 mm 
sodium phosphate buffer (pH 7.4, containing 50 mm 
NaCl). The resulting milky suspension was transferred to LiposoFast apparatus (Avestin; Ottawa, Canada), ex- 
truded 19 times back and forth through a polycarbonate membrane (100 nm pore size), and diluted with the same 
buffer to give a final PC concentration of 10 mm. After preincubating for 5 min at 37°C in a shaking water 
bath, either AAPH (a final concentration of 2 mm) or a mixture of Fe(III)-EDTA and ascorbic acid (final concen- 
trations of 0.1 mm and 2 mm, respectively) was added to the liposomes. All experiments were conducted at least three times.

Quantification. At given time points (0–4 h), a 500-μl 
aliquot of each reaction mixture was withdrawn, 25 μl 
of 0.2 M Na$_2$EDTA was added, and the mixture extract- 
ted twice with two volumes of hexane-2-propanol (3:2, 
v/v). Each extract was evaporated to dryness in vacuo, 
and the lipid residue dissolved in ethanol. PC-OOH, α- 
tocopherol, and 8a-(PC-dioxy)-α-tocopherones were 
quantified by reversed-phase HPLC with UV or fluores- 
cence detection. To analyze the other products of 
α-tocopherol, PC and its oxidation products were re- 
moved by solid-phase extraction before the HPLC analy- 
ysis. HPLC was done with a μBondapack 5 μC8 
column (3.9 × 150 mm, Nihon Waters; Tokyo, Japan), 
using methanol-acetonitrile-water (70:25:5, v/v) at 
1.0 ml/min and monitoring for absorbance at 280 nm, 
and γ-tocopherol was used as the internal standard. In 
another experiment, each reaction mixture (500-μl) was treated with 1 m HCl (0.1 ml) prior to extraction and the 
hydrolysis products were then analyzed to detect oxid- 
ized α-tocopherol present as tocopherone com- 
pounds.

Results

Isolation and characterization of 8a-(PC-dioxy)-α- 
tocopherones

Figure 1 shows HPLC traces of the reaction products of 
α-tocopherol with peroxy radicals derived from PCs in 
the presence of AMVN. Along with a peak corre- 
sponding to the α-tocopherol spirodiene dimer, peaks 
corresponding to the addition products of α-tocopherol 
with each PC appear on the chromatograms. Their struc- 
tures were identified as described in the Materials and 
Methods section; two positional isomers of 1-palmityl-
2-[9-(8a-dioxy-α-tocopherol)-octadecadienoyl]-
and 1-palmityl-2-[13-(8a-dioxy-α-tocopherol)-octadeca-
dienoyl]-3-sn-PCs (1), four positional isomers of 1-
palmityl-2-[8a-dioxy-α-tocopherol]-octadecatrien-
yl]-3-sn-PC (2), and six positional isomers of 1-
palmityl-2-[8a-dioxy-α-tocopherol]-eicosatetraen-
yl]-3-sn-PC (3) or 1-stearoyl-2-[8a-dioxy-α-tocophe-
rol]-eicosatetraenyl]-3-sn-PC (4), respectively (Fig. 2).

Peaks 1a and 1b, 3a-d, and 4a-d were isolated by 
reversed-phase HPLC and their isomeric compositions 
were analyzed by normal-phase HPLC after NaNBH$_4$ 
reduction and transmethylation. Peak 1a contained 
the 13-isomer and 1b the 9-isomer; peak 3a or 4a con- 
tained the 15-isomer, 5b or 4b the 11- and 12-isomers, 3c 
or 4c the 8- and 9-isomers, and 3d or 4d the 5-isomer of 
the corresponding 8a-(PC-dioxy)-α-tocopherone (data 
not shown).

Reaction of α-tocopherol during the peroxidation of 
PC in liposomes

The unilamellar liposomes of DMPC, PLPC and 
PACP containing α-tocopherol were reacted with peroxy- 
radicals that had been generated in the aqueous phase 
by the thermal decomposition of AAPH. Figure 3 shows 
the results of the formation of PC-OOH, α- 
tocopherol consumption, and production of the 
α-tocopherol products. α-Tocopherol suppressed the for- 
mation of PC-OOH until all the α-tocopherol had been 
depleted in the PLPC and PACP liposomes (Figs. 3B 
and 3C). The detected reaction products of α-tocophe-
rol were 8a-(PC-dioxy)-α-tocopherones, α-toco-
pheroylquinone, and 2,3- and 5,6-epoxy-α-tocopheryl-
quinones. The spirodiene dimer of α-tocopherol did not 
appear under the present experimental conditions.
Tocopherolquinone and epoxy-α-tocopherolquinones were also detected in saturated DMPC liposomes (Fig. 3A), so these products might have been produced from the reaction between α-tocopherol and the AAPH-derived peroxyl radicals. On the other hand, the reaction products of α-tocopherol with the PC-derived peroxyl radicals, 8a-(PC-dioxy)-α-tocopherones, appeared in the PLPC and PAPC liposomes (Figs. 3B and 3C). In another experiment, the sample was treated with 1 M HCl prior to extraction and the amounts of α-tocopherol and its oxidation products were determined (Table 1). Treatment with HCl released additional α-tocopherolquinone and epoxy-α-tocopherolquinones, presumably from the 8a-substituted α-tocopherol and epoxy-α-tocopherone precursors.14

Incubation of the PC liposomes with Fe(III)-EDTA and ascorbic acid caused α-tocopherol consumption (Fig. 4). The α-tocopherol in the DMPC liposomes grad-
Fig. 3. Reaction of α-Tocopherol during the AAPH-Initiated Peroxidation of PC in Liposomes.

Large unilamellar liposomes of DMPC (A), PLPC (B), and PAPC (C) containing 0.1 mol% α-tocopherol were incubated at 37°C under air. Peroxidation was initiated by 2 mM AAPH. The formation of PC-OOH with (●) or without (○) α-tocopherol, the consumption of α-tocopherol (○), and the formation of α-tocopherol products 8a-(PC-dioxy)-α-tocopherones (●), α-tocopherylquinone (◇), 2,3-epoxy-α-tocopherylquinone (△), and 5,6-epoxy-α-tocopherylquinone (▼) are shown. Each value is expressed as the mean ± standard deviation of three separate experiments.

Fig. 4. Reaction of α-Tocopherol during the Fe(III)-EDTA and Ascorbate-Initiated Peroxidation of PC in Liposomes.

Large unilamellar liposomes of DMPC (A), PLPC (B), and PAPC (C) containing 0.1 mol% α-tocopherol were incubated at 37°C under air. Peroxidation was initiated by a mixture of 0.1 mM Fe(III)-EDTA and 2 mM ascorbic acid. The formation of PC-OOH with (●) or without (○) α-tocopherol, the consumption of α-tocopherol (○), and the formation of α-tocopherol products 8a-(PC-dioxy)-α-tocopherones (●), α-tocopherylquinone (◇), 2,3-epoxy-α-tocopherylquinone (△), and 5,6-epoxy-α-tocopherylquinone (▼), are shown. Each value is expressed as the mean ± standard deviation of three separate experiments.

**U**naturally disappeared and α-tocopherylquinone was formed as the reaction product (Fig. 4A). Almost the same amounts of α-tocopherylquinone also appeared in the PLPC and PAPC liposomes (Figs. 4B and 4C). In addition, α-tocopherol inhibited the formation of PC-OOH in these unsaturated PC liposomes, and 8a-(PC-dioxy)-α-tocopherones and 2,3-epoxy- and 5,6-epoxy-α-tocopherylquinones were detected. The products formed in the 4-h samples of PLPC and PAPC only accounted for 31% and 27% of the consumed α-tocopherol, respectively. In particular, the 8a-(PC-dioxy)-α-tocopherones had low yields compared with
those in the AAPH-initiated peroxidation systems.

Discussion

α-Tocopherol is well known for acting as the major lipid-soluble chain-breaking antioxidant in biological systems by virtue of its ability to react with lipid peroxyl radicals to terminate the peroxidative process.4-6 Much interest has been shown in the products of its reactions with lipid peroxyl radicals.7-18 We have already isolated and characterized 8a-(lipid-dioxo)-α-tocopherones as reaction products of the α-tocopheroxyl radical with lipid peroxyl radicals in a solution.10,11,15 Bulk phase,11,15 and liposomes.16,17 In the DLPC liposomal systems, the DLPC-derived peroxyl radicals reacted with α-tocopherol to yield the 8a-substituted α-tocopherones.16 However, DLPC is relatively rare in a biological membrane when compared to molecular species of PC with a saturated fatty acid esterified at the sn-1 position of glycerol and an unsaturated fatty acid at the sn-2 position.22 In the present study, we prepared and characterized the 8a-(PC-dioxo)-α-tocopherones as reaction products of α-tocopheroxyl radicals with peroxyl radicals of PLPC, PLnPC, PCAP and SAPC. The PC-peroxyl radicals derived from each PC species could attack the tocopheroxyl radicals to form the corresponding 8a-(PC-dioxo)-α-tocopherones (Figs 1 and 2).

Autoxidation of the unsaturated lipid (LH) in the presence of an initiator (In) and inhibition by α-tocopherol (TH) in liposomes proceeds by the following reactions:14,35

\[
\begin{align*}
\text{Initiation} & : \text{LH} + \text{In} \rightarrow \text{L}^\cdot + \text{InH} \quad (1) \\
\text{Propagation} & : \text{L}^\cdot + \text{O}_2 \rightarrow \text{LOO}^\cdot \quad (2) \\
\text{Termination} & : \text{LOO}^\cdot + \text{LH} \rightarrow \text{LOOH} + \text{L}^\cdot \quad (3) \\
& : \text{LOO}^\cdot + \text{TH} \rightarrow \text{LOOH} + \text{T}^\cdot \quad (4) \\
& : \text{LOO}^\cdot + \text{T}^\cdot \rightarrow \text{non-radical products} \quad (5)
\end{align*}
\]

If LH is PC, peroxyl radicals (LOO·) can be produced by oxygen addition of the pentadienyl radicals (L·) of PC:25 9- and 13-peroxyl radicals of the linoleoyl moiety from PLPC, 9-, 12-, 13- and 16-peroxyl radicals of the linolenoyl moiety from PLnPC, and 5-, 8-, 9-, 11-, 12- and 15-peroxyl radicals of the arachidonoyl moiety from PCAP and SAPC. α-Tocopherol (TH) can terminate the propagation reaction by reactions 4 and 5. Reaction 5 results the formation of addition products of the α-tocopheroxyl radical (T·) with the peroxyl radicals of PC (LOO·). Our results indicate that the peroxyl radicals of PLPC, PLnPC, PCAP and SAPC can attack the tocopherylxyl radicals at the 8a-position to form all possible 8a-(PC-dioxo)-α-tocopherones as non-radical products (Fig. 2). Therefore, this mechanism is consistent with the behavior of α-tocopherol as a chain-breaking antioxidant in PC liposomes. Alternatively, there is another pathway to scavenge peroxyl radicals by α-tocopherol rather than the formation of 8a-(PC-dioxo)-α-tocopherones which involves the formation of 2,3- and 5,6-epoxy-α-tocopherylquinones.15,14,17,36 Epoxy formation is thought to proceed via the initial addition of a peroxyl radical to either the 5- or 7-position of the α-tocopheroxyl radical, with subsequent elimination of an alkoxyl radical and the addition of another peroxyl radical to the 8a-position of the epoxy-α-tocopheryl radical.15 The formation of these epoxy compounds results in the net consumption of two peroxyl radicals.37 The major oxidative pathway for α-tocopherol varies between model systems, although the mechanism is still unclear. In a polar medium or a liposome system, epoxy-α-tocopherones and their precursors, epoxyhydroperoxy-α-tocopherones, are the major products,12,14,36 whereas 8a-substituted α-tocopherones predominate in a non-polar medium.10

The employment of large unilamellar vesicles in peroxidation studies has been widely documented in the literature, because they are considered appropriate structural models for biological membranes and suitable as targets for peroxidation.31,38 When membrane peroxidation is initiated by a constant flux of peroxyl radicals from the thermal decomposition of water-soluble AAPH, α-tocopherol could scavenge the AAPH-derived peroxyl radicals to form α-tocopherylquinone and epoxy-α-tocopherylquinones in DMPC liposomes (Fig. 3A). Since PLPC and PCAP exist in larger amounts than α-tocopherol, most of the AAPH-derived peroxyl radicals would attack the PC molecules, and the resulting PC-peroxyl radicals would react with α-tocopherol.11,16 However, the results here indicate that the formation of 8a-(PC-dioxo)-α-tocopherones was only 6-10% of the consumed α-tocopherol (Figs 3B and 3C). The mobility of α-tocopherol and of the PC-peroxyl radicals in the liposomes is restricted by their long side chains. Thus, the AAPH-derived peroxyl radicals produced in the aqueous phase might have been scavenged by α-tocopherol before the reaction with unsaturated PC to form the addition products of α-tocopherol with AAPH-derived peroxyl radicals. Although we could not detect such compounds under the present HPLC conditions, the additional formation of α-tocopherylquinone and epoxy-α-tocopherylquinones by the HCl treatment indicates the presence of such 8a-substituted tocopherone and epoxytocopherone precursors (Table 1). Moreover, the amount of 8a-(PC-dioxo)-α-tocopherones formed in PLPC liposomes was about half that in the previous DLPC liposomes.17 The liposomal concentration of linoleate is two times higher for DLPC than for PLPC, and the formation of 8a-(PC-dioxo)-α-tocopherones might thus be favored for DLPC.

The oxidation system of Fe(III)-EDTA and ascorbate is a more accurate model of biological lipid peroxidation39-41 than of lipid peroxidation initiated by a radical initiator. Iron-dependent α-tocopherol oxidation in the unsaturated PC liposomes yielded 8a-(PC-dioxo)-α-tocopherones, α-tocopherylquinone, and epoxy-α-tocopherylquinones (Fig. 4), all of which were formed in the AMVN- and AAPH-initiated oxidation processes.33,17 Thus, α-tocopherol in the iron-dependent system is oxidized by peroxyl radicals of the unsaturated PC molecules. On the other hand, the production of α-tocopherylquinone in both the saturated and unsaturated PC liposomes was almost the same (Fig. 4). Yamamoto and Nik15 have reported that α-tocopherol incorporated into liposomal membranes could reduce the Fe(III) ion to give the Fe(II) ion. Therefore, α-
tocopherolquinone detected in the present iron-dependent system might have been produced from the oxidation of α-tocopherol by the Fe(III) ion. The products formed in the iron-dependent system only accounted for a small part of the α-tocopherol that was consumed. Thus, the produced products might have been decomposed immediately by iron-catalyzed oxidation.

The antioxidative reaction of α-tocopherol in a perfused rat liver model has recently been reposted. The principal products formed were 8a-substituted α-tocopherones and epoxy-α-tocopherones, rather than their hydrolysis products. However, this finding was only evident from the release of additional α-tocopherolquinone or epoxy-α-tocopherolquinones in HCl-treated samples. Further studies on the identification of 8a-substituted α-tocopherones in an intact organ system are needed to elucidate the antioxidative activity of α-tocopherol.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 08660151) from the Ministry of Education, Science, Sports and Culture of Japan.

References


