Inhibitory Effects of Catechol Derivatives on Oxidation of Soybean Phosphatidylcholine Liposomes Induced by Hydrophilic and Hydrophobic Free Radical Initiators

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Effects of pyrocatechol and six of its monosubstituents on oxidation of soybean phosphatidylcholine liposome induced by hydophilic free radical initiator, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), and hydrophobic free radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), were determined and relationships with their redox potentials and hydrophobic parameters were studied for their inhibitory effects by regression analysis. Inhibitory potencies of the catechol compounds on hydrophilic AAPH-induced oxidation clearly correlated with their redox potentials, whereas no significant effects of the hydrophobicities of the compounds were found. These results indicated that scavenging activities of these compounds on AAPH-initiated radical chain reactions were controlled by their electron donor activities. Inhibitory potencies of these compounds on hydrophobic AMVN-induced oxidation of liposome, in contrast, were significantly correlated with their hydrophobicity, although regression analysis revealed that their electron donor activities were also important.

Key words lipid peroxidation; catechol derivative; structure–activity relationship; radical scavenger; redox potential; hydrophobicity

The nonenzymatic, free radical-mediated oxidations of biological membranes have been suggested to be associated with the pathogenesis of various tissue injuries.[1,2] It has been revealed that various phenolic antioxidants like flavonoids, tannins and α-tocopherol scavenge free radicals such as superoxide anion radicals and lipid peroxyl radicals and thus prevent cell damage.[3–6] These compounds are viewed as promising therapeutic drugs for free radical pathologies like ischemia, anemia and arthritis.[5] In order to identify the mechanisms of free radical-mediated oxidations of biological membranes and inhibitory mechanisms of antioxidants against the oxidation, artificial phospholipid liposome has been a good model system.[7,8] It has been found that the phospholipids in liposomal membranes are oxidized by a free radical chain mechanism and that antioxidants such as α-tocopherol suppress the peroxidation.[9–11]

Although there have been many studies on free radical scavenging activities of these antioxidants and their scavenging mechanisms, there have been few reports on the physico-chemical properties of these compounds which govern their free radical scavenging abilities. We previously stated that superoxide anion scavenging activities of catechol derivatives are controlled by their electron donor activities.[12] For inhibitory activities of antioxidants, which interact with peroxyl radicals, against free radical-induced oxidation of membrane lipids, two physico-chemical properties seem to be important: strong electron donor activities and large partition coefficients between water and lipid bilayer are apparently essential for their free radical scavenging processes. However, which property is dominant in the inhibition of the antioxidants on free radical-induced lipid peroxidation may depend on the environment in which free radical reactions are initiated; that is, it may be important whether the free radical is generated in the aqueous phase or in the lipid bilayer. In this work we attempted to clarify the inhibitory effects of monosubstituents of pyrocatechol on hydrophilic and hydrophobic free radical-induced lipid peroxidation using 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as a water-soluble and oil-soluble radical initiator, respectively. A water-soluble AAPH generates aqueous free radicals and induces chain oxidation of lipids, whereas a lipid-soluble AMVN generates free radicals in the lipid bilayer and induces chain oxidation of lipids.[13] In this work the relationships of the inhibitory effects of monosubstituents of pyrocatechol on the free radical-mediated chain oxidation of soybean phosphatidylcholine liposome were examined quantitatively by regression analysis using two physicochemical parameters of each compound, redox potentials and hydrophobic parameter.

Experimental

Materials Azo compounds, AAPH and AMVN, and sodium salt of L-ascorbic acid were purchased from Wako Pure Chemical Industries (Osaka). All catechol compounds were from Wako Pure Chemicals and Nakarai Tesque, Inc. (Kyoto, Japan). Commercial soybean phosphatidylcholine purchased from Daigo Chemical Co. (Osaka) was purified by silica-gel columns and its purity was ascertained on thin layer chromatography.[14]

Preparation of Liposome Purified phosphatidylcholine mentioned above was dissolved in chloroform either with or without AMVN. Chloroform was removed by evacuation to obtain a thin film on a flask wall. Appropriate amount of medium (100 mM NaCl, citric acid–sodium phosphate buffer (pH 7.0)) was added and liposomes of the lipids were prepared by vortex mixing, followed by probe-type sonication at a power of 50W for 10 min at 0 °C under a stream of nitrogen.

Measurement of Lipid Peroxidation When the water-soluble initiator, AAPH, was used, it was injected into the liposome suspension to be a final concentration of 37 mM. When AMVN was used, its final concentration was 1.88 mM. Final lipid concentration in liposome suspension was 3.75 mM. The oxidation induced by AAPH or AMVN was carried out at either 37 or 50 °C, respectively. As soon as all the reagents had been added, the reaction vessel was sealed off from the atmosphere. The reaction mixture was agitated with a magnetic stirrer and the rate of oxygen consumption was measured continuously by an oxygen meter, Oxygraph Model 8 (Central Kagaku Co., Tokyo), equipped with an oxygen electrode. Oxygen consumption in the absence

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of liposome due to thermal degradation of the radical initiators was negligible.

Results

Inhibitory Effects of Catechol Derivatives on AAPH-Induced Lipid Peroxidation in Liposome We first examined the effects of pyrocatechol and six of its monosubstituents on oxidation of soybean phosphatidylcholine liposome induced by hydrophilic free radical initiator, AAPH. As shown in Fig.1 for pyrocatechol, oxidation of lipids induced by AAPH was inhibited by catechol derivatives dose dependently. Oxidation rate was reduced by these compounds. The concentrations of the catechol compounds causing 50% reduction in oxygen consumption rate, IC_{50} (AAPH), are shown in Table 1 together with their redox potential, E_0, and hydrophobic parameter, π. Among catechol derivatives tested, the activity of 3-OH substituent (pyrogallol) was strongest, although it was less than that of the polyhydric phenol, quercetin. Similar order of the inhibitory potencies of these catechol compounds was also observed at lower concentration ratios of AAPH and soybean phosphatidylcholine and in the presence of cholesterol in liposome (data not shown).

We next sought to determine the relation of the physicochemical properties of the derivatives was examined with their inhibitory potencies on lipid peroxidation. The correlation between their inhibitory potencies with two parameters of the derivatives: redox potential and hydrophobic parameter by regression analysis. Inhibitory potencies of the catechol derivatives were expressed as logarithm values of $1/IC_{50}$(AAPH). The values of redox potential, E_0, of seven catechol derivatives including 4-CH=CH–COO^- (caffeic acid) were cited from Horner and Geyer[15] and the values of the hydrophobic parameter, π, of the six derivatives were taken from Leo *et al.*[16] The relation between the inhibitory potencies and these parameters are shown in Eqs.1 through 4:

$$\log(1/IC_{50}(\text{AAPH}))(M^{-1}) = -3.51E_0 + 7.85 \quad (n=7,\ r=0.97,\ s=0.20,\ F=80.4) \tag{1}$$

When datum of caffeic acid was excluded,

$$\log(1/IC_{50}(\text{AAPH}))(M^{-1}) = -3.45E_0 + 7.81 \quad (n=6,\ r=0.97,\ s=0.18,\ F=66.7) \tag{2}$$

$$\log(1/IC_{50}(\text{AAPH}))(M^{-1}) = 0.029\pi + 5.18 \quad (n=6,\ r=0.32,\ s=0.02,\ F=0.46) \tag{3}$$

$$\log(1/IC_{50}(\text{AAPH}))(M^{-1}) = -3.75E_0 - 0.016\pi + 8.04 \quad (n=6,\ r=0.98,\ s=0.09,\ F=46.7) \tag{4}$$

Here, n is the number of compounds tested, r is the correlation coefficient, s is the standard deviation, and F is the ratio between regression and residual variances. Equations 1—4 and Fig. 2 reveal that the inhibitory potencies of the catechol derivatives clearly correlate with their redox potential and that the derivatives with lower redox potentials have stronger inhibitory effect on lipid peroxidation. On the other hand, the inhibitory potencies did not depend on the hydrophobicity of the compounds. These results indicate that inhibitory potencies of the catechol derivatives on lipid peroxidation initiated by hydrophilic free radical initiator are controlled by their electron donor activities.

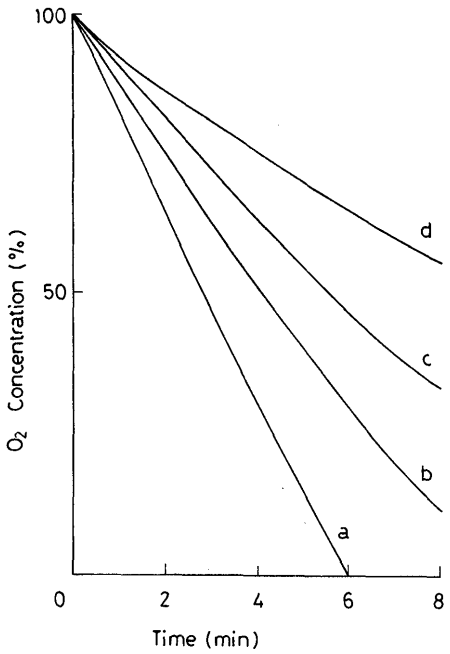
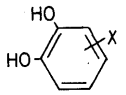


Fig. 1. Dose-Dependent Inhibition of AAPH-Induced Peroxidation of Soybean Phosphatidylcholine Liposome by Pyrocatechol

a, control; b, 5 μM; c, 10 μM; d, 20 μM.

Table 1. Concentrations of Catechol Derivatives Causing 50% Reduction in Oxygen Consumption Rate Induced by AAPH, IC_{50}(AAPH), Those by AMVN, IC_{50}(AMVN), Their Redox Potentials, E_0, and Their Hydrophobic Parameter, π[a]



X	IC_{50}(AAPH) (μM)[b]	IC_{50}(AMVN) (μM)[b]	E_0 (V)[c]	π[d]
H	10.4±2.1 (4)	25.4±1.3 (3)	0.795	0.00
4-CH_3	5.9±1.3 (4)	6.7±1.2 (3)	0.753	0.56
4-$C(CH_3)_3$	5.3±1.3 (3)	3.9±0.6 (3)	0.732	1.98
4-Cl	9.5±0.6 (4)	10.5±0.8 (3)	0.801	0.71
3-OH	3.3±0.5 (3)	12.4±0.6 (3)	0.680	−0.67
4-COOH	10.2±1.4 (4)	350 ±14 (3)	0.833	−4.36
4-CH=CH–COOH	9.3±0.5 (4)	169 ±12 (3)	0.794	—
Ascorbic acid	9.0±0.4 (3)[e]	n.o.[f]		
Quercetin	0.26±0.04 (4)	3.0±0.7 (3)		

a) Inhibitory potencies of ascorbic acid and quercetin are also listed for comparison. *b*) Numbers represent means±S.D. of results from 3—4 (numbers in parentheses) different preparations. *c*) Cited from reference 15. *d*) Cited from reference 16. *e*) Concentration causing two-fold increase in time to complete oxygen consumption was defined as IC_{50} value. *f*) 50% inhibition was not observed at any concentration less than 2 mM.

Inhibitory Effects of Catechol Derivatives on AMVN-Induced Lipid Peroxidation in Liposome We next examined the effects of the same compounds on oxidation of soybean phosphatidylcholine liposome induced by the hydrophobic radical initiator, AMVN. As shown in Table 1 for concentrations which caused 50% decrease of oxygen consumption rate, IC_{50} (AMVN), inhibitory potency of the 4-$C(CH_3)_3$ substituent was strongest, while, those of anionic 4-COO^- substituent (protocatechuic acid) and

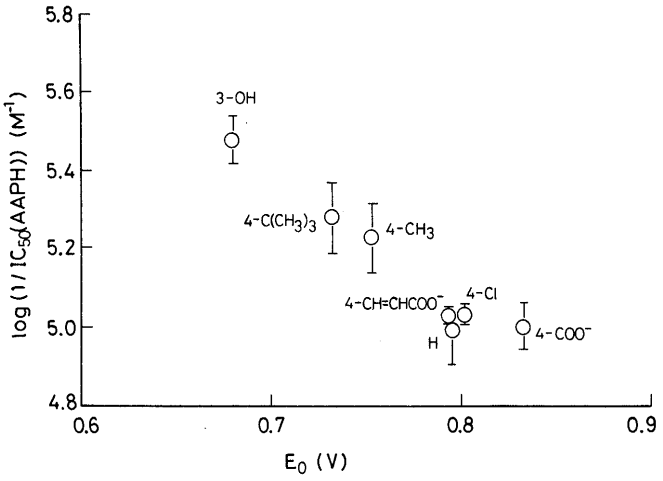


Fig. 2. Relation between Values of Redox Potential, E_0, of Substituents of Pyrocatechol and Their Inhibitory Effects on Lipid Peroxidation Induced by Hydrophobic Radical Initiator, AAPH, Expressed as $1/IC_{50}$(AAPH) Values

4-CH=CH–COO$^-$ substituent (caffeic acid) were much weaker than others. Inhibitory potencies of these anionic substituents on AMVN-induced oxidation were also very weak compared with those of the same substituents on hydrophilic AAPH-induced lipid peroxidation, just like ascorbic acid shown in Table 1. Similar order of the inhibitory potencies of these catechol compounds was also observed at lower ratio of concentrations of AMVN and soybean phosphatidylcholine and in the presence of cholesterol in liposome (data not shown).

Like the AAPH-induced oxidation of liposomes described above, we examined the correlation between the inhibitory potencies of the catechol derivatives with their redox potential and hydrophobic parameter (Eqs. 5 through 8).

$$\log(1/IC_{50}(AMVN))(M^{-1}) = -9.33E_0 + 11.79$$
$$(n=7,\ r=0.66,\ s=1.39,\ F=3.77) \quad (5)$$

When datum of caffeic acid was excluded,

$$\log(1/IC_{50}(AMVN))(M^{-1}) = -8.18E_0 + 11.02$$
$$(n=6,\ r=0.65,\ s=1.02,\ F=2.97) \quad (6)$$

$$\log(1/IC_{50}(AMVN))(M^{-1}) = 0.307\pi + 4.85$$
$$(n=6,\ r=0.96,\ s=2.23,\ F=53.42) \quad (7)$$

$$\log(1/IC_{50}(AMVN))(M^{-1}) = -3.16E_0 + 0.269\pi + 7.26$$
$$(n=6,\ r=0.99,\ s=1.17,\ F=72.6) \quad (8)$$

Equations 5 through 8 and Fig. 3 reveal that catechol derivatives with larger hydrophobicity have stronger inhibitory effects on hydrophobic AMVN-induced lipid peroxidation in liposome. As shown in Fig. 4 and Eqs. 5 and 6, the tendency was also found that derivatives with lower redox potential have greater inhibitory effect, although the correlation was not as good as that between inhibitory effects and hydrophobic parameter. Therefore, as shown in Eq. 8, the addition of the redox potential term improved the quality of the correlation. Thus, the inhibitory potencies of the catechol derivatives on lipid peroxidation of liposome initiated by hydrophobic free radical, which is present in the lipid bilayer, are controlled by their hydrophobicity as well as their electron donor activities.

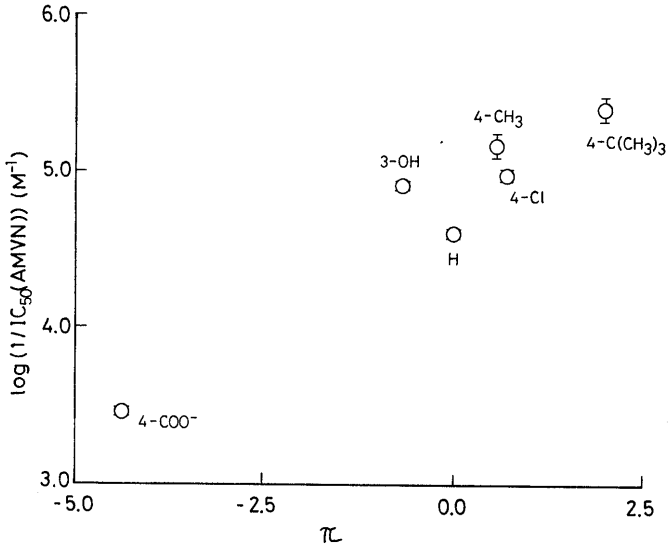


Fig. 3. Relation between Values of Hydrophobic Parameter, π, of Substituents of Pyrocatechol and Their Inhibitory Effects on Lipid Peroxidation Induced by Hydrophobic Radical Initiator, AMVN, Expressed as $1/IC_{50}$(AMVN) Values

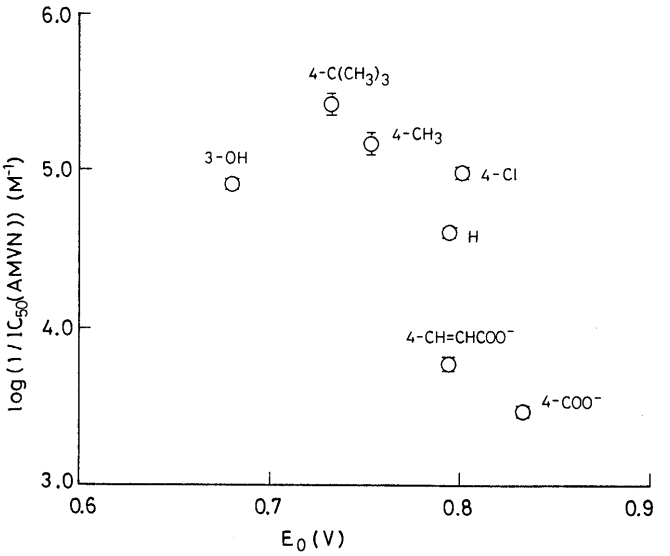


Fig. 4. Relation between Values of Redox Potential, E_0, of Substituents of Pyrocatechol and Their Inhibitory Effects on Lipid Peroxidation Induced by Hydrophobic Radical Initiator, AMVN, Expressed as $1/IC_{50}$(AMVN) Values

Discussion

Azo compounds decompose thermally to give carbon-centered radicals,

$$RN{=}NR \rightarrow N_2 + 2R\cdot \quad (9)$$

When water-soluble AAPH is used as a radical initiator, the radicals are formed initially in an aqueous phase by its thermal decomposition. When hydrophobic AMVN is used, the radicals are generated within the lipid bilayer. Most of the carbon radicals formed react with oxygen to give peroxyl radicals,[7,8,11,17]

$$R\cdot + O_2 \rightarrow ROO\cdot \quad (10)$$

These peroxyl radicals attack lipids in liposomes and abstract active, doubly-allylic hydrogens from the lipids to initiate free radical chain oxidation,[7,11,17]

$$ROO\cdot + LH \rightarrow ROOH + L\cdot \quad (11)$$

$$L\cdot + O_2 \rightarrow LOO\cdot \quad (12)$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot \quad (13)$$

Antioxidants such as catechol derivatives interact with these peroxyl radicals shown as InH below,[7,8,11,17]

$$ROO\cdot(LOO\cdot) + InH \rightarrow ROOH(LOOH) + In\cdot \quad (14)$$

These compounds stop the radical chain reaction by attacking either alkyl peroxyl radicals or lipid peroxyl radicals.[7,8,11,17]

It has been reported that the water-soluble, chain-breaking antioxidants such as ascorbic acid and uric acid scavenge the oxygen radicals residing in the aqueous phase, as also shown in this study, while lipid-soluble, chain-breaking antioxidants like α-tocopherol scavenge the radicals predominantly within the membrane lipid bilayer.[18,19] Amphiphilic catechol compounds seem to be able to scavenge free radicals in both water and lipid bilayer. Therefore, when free radicals are initiated by water-soluble AAPH, these catechol compounds seem to interact with AAPH-initiated water-soluble peroxyl radicals in aqueous phase or lipid peroxyl radicals in lipid bilayer which are generated by the radical chain reactions described above. Analysis based on quantitative structure–activity relationship of catechol derivatives on AAPH-induced oxidation of lipids in liposome revealed that inhibitory potencies of the derivatives tested clearly depended on their electron donor activities. No significant effects of their hydrophobicity were observed. On the other hand, inhibitory potencies of the catechol compounds on lipid peroxidation induced by lipid soluble radical initiator depended on hydrophobicity of the compounds as well as on their electron donor activities. These results suggested that in hydrophilic AAPH-initiated lipid peroxidation interaction of the compounds with peroxyl radicals occurred mainly in the aqueous environment. Therefore, interaction with carbon radical-derived peroxyl radicals seems to control the inhibitory potencies of catechol compounds.

When free radicals are initiated by hydrophobic AMVN, however, catechol compounds interact with both hydrophobic peroxyl radicals derived from AMVN and lipid peroxyl radicals in a lipid environment. Since in AMVN-initiated lipid peroxidation such antioxidants as catechol compounds must interact peroxyl radicals in the lipid bilayer, dependence on hydrophobicity of the compounds in AMVN-initiated lipid peroxidation is reasonable. Dependence on hydrophobicity of catechol compounds for the activities in free radical related reactions in a hydrophobic environment is also reported for their inhibitory effects on 5-lipoxygenase activities.[20]

The results obtained here indicated that different physico-chemical property of antioxidants is required when free-radical reactions are initiated either in an aqueous environment or a hydrophobic environment. This finding should be kept in mind as useful antioxidants are developed to prevent free radical-induced disease.

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