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Structure-activity relationship of antioxidants for inhibitors of linoleic acid hydroperoxide-induced toxicity in cultured human umbilical vein endothelial cells

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Abstract

Structure-activity relationship of antioxidants for the protective effects on linoleic acid hydroperoxide (LOOH)-induced toxicity were examined in cultured human umbilical vein endothelial cells. α-Tocopherol, 2,2,5,7,8-pentamethylchroman-6-ol, butylated hydroxytoluene, probucol, and fatty acid esters of ascorbic acid provided efficient protection against the cytotoxicity of LOOH in pretreatment, but phenols without alkyl groups at the *ortho* positions and hydrophilic antioxidants such as Trolox and ascorbic acid provided no protection. Probably, the effectiveness of the protection against cytotoxicity by these antioxidants depends primarily on their rate of incorporation into cells due to their lipophilicity, secondly on their antioxidant activity, and thirdly on their orientation in biomembranes. On the other hand, flavones, such as baicalein and luteolin bearing 3 to 5 hydroxyl groups, and flavonols showed a protective effect against LOOH cytotoxicity when added together with LOOH, but not by pretreatment. Among catechins, (+)-catechin and (–)-epigallocatechin gallate monoglucoside and diglucoside were effective in suppressing LOOH-induced cytotoxicity, but their effects were not so strong. The structure-activity relationship of flavonoids revealed the presence of either the *ortho*-dihydroxy structure in the B ring of flavonoids or the 3-hydroxyl and 4-oxo groups in the C ring to be important for the protective activities. Furthermore, coumarins such as esculetin containing the *ortho* catechol structure had protective effects in both pretreatment and concurrent treatment. These results suggest that *ortho* catechol moiety of flavonoids, catechins, and coumarins is an important structure in the protection against LOOH-induced cytotoxicity, and that the alkyl groups of monophenols are critical for protection.

Abbreviations: BHT, butylated hydroxytoluene; C, (+)-catechin; EC, (–)-epicatechin; ECg, (–)-epicatechin gallate; ECGF, endothelial cell growth factor; EGC, (–)-epigallocatechin; EGCg, (–)-epigallocatechin gallate; EGCg-G1, (–)-epigallocatechin gallate monoglucoside; EGCg-G2, (–)-epigallocatechin gallate diglucoside; EGF, epidermal growth factor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; LOOH, linoleic acid hydroperoxide; NDGA, nordihydroguaiaretic acid; PMC, 2,2,5,7,8-pentamethylchroman-6-ol; WST-1, 4-[3(4-iodophenyl)-2-(4-nitrophenyl)-*2H*-5-tetrazolio]-1,3-benzene disulfonate.

Introduction

Active oxygen species are thought to be the causative agents of various diseases, including cardiovascular diseases and cancer, and even accelerating factors in the aging process (Horton and Fairhurst, 1987; Esterbauer and Cheeseman, 1987). Today, vascular diseases and cancer are major causes of death in the aged. If excess active oxygen species are not scavenged by biological antioxidant defense systems, they can cause

oxidative damage to *in vivo* components such as lipids, proteins, and DNA. For example, according to the response to injury hypothesis, injury to the endothelial cells lining blood vessel walls by oxidative damage has been proposed as the initial event in the etiology of atherosclerosis (Ross and Glomset, 1993).

It has become apparent that endothelial cells are damaged by hydrogen peroxide (Weiss et al., 1981), lipid hydroperoxides (Sasaguri et al., 1984), and oxidized low-density lipoprotein (Morel et al., 1984). We have also reported that linoleic acid hydroperoxide (LOOH) and its degradation products, unsaturated aliphatic aldehydes, injure cultured human umbilical vein endothelial cells (HUVEC) (Kaneko et al., 1988), and that phosphatidylcholine hydroperoxides show little or no toxicity in HUVEC (Kaneko et al., 1994a, 1996). Exploration for compounds that prevent the damage is of great importance, since oxidative damage caused by these species is thought to play an important role in a variety of the degenerative changes just described.

The most important biological antioxidants are α-tocopherol (vitamin E) (Burton et al., 1983) and ascorbic acid (vitamin C) (Tappel, 1968). α-Tocopherol has a phenolic hydroxyl group in its structure; it is located in cellular membranes and acts mainly as a protecting agent against the oxidation of unsaturated lipids. It has been considered that the antioxidant action of phenols depends on the hydrogen-donating capacity of a hydroxyl group in each molecule (Burton et al., 1985; Mukai et al., 1988). Since LOOH acts *in vivo* as an oxygen radical-generating agent, the protective effects of tocopherol homologs on LOOH-induced cytotoxicity are also presumed to depend on their hydrogen-donating capacity. On the other hand, ascorbic acid, which is hydrophilic, plays a role as an antioxidant in the aqueous phase, such as in plasma or cytosol. It may be difficult for ascorbic acid to trap radicals produced from lipid peroxides in biomembranes, but it might be effective as a scavenger of exogeous radicals or by interacting with α-tocopherol in the membranes. Furthermore, lipophilic derivatives of ascorbic acid might be effective in protecting against the toxicity of lipophilic LOOH.

Flavonoids comprise a group of natural polyphenolic compounds found in a wide variety of plant sources including vegetables, fruits, and teas. They have recently provoked interest because of their broad pharmacological activities. Flavonoids have been reported to be chain-breaking inhibitors of the peroxidation process due to their ability to scavenge oxygen radicals or intermediate alkoxyl radicals (Torel et al., 1986; Sichel et al., 1991). Several recent studies have reported that specific flavonoids, especially quercetin, scavenge superoxide radicals (Bors et al., 1990a) and hydroxyl radicals (Hanasaki et al., 1994; Zhou and Zheng, 1991), reduce lipid peroxyl radicals (Jovanovie et al., 1994), and inhibit lipid peroxidation (Fraga et al., 1987; Negre-Salvayre et al., 1991; Terao et al., 1994). Furthermore, quercetin, kaempferol, and taxifolin suppress the cytotoxicity of hydrogen peroxide in Chinese hamster V79 cells (Nakayama et al., 1993), and quercetin prevents the cytotoxicity of oxidized low-density lipoproteins in lymphoid cell lines (Negre-Salvayre and Salvayre, 1992). Catechins are one group of flavonoids contained in green tea leaves at concentrations as high as 14 to 18%. In recent years, catechins have also attracted much attention because of their antimutagenic and anticarcinogenic activities (Yoshizawa et al., 1987; Xu et al., 1992; Fujiki and Okuda, 1992). Many studies have indicated that catechins suppress lipid peroxidation in phospholipid bilayers (Terao et al., 1994), biological tissues (Fraga et al., 1987), and subcellular fractions (Okuda et al., 1983; Namiki and Osawa, 1986; Negre-Salvayre et al., 1991; Decharneux et al., 1992). Furthermore, catechins have been reported to protect against the cytotoxicity of hydrogen peroxide in cultured cells (Nakayama, 1994) and oxidative damage of rat liver homogenates or heme proteins caused by *tert*-butyl hydroperoxide (Chen and Tappel, 1995; Chen and Tappel, 1996). As mentioned above, numerous researchers have investigated the antioxidant ativity of flavonoids, and several attempts have been made to elucidate their structure-activity relationships. There is general agreement that flavonoids inhibit lipid peroxidation either by chelating transition metals (Afanas'ev et al., 1989) or by scavenging free radicals through the formation of less-reactive flavonoid aroxyl radicals (Afanas'ev et al., 1989; Cotelle et al., 1992). A number of flavonoids have been assayed as antioxidants according to their abilities to inhibit lipid peroxidation induced enzymatically or nonenzymatically (van Acker et al., 1996) and to react with chemically generated peroxyl radicals (Torel et al., 1996). Coumarins are also a group of phenolic compounds widely distributed in nature (Egan et al., 1990). Among these compounds, esculetin (6,7-dihydroxycoumarin) has been reported to protect against oxidative damage by acting as an inhibitor or quencher of reactive oxygen species (Hiramoto et al., 1996; Martín-Aragón et al., 1998). However, little is

known about effects of these antioxidants on the toxicity of previously prepared polyunsaturated fatty acid hydroperoxides, a primary product of lipid peroxidation, much less structure-activity relationship of antioxidants for inhibitors of the hydroperoxide-induced cytotoxicity. In this study, we examine the protective effects of these antioxidants against linoleic acid hydroperoxide (LOOH)-induced toxicity to cultured HUVEC and try to elucidate the structure-activity relationship of antioxidants.

Materials and methods

Materials

Butylated hydroxytoluene (BHT), 6-*O*-stearoylascorbic acid, kaempferol, quercetin, morin, and rutin were purchased from Wako Pure Chemical Industries (Osaka, Japan). RRR-α-Tocopherol, ascorbic acid, 7,8-dihydroxyflavone, and taxifolin were obtained from Sigma (St. Louis, MO, USA) and quercetagetin from Extrasynthèse (Genay, France). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), curcumin, nordihydroguaiaretic acid (NDGA), 2-*tert*-butyl-4-methylphenol, 2,4,6-trimethylphenol, 6-*O*-palmitoylascorbic acid, and esculetin were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 2-*tert*-Butyl-6-methylphenol, 2,6-di-*tert*-butylphenol and 2,6-*O*-dipalmitoylascorbic acid were purchased from Tokyo Chemical Industries (Tokyo, Japan). (+)-Catechin (C), (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECg), and (–)-epigallocatechin gallate (EGCg) were purchased from Kurita Kogyo Co. (Tokyo, Japan); (–)-epigallocatechin gallate monoglucoside (EGCg-G1) and (–)-epigallocatechin gallate diglucoside (EGCg-G2) were obtained from Kikkoman Co. (Chiba, Japan). An assay kit using 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-*2H*-5-tetrazolio]-1,3-benzene disulfonate (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate to measure cell viability was from Dojindo Laboratories (Kumamoto, Japan). Epidermal growth factor (EGF) was obtained from Collaborative Research Inc. (Walton, MA, USA), MCDB-104 medium was from Nihon Pharmaceutical Co. (Tokyo, Japan); and fetal bovine serum (FBS) was from Moregate Laboratories (Melbourne, Australia). Endothelial cell growth factor (ECGF) was isolated from newborn bovine brains by the method of Lobb and Fett (1984). Linoleic acid hydroperoxide (LOOH) (Kaneko and Matsuo, 1984) and 2,2,5,7,8-pentamethylchroman-6-ol (PMC) (Nilson et al., 1968) were prepared as described previously. Probucol and 2-*O*-octadecylascorbic acid (CV3611) were kindly supplied by Daiichi Seiyaku Co. (Tokyo, Japan) and Takeda Chemical Industries (Osaka, Japan), respectvely.

Cell culture

Endothelial cells were isolated from the veins of human umbilical cords as described previously (Kaneko et al., 1988). Cells were grown in gelatin-coated dishes at 37 °C under 5% CO_2 and 95% air in MCDB-104 medium supplemented with 10% FBS, 30 ng ml^{-1} ECGF, 10 ng ml^{-1} EGF, and 10 ng ml^{-1} heparin. Cells at passages 6–14 were used for experiments.

Estimation of the extent of cell injury

Endothelial cells were seeded at a density of 500 to 1000 cells per well in 96-multiwell plates and cultivated for 3 or 4 days. For preincubation studies, endothelial cells at confluency were treated for 24 h in culture medium containing antioxidants. Each antioxidant was dissolved in ethanol and diluted with the medium to a final concentration of 50 μM. Control cultures were treated with ethanol without antioxidants. The final ethanol concentration was less than 0.1% and had no effect on the cells. After treatment with antioxidants, the medium was changed to Earle's solution containing LOOH (50 μM) and the cells were further incubated for 3 h. In the case of concurrent treatment, cells were treated for 3 h in Earle's solution containing both LOOH and an antioxidant. Cell survival rates were determined by counting with a Coulter Counter ZM (Coulter Electronics, Hialeah, FL, USA) and the trypan blue exclusion test on cells removed from the dish by trypsinization (Kaneko et al., 1987) or by using a Cell Counting Kit (Dojindo Laboratories; Kumamoto, Japan) utilizing a coloration reaction based on the formation of a non-toxic formazan from the metabolic reduction of WST-1 by dehydrogenase activities in viable cells. The cytotoxic effect of LOOH and the protective effect of antioxidants against LOOH-induced cytotoxicity were assessed by comparing the cell survival rates with those of cells from the control experiment.

Statistical analysis

Statistical analyses were performed using Student's *t*-test. Experimental values are the means ± SD for the numbers of experiments indicated in the legends. *p*-values < 0.05 were considered statistically significant.

Results

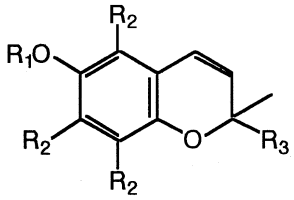
When HUVEC were pretreated with tocopherol homologs, α-tocopherol and PMC provided efficient protection against the cytotoxicity of LOOH, but tocol, Trolox, and α-tocopherol nicotinate showed no protection (Table 1) (Kaneko et al., 1994b). Under concurrent treatment of cells with both tocopherol homologs and LOOH, only PMC had a weak protective effect. The results of preincubation experiments in which α-tocopherol shows a protective effect while tocol does not suggest that the presence of alkyl groups on the aromatic ring is critical for the exertion of the protective effect of tocopherol homologs. In order to see the relationship between the protective effects of phenols and their alkyl substitutions, we examined the protective effects of simple, alkylated phenols on the cytotoxicity. As shown in Table 2, 2-*tert*-butyl-6-methylphenol and 2,6-di-*tert*-butylphenol, which have two alkyl groups at both positions *ortho* to a hydroxyl group, were slightly but significantly protective. BHT, which has two *tert*-butyl groups at the *ortho* positions and a methyl group at the *para* position, was very effective, although 2,4,6-trimethylphenol showed weak protection. On the other hand, in the case of concurrent treatment, 2-*tert*-butyl-6-methylphenol and 2,4,6-trimethylphenol were effective, while phenols having two *tert*-butyl groups at positions *ortho* to a hydroxyl group were quite ineffective. Propyl gallate, which has three phenolic hydroxyl groups side by side on its aromatic ring, was nearly without effect (Table 3) (Kaneko et al., 1994b). We also examined the protective effects of symmetric phenols with two aromatic rings at both molecular ends. Probucol showed a protective effect that was stronger than that of BHT but weaker than that of α-tocopherol. In the case of concurrent treatment with alkylphenols and polyhydroxyphenols, only curcumin showed weak protection.

Since ascorbic acid is hydrophilic, it was expected that it would provide some protection against the toxicity of LOOH when present in medium containing LOOH. Ascorbic acid itself had no protective effect even at even a concentration of 500 μM in either concurrent treatment or pretreatment; however, esterized derivatives, such as 6-*O*-palmitoylascorbic acid, 6-*O*-stearoylascorbic acid, and 2,6-*O*-dipalmitoylascorbic acid, and an alkylated derivative, CV3611, 2-*O*-octadecylascorbic acid, provided some protection against the toxic effect of LOOH (Table 4) (Kaneko et al., 1993). Dipalmitoylascorbic acid and CV3611 were more effective than the monoesters.

Table 5 shows the protective effects of flavonoids against LOOH-induced cytotoxicity (Kaneko and Baba, 1999). When cells were treated concurrently with flavonoids and LOOH, flavone, the fundamental skeleton with no hydroxyl groups in its structure, had no effect. Although 7-hydroxyflavone, chrysin, and apigenin also failed to protect, 7,8-dihydroxyflavone, baicalein, and luteolin protected against LOOH-induced toxicity. In particular, luteolin provided almost complete protection at 50 μM and apparent protection even at 2 μM (Figure 1). Flavonols are flavones bearing a hydroxyl group at the 3-position of the C ring. All the flavonols examined had a protective effect against the cytotoxicity of LOOH. Flavonols bearing three to five hydroxyl groups showed strong protection; in particular, quercetin was an extremely good protector of cells against LOOH-induced toxicity. Quercetin and kaempferol had protective effects at concentrations above 5 μM, and quercetin provided complete protection at 25 μM (Figure 1). However, the protective effects of quercetagetin and myricetin, which have six hydroxyl groups, were unexpectedly weak. The substitution of rutinose for a hydroxyl group at the 3-position of quercetin, that is rutin, resulted in the loss of the protective effect. Flavanones lacking the double bond between the 2- and 3-positions of the C ring had no protective effect in these treatments. In contrast, when cells were incubated with flavonoids for 24 h prior to treatment with LOOH, none of the flavonoids protected cells from LOOH injury.

Table 6 shows the survival rates of cells treated with LOOH and catechins (Kaneko et al., 1998). EGCg-G1 and EGCg-G2, which are prepared by substituting glucose for the 4′-hydroxyl group and the hydroxyl group of gallate at the 3-position, respectively, have been reported to shows increased stabilities in aqueous solution (Kitao et al., 1995). Thus, significant protection was expected by these glucoside derivatives. C, EGCg-G1, and EGCg-G2 showed significant protection against LOOH-induced cytotoxicity, while EC and ECg were weakly protective. Unexpectedly,

Table 1. Protective effect of tocopherols on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]



Compound	R_1	R_2	R_3	% Survival	
				Concurrent treatment	Pretreatment
EtOH	–	–	–	6.1±3.4	5.7±3.8
α-Tocopherol	H	CH_3	$C_{16}H_{33}$	9.8±8.5	77.8±9.6[b]
α-Tocopherol nicotinate	Nicotinate	CH_3	$C_{16}H_{33}$	2.7±1.4	10.1±3.4
PMC	H	CH_3	CH_3	23.3±4.9[b]	67.2±13.0[b]
Tocol	H	H	$C_{16}H_{33}$	5.4±2.0	9.1±8.5
Trolox	H	CH_3	COOH	7.5±6.3	8.8±2.8

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of five separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.

EGCg, which has been reported to have strong antioxidant capability, had no effect under our conditions. Similar to flavonoids, preincubation with catechins provided no protective effect on LOOH-induced toxicity to HUVEC.

The protective effects of coumarins on LOOH-induced cytotoxicity are shown in Table 7. Coumarin with no hydroxyl groups in its structure had no effect. Coumarins, such as 4-hydroxycoumarin, 7-hydroxycoumarin, scopoletin, and 8-acetyl-6-hydroxy-7-methoxycoumarin, having one hydroxyl group, were also ineffective. Esculetin, which has an *ortho* catechol (6,7-dihydroxy) structure in its molecule, provided strong protection in both concurrent treatment and pretreatment.

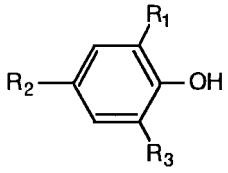
Discussion

Protection of the cellular environment from oxidative stress is dependent on the concentrations of antioxidants and the activities of antioxidant enzymes in cells. Among biomolecules, polyunsaturated fatty acids are among the most liable to undergo oxidation. Their hydroperoxides are the primary products of lipid peroxidation and are thought to be the precursors of alkoxyl and alkylperoxyl radicals formed during decomposition by transition metals, light, or heat. In fact, polyunsaturated fatty acid hydroperoxides are strongly toxic in cultured HUVEC (Kaneko et al., 1988). We have explored protective antioxidants against the cytotoxicity of LOOH. In this study, to avoid reactions of LOOH with components in the culture medium such as serum proteins, Earle's solution was used to treat the cells with LOOH.

Among tocopherol homologs, α-tocopherol and PMC were effective in protecting against the cytotoxicity of LOOH in the case of pretreatment, while tocol, Trolox, and α-tocopherol nicotinate were ineffective. PMC is an α-tocopherol model compound without a long isoprenoid side chain. Tocol is an α-tocopherol model compound without three methyl groups on its aromatic ring. Trolox is a water-soluble α-tocopherol model compound. α-Tocopherol is present in cultured HUVEC at 0.2±0.1 nmol mg^{-1} protein, but this concentration appears to be ineffective for protecting against the cytotoxicity of 50 μM LOOH. After pretreatment for 24 h, α-tocopherol and PMC were incorporated into the cells at 16.6±2.7 and 0.5±0.1 nmol mg^{-1} protein, respectively (Kaneko et al., 1991). Trolox was not incorporated at all. α-Tocopherol nicotinate, which has no hydroxyl group, had no protective effect on the cytotoxicity. It seems that the nicotinate cannot be hydrolyzed in HUVEC under these conditions.

Effects of alkyl substitution to the aromatic ring of alkylated phenols on their protective effects against

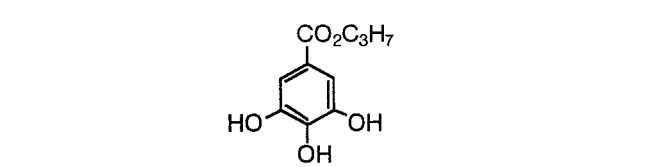
Table 2. Protective effect of simple phenols on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]



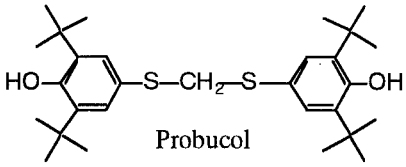
Compound	R_1	R_2	R_3	% Survival	
				Concurrent treatment	Pretreatment
EtOH	–	–	–	6.2±3.4	5.5±2.0
p-Cresol	H	CH_3	H	5.1±1.5	5.8±1.3
2-*tert*-Butyl-4-methylphenol	*t*-C_4H_9	CH_3	H	10.1±5.6	5.7±2.8
2-*tert*-Butyl-6-methylphenol	*t*-C_4H_9	H	CH_3	48.6±13.6[b]	18.5±11.4[c]
2,6-di-*tert*-Butylphenol	*t*-C_4H_9	H	*t*-C_4H_9	1.5±1.4	19.9±2.8[b]
2,4,6-Trimethylphenol	CH_3	CH_3	CH_3	33.7±9.7[b]	16.9±8.5
BHT	*t*-C_4H_9	CH_3	*t*-C_4H_9	1.7±1.2	45.1±9.5[b]

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of five separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.
[c] $p < 0.05$ compared with EtOH.

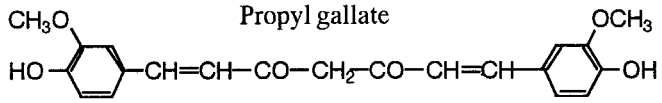
Table 3. Protective effect of polyhydroxyphenols on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]



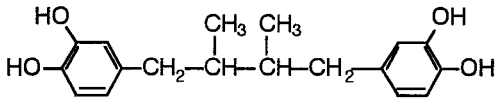
Propyl gallate



Probucol



Curcumin

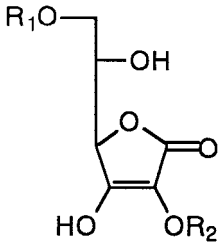


NDGA

Compound	% Survival	
	Concurrent treatment	Pretreatment
EtOH	6.2±3.4	5.5±2.0
Propyl gallate	12.1±4.4	10.0±6.7
Probucol	8.9±2.7	59.3±6.3[b]
Curcumin	20.7±8.5[b]	1.9±1.8
NDGA	6.1±4.0	21.5±9.8[b]

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of five separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.

Table 4. Protective effect of ascorbates on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]



Compound	R_1	R_2	% Survival	
			Concurrent treatment	Pretreatment
EtOH	–	–	6.4±5.1	6.4±4.4
Ascorbic acid	H	H	6.1±5.4	6.5±1.8
6-*O*-Palmitoylascorbic acid	$COC_{15}H_{31}$	H	12.5±1.8	29.7±9.2[b]
6-*O*-Stearoylascorbic acid	$COC_{17}H_{35}$	H	15.7±6.5[c]	29.0±6.9[b]
2,6-*O*-Dipalmitoylascorbic acid	$COC_{15}H_{31}$	$COC_{15}H_{31}$	23.1±12.8[b]	45.0±12.1[b]
CV3611	H	$OC_{18}H_{37}$	12.6±9.3	43.8±20.3[b]

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of four separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.
[c] $p < 0.05$ compared with EtOH.

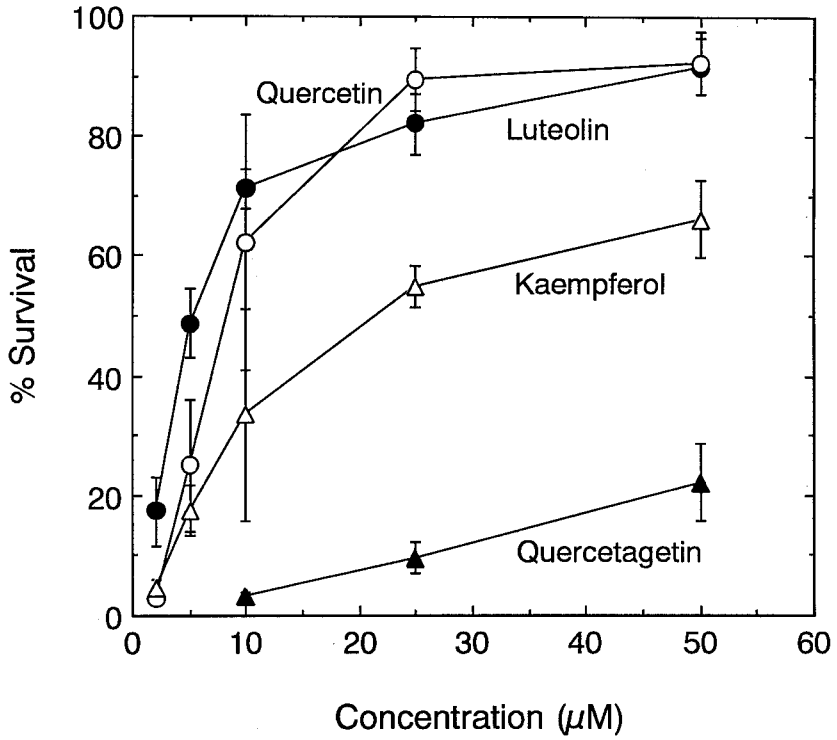
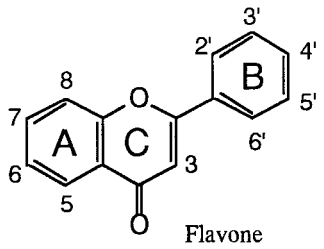
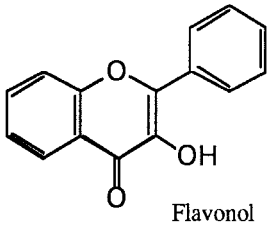
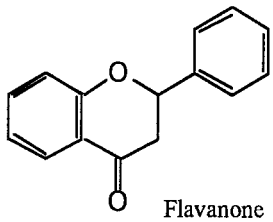


Figure 1. Effect of flavonoids on linoleic acid hydroperoxide-induced toxicity to human umbilical vein endothelial cells. Cells were incubated in Earle's solution containing 2–50 μM of a flavonoid and 50 μM LOOH for 3 h. Results represent the means ± SD of three experiments.

Table 5. Protective effect of flavonoids on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]







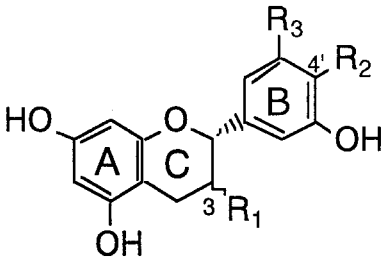
Class	Compound	Position of OH group	% Survival	
			Concurrent treatment	Pretreatment
Control	EtOH		2.2±1.8	2.6±1.1
Flavone	Flavone		3.0±2.7	2.7±1.7
	7-Hydroxyflavone	7	2.7±0.8	2.3±1.4
	Chrysin	5,7	3.6±1.9	1.9±1.4
	7,8-Dihydroxyflavone	7,8	29.5±7.1[b]	7.2±1.7
	Apigenin	5,7,4′	2.5±1.0	2.1±1.3
	Baicalein	5,6,7	28.9±4.5[b]	3.1±1.7
	Luteolin	5,7,3′,4′	91.7±4.7[b]	4.8±2.7
Flavonol	3-Hydroxyflavone	3	35.3±4.8[b]	2.3±1.4
	Galangin	3,5,7	61.7±10.9[b]	2.5±1.7
	Kaempferol	3,5,7,4′	66.2±6.4[b]	2.8±1.2
	Fisetin	3,7,3′,4′	79.4±4.5[b]	4.0±1.9
	Morin	3,5,7,2′,4′	63.8±11.0[b]	3.3±1.3
	Quercetin	3,5,7,3′,4′	92.3±5.1[b]	3.4±2.1
	Rutin	5,7,3′,4′,3-ORut	6.3±1.5	2.8±1.3
	Quercetagetin	3,5,6,7,3′,4′	21.9±6.5[b]	3.6±1.3
	Myricetin	3,5,7,2′,3′,4′	8.3±2.2	2.4±1.8
Flavanone	Naringenin	5,7,4′	6.8±2.1	2.0±1.2
	Hesperetin	5,7,3′,4′-OCH_3	2.8±1.4	3.1±1.3
	Hesperidin	5,3′,7-ORut,4′-OCH_3	3.5±0.9	1.8±0.9
	Taxifolin	3,5,7,3′,4′	6.6±1.1	7.2±1.7

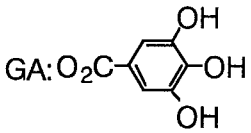
[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of four separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.

the cytotoxicity of LOOH were examined. As seen in Table 2, the presence of two alkyl groups at positions *ortho* to a hydroxyl group and an alkyl group at the *para* position is important for the exertion of the protective effect under these conditions. Alkyl groups have electron-donating characteristics. Since a *tert*-butyl group is bulky, a steric effect might be produced that stabilized the phenoxyl radical generated by hydrogen abstraction. This stabilization is thought to enhance antioxidant activity. Probucol also provided strong protection against LOOH-induced cytotoxicity. Probucol has two *tert*-butyl groups on positions *ortho* to each phenolic hydroxyl group.

Since hydrophilic antioxidants are easily dissolved in aqueous solution, it was expected that they would provide some protection against the cytotoxicity of LOOH in the case of concurrent treatment. However, Trolox and ascorbic acid were unable to protect cells from the toxicity of LOOH while lipophilic ascorbic acid derivatives are effective protectors. On the other hand, lipophilic derivatives of ascorbic acid protected against the toxicity of LOOH to some extent (Table 4).

Table 6. Protective effect of catechins on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]





GL: O-α-D-Glucose

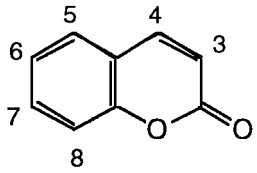
Compound		R_1	R_2	R_3	% Survival	
					Concurrent treatment	Pretreatment
EtOH					4.5±2.2	7.8±2.9
C		OH	OH	H	32.9±13.4[b]	13.0±2.3
EC		OH	OH	H	10.4±1.3	10.5±2.4
EGC		OH	OH	OH	4.2±1.6[b]	9.7±3.1
ECg		GA	OH	H	13.7±2.6	10.1±1.4
EGCg		GA	OH	OH	8.0±1.4	12.7±3.3
EGCg-G1		GA	GL	OH	26.2±5.3[b]	11.0±0.5
EGCg-G2		GAGL	GL	OH	36.2±6.5[b]	11.9±0.3

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of four separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.

Fatty acid esters of ascorbic acid have been shown to act as chain-braking antioxidants and suppress the oxidation of methyl linoleate (Takahashi et al., 1986). It is well known that ascorbic acid and its derivatives containing an enediol structure are oxidized through a two-step reaction (Lohmann et al., 1984). Neither 2,6-*O*-dipalmitoylascorbic acid nor CV3611 has the enediol structure in the molecule; they may act as antioxidants by donating the hydrogen atom of a hydroxyl group at the 3-position to radicals prepared from the decomposition of LOOH.

In recent years, flavonoids have sparked interest as therapeutic agents against a wide variety of diseases involving radical damage (Havsteen, 1983). The mechanism and structural requirements are not yet fully understood, although both metal-chelating and free radical-scavenging activities have been recognized as mechanisms for the antioxidative effects of flavonoids in biological systems (Afanas'ev et al., 1989). Flavonoid aglycons are somewhat lipophilic among plant antioxidants, but are more hydrophilic than α-tocopherol. α-Tocopherol is thought to be located within membranes with its chromane ring stabilized by hydrogen bonding with ester carbonyl groups of phospholipids, while flavonoid aglycons are assumed to be localized near the membrane surface (Ratty and Das, 1988) in a region suitable for scavenging aqueous oxygen radicals and lipophilic radicals incorporated into the membranes. The protective effect of flavonoids does not depend simply on the number of free hydroxyl groups present in the molecule, although the presence of OH groups is essential. Although cells received no protection from the cytotoxicity of LOOH by pretreatment with flavonoids, flavones bearing an *ortho* catechol moiety and the flavonols examined showed a protective effect against the cytotoxicity of LOOH in the case of concurrent treatment. A catechol moiety in the B ring lends high stability to the aroxyl radical via hydrogen bonding (Bors et al., 1990b) or by expanded electron delocalization (Bors et al., 1990a). The *ortho*-dihydroxy structure in the A ring also seems to be effective on the basis

Table 7. Protective effect of coumarins on linoleic acid hydroperoxide-induced toxicity to cultured human umbilical vein endothelial cells[a]



Compound	% Survival	
	Concurrent treatment	Pretreatment
EtOH	3.0±1.1	3.4±0.9
Coumarin	3.1±1.3	3.8±0.6
4-Hydroxycoumarin	2.3±0.5	3.3±1.0
7-Hydroxycoumarin	3.2±1.3	3.5±1.6
Esculetin (6,7-Dihydroxycoumarin)	65.2±14.9[b]	61.8±8.4[b]
Scopoletin (7-hydroxy-6-methoxycoumarin)	5.5±2.9	3.3±0.6
8-Acetyl-6-hydroxy-7-methoxycoumarin	3.5±1.7	3.2±0.6

[a] Cells were incubated in Earle's solution containing LOOH (50 μM) for 3 h. Antioxidants (50 μM) were present concurrently with LOOH for 3 h or previously in the culture medium for 24 h. Values are means ± SD of four separate experiments performed in duplicate.
[b] $p < 0.01$ compared with EtOH.

of the data for 7,8-dihydroxyflavone and baicalein. Some flavonols such as 3-hydroxyflavone, galangin, kaempferol, and morin, are protective against LOOH-induced cytotoxicity, despite having no *ortho* catechol moiety. The *ortho*-dihydroxy structure of the B ring is important, but it seems not to be essential for the antioxidative effects of flavonoids. On the other hand, as seen from the results using quercetagetin and myricetin, a pyrogallol moiety instead of a catechol moiety seems to lower the protective activity. Quercetin and taxifolin have identical numbers of hydroxyl groups in the same positions. Quercetin has the 2,3-double bond and the 4-oxo group in the C ring, while taxifolin lacks the 2,3-double bond. Although quercetin shows strong protection against LOOH-induced cytotoxicity, taxifolin does not. Many authors agree on the importance of this substituent in compounds with a 2,3-double bond. 3-Hydroxyflavone was unexpectedly active, which indicates the importance and probably the oxidizability of the 3-hydroxyl group. Blocking this group has been described as masking the antioxidative activity (Ratty and Das, 1988; Cholbi et al., 1991). Actually, rutin, glycosylated on the 3-hydroxyl group of the C ring, decreased the protective activity, although it has four hydroxyl groups at the same positions as does luteolin. The low protective activity might be due to the decrease in the lipophilicity by the substitution of glucose at 3-hydroxyl group. Furthermore, from equivalent activities of luteolin and quercetin, the importance of 3-hydroxyl group seems to decrease in the flavonoids having a catechol moiety in the B ring and 2,3-double bond in the C ring. On the other hand, a hydroxyl group at the 5- or 7-position of the A ring does not greatly influence the scavenging activity, as can be seen from the results for 7-hydroxyflavone, chrysin, and apigenin.

Catechins are a class of flavonoids. Although they are flavan-3-ols containing *ortho*-dihydroxyl structures in the B-ring and 3- and 5-hydroxyl groups in the A-ring, they do not satisfy the determinants of the 2,3-double bond in conjugation with a 4-oxo function. Catechins are thought to localize on the polar surfaces of phospholipid bilayers as well as flavonoids (Ratty and Das, 1988). In the case where cells were treated concurrently with catechins and LOOH, C, EGCg-G1, and EGCg-G2 were able to protect the cells against the cytotoxicity of LOOH. The structural difference between C and EC lies only in the configuration at the 3-position of the ring C. The quasi-equatorial hydroxyl group at the 3-position of C seems to be important for the protective effect. EGCg is well known as a strong antioxidant, and it has been reported to be effective for scavenging free radicals (Guo et al., 1999). However, EGCg showed no protective effect under our experimental conditions. The lability of EGCg is presumed to be responsible for the inefficiency. The protective

effects of EGCg-G1 and EGCg-G2 should be based on structural stabilization by glucosylation of EGCg.

Coumarins are also a class of natural antioxidant compounds. Among the coumarins examined, esculetin bearing a 6,7-dihydroxyl moiety showed a protective effect on LOOH-induced cytotoxicity in both pretreatment and concurrent treatment. Esculetin is known as a strong lipoxygenase inhibitor. Furthermore, it has recently been reported that the amount of thiobarbituric acid-reactive substances (TBARS) in the livers of esculetin-treated mice was lower than in control mice (Martín-Aragón et al., 1998). The *ortho* catechol structure is also critical for the protection of cells against LOOH-induced cytotoxicity.

The effectiveness of antioxidants should be dependent on the experimental conditions and their natures such as reactivity, solubility, etc. The chance of an encounter between antioxidants and radicals should be taken into account when trying to understand the effectiveness of antioxidants. Although hydrophilic antioxidants such as ascorbates and Trolox were expected to provide some protection against LOOH in the medium, they showed no protection. It may be difficult to scavenge effectively lipophilic LOOH in the medium. LOOH is considered to show its cytotoxicity through the production of radicals during degradation in cell membranes, since lipid hydroperoxides added to a culture medium are rapidly incorporated into cells (Kaneko et al., 1996). Actually, lipophilic antioxidants were protective against the LOOH-induced toxicity under our experimental conditions. However, their effects are different among them. α-Tocopherol, BHT, and probucol showed a protective effect only in pretreatment, while flavonoid aglycons were effective only in concurrent treatment. α-Tocopherol, probucol, etc. are thought to be located within cell membranes, while flavonoid aglycons are assumed to be localized near the membrane surface. The lack of protective effect of flavonoid aglycons in pretreatment may be due to their quick metabolism (Shimoi et al., 1998). Esculetin had a protective effect in both pretreatment and concurrent treatment. The protective effect of antioxidants against LOOH-induced cytotoxicity may depend on their lipophilicity, antioxidant activity, stability, and position in membranes. The ingestion of foods containing α-tocopherol, flavonoids, and esculetin may be partly effective in inhibiting damage to the endothelium caused by lipid peroxides.

References

Afanas'ev IB, Dorozhko AI, Brodskii AV, Kostyuk VA and Potapovitch AI (1989) Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. Biochem Pharmacol 38: 1763–1769.

Bors W, Heller W, Michel C and Saran M (1990a) Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Methods Enzymol 186: 343–354.

Bors W, Heller W, Michel C and Saran M (1990b) Radical chemistry of flavonoid antioxidants. In: Emerit I (ed.) Antioxidants in Therapy and Preventive Medicine (pp 165–170) Plenum Press, New York.

Burton GW, Joyce A and Ingold KU (1983) Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human plasma and erythrocyte membrane? Arch Biochem Biophys 221: 281–290.

Burton GW, Doba T, Gabe EG, Hughes L, Lee FL, Prasad L and Ingold KU (1985) Autoxidation of biological molecules. 4. Maximizing the antioxidants activity of phenols. J Am Chem Soc 107: 7053–7065.

Chen H and Tappel A (1995) Vitamin E, selenium, trolox C, ascorbic acid palmitate, acetylcysteine, coenzyme Q, b-carotene, canthaxanthin, and (+)-catechin protect against oxidative damage to kidney, heart, lung and spleen. Free Radic Res 22: 177–186.

Chen H and Tappel A (1996) Protection by multiple antioxidants against lipid peroxidation in rat liver homogenate. Lipids 31: 47–50.

Cholbi MR, Paya M and Alcaraz MJ (1991) Inhibitory effects of phenolic compounds on CCl4-induced microsomal lipid peroxidation. Experientia 47: 195–199.

Cotelle N, Bernier JL, Hénichart JP, Catteau JP, Gaydou E and Wallet JC (1992) Scavenger and antioxidant properties of ten synthetic flavones. Free Radic Biol Med 13: 211–219.

Decharneux T, Dubois F, Beauloye C, Coninck SW and Wattiaux R (1992) Effect of various flavonoids on lysosomes subjected to an oxidative or an osmotic stress. Biochem Pharmacol 44, 1243–1248.

Egan D, O'Kennedy R, Moran E, Cox D, Prosser E and Thornes RD (1990) The pharmacology, metabolism, analysis, and applications of coumarin and coumarin-related compounds. Drug Metabol Rev 22: 503–529.

Esterbauer H and Cheeseman KH (1987) Lipid peroxidation: Part II Pathological implications. Chem Phys Lipids 45: 103–364.

Fraga CG, Martino VS, Ferraro GE, Coussio JD and Boveris A (1987) Flavonoids as antioxidants evaluated by *in vitro* and *in situ* liver chemiluminescence. Biochem Pharmacol 36: 717–720.

Fujiki H and Okuda T (1992) (–)-Epigallocatechin gallate. Green tea polyphenols, anticarcinogens and antioxidants. Drug Future 17: 462–464.

Guo O, Zhao B, Shen S, Hou J and Xin W (1999) ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. Biochim Biophys Acta 1427: 13–23.

Hanasaki Y, Ogawa S and Fukui S (1994) The correlation between active oxygen scavenging and antioxidative effects of flavonoids. Free Radic Biol Med 16: 845–850.

Havsteen B (1983) Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 32: 1141–1148.

Hiramoto K, Ojima N, Sako K and Kikugawa K (1996) Effect of plant phenolics on the formation of the spin-adduct of hydroxyl radical and the DNA strand breaking by hydroxyl radical. Biol Pharm Bull 19: 558–563.

Horton AA and Fairhurst S (1987) Lipid peroxidation and mechanisms of toxicity. Crit Rev Toxicol 18: 27–79.

Jovanovie SV, Steenken S, Tosic M, Marjanovie B and Simic MG (1994) Flavonoids as antioxidants. J Am Chem Soc 116: 4846–4851.

Kaneko T and Matsuo M (1984) Isomerization of linoleic acid hydroperoxides under argon and under degassed condition. Chem Pharm Bull (Tokyo) 32: 332–335.

Kaneko T, Honda S, Nakano S and Matsuo M (1987) Lethal effects of a linoleic acid hydroperoxide and its autoxidation products, unsaturated aliphatic aldehydes, on human diploid fibroblasts. Chem Biol Interact 63: 127–137.

Kaneko T, Kaji K and Matsuo M (1988) Cytotoxicities of a linoleic acid hydroperoxide and its related aliphatic aldehydes toward cultured human umbilical vein endothelial cells. Chem Biol Interact 67: 295–304.

Kaneko T, Nakano S and Matsuo M (1991) Protective effect of vitamin E on linoleic acid hydroperoxide-induced injury to human endothelial cells. Lipids 26: 345–348.

Kaneko T, Kaji K and Matsuo M (1993) Protective effect of lipophilic derivatives of ascorbic acid on lipid peroxide-induced endothelial injury. Arch Biochem Biophys 304: 176–180.

Kaneko T, Baba N and Matsuo M (1994a) Phospholipid hydroperoxides are significantly less toxic to cultured endothelial cells than fatty acid hydroperoxides. Life Sciences 55: 1443–1449.

Kaneko T, Kaji K and Matsuo M (1994b) Protection of linoleic acid hydroperoxide-induced cytotoxicity by phenolic antioxidants. Free Radic Biol Med 16: 405–409.

Kaneko T, Baba N and Matsuo M (1996) Cytotoxicity of phosphatidylcholine hydroperoxides is exerted through decomposition of fatty acid hydroperoxide moiety. Free Radic Biol Med 21: 173–176.

Kaneko T, Matsuo M and Baba N (1998) Inhibition of linoleic acid hydroperoxide-induced toxicity in cultured human umbilical vein endothelial cells by catechins. Chem Biol Interact 114: 109–119.

Kaneko T and Baba N (1999) Protective effect of flavonoids on endothelial cells against linoleic acid hydroperoxide-induced toxicity. Biosci Biotechnol Biochem 63: 323–328.

Kitao S, Matsudo T, Saitoh M, Horiuchi T and Sekine H (1995) Enzymatic synthesis of two stable (–)-epigallocatechin gallate-glucosides by sucrose phosphorylase. Biosci Biotechnol Biochem 59: 2167–2169.

Lobb RR and Fett JW (1984) Purification of two distinct growth factors from bovine neutral tissue by heparin affinity chromatography. Biochemistry 23: 6295–6299.

Lohmann W, Pagel D and Penka V (1984) Structure of ascorbic acid and its biological function. Determination of the conformation of ascorbic acid and isoascorbic acid by infrared and ultraviolet investigations. Eur J Biochem 138: 479–480.

Martín-Aragón S, Bebedí JM and Villar AM (1998) Effects of the antioxidant (6,7-dihydroxycoumarin) esculetin on the glutathione system and lipid peroxidation in mice. Gerontology 44: 21–25, 1998.

Morel DW, DiCorleto PE and Chisolm GM (1984) Endothelial and smooth muscle cells alter low density lipoprotein *in vitro* by free radical oxidation. Arteriosclerosis 4: 357–364.

Mukai K, Fukuda D, Tajima K and Ishizu K (1988) A kinetic study of reactions of tocopherols with a substituted phenoxyl radicals. J Org Chem 53: 430–432.

Nakayama T, Yamada M, Osawa T and Kawanishi S (1993) Suppression of active oxygen-induced cytotoxicity by flavonoids. Biochem Pharmacol 45: 265–267.

Nakayama T (1994) Suppression of hydroperoxide-induced cytotoxicity by polyphenols. Cancer Res 54: 1991s–1993s.

Namiki, M and Osawa T (1986) Antioxidants/antimutagens in foods. Basic Life Sci 39: 131–142.

Negre-Salvayre A, Alomar Y, Troly M and Salvayre R (1991) Ultraviolet-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cultured cells. III. The protective effect of antioxidants (probucol, catechin, vitamin E) against the cytotoxicity of oxidized LDL occurs in two different ways. Biochim Biophys Acta 1096: 291–300.

Negre-Salvayre A and Salvayre R (1992) Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cells lines. Free Radic Biol Med 12: 101–106.

Nilson JLG, Sievertsson H and Selander H (1968) Synthesis of methyl substituted 6-hydroxychromans, model compounds of tocopherols. Acta Chem. Scand. 22: 3160–3170.

Okuda T, Kimura Y, Yoshida T, Hatano T, Okuda H and Arichi S (1983) Studies on the activities of tannins and related compounds from medicinal plants and drugs. I. Inhibitory effects on lipid peroxidation in mitochondria and microsomes of liver. Chem Pharm Bull (Tokyo) 31: 1625–1631.

Payá M, Halliwell B and Hoult JRS (1992) Interactions of a series of coumarins with reactive oxygen species. Biochem Pharmacol 44: 205–214.

Payá M, Ferrándiz F, Miralles C, Montesinos C, Ubeda A and Alcaraz MJ (1993) Effects of coumarin derivatives on superoxide anion generation. Arzneimittel Forsch 43: 655–658.

Ratty AK and Das NP (1988) Effects of flavonoids on nonenzymatic lipid peroxidation: Structure-activity relationship. Biochem Med Metab Biol 39: 69–79.

Ross R and Glomset JA (1993) The pathogenesis of atherosclerosis: A perspective for the 1990s. Nature 362: 801–809.

Sasaguri Y, Nakashima T, Morimatsu M and Yagi K (1984) Injury to cultured endothelial cells from human umbilical vein by linoleic acid hydroperoxide. J Appl Biochem 6: 144–150.

Shimoi K, Okada H, Furugori M, Goda T, Takase S, Suzuki M, Hara Y, Yamamoto H and Kinae N (1998) Intestinal absorption of luteolin and luteolin 7-O-b-glucoside in rats and humans. FEBS Lett 438: 220–224.

Sichel G, Corsaro C, Scalia M, Di Bilio AJ and Bonomo R (1991) *In vitro* scavenger activity of some flavonoids and melanins against O_2^-. Free Radic Biol Med 11: 1–8.

Takahashi M, Niki E, Kawakami A, Kumasa A, Kamiya Y and Tanaka K (1986) Oxidation of lipids. XIV. Inhibition of oxidation of methyl linoleate by fatty acid esters of L-ascorbic acid. Bull Chem Soc Jpn 59: 3179–3183.

Tappel AL (1968) Will antioxidant nutrients slow aging processes? Geriatrics 23: 97–105.

Terao J, Piskuli M and Yao Q (1994) Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. Arch Biochem Biophys 308: 278–284.

Torel J, Cillard J and Cillard P (1986) Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochemistry 25: 383–385.

Torel J, Cillard J and Cillard P (1996) Antioxidant activity of flavonoids and reactivity with peroxy radical. Phytochemistry 25: 383–385.

van Acker SABE, van den Berg DJ, Tromp MNJL, Griffioen DH, van Bennekom WP, van der Vijgh WJF and Bast A (1996) Structural aspects of antioxidant activity of flavonoids. Free Radic Biol Med 20: 331–342.

Weiss SJ, Young J, LoBuglio AF, Slivka A and Nimeh NF (1981) Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. J Clin Invest 68: 714–721.

Xu Y, Ho CT, Amin S, Han C and Chung FL (1992) Inhibition of tobacco-specific nitrosoamine-induced lung tumorigenesis in

A/J mice by green tea and its major polyphenols as antioxidants. Cancer Res 52: 3875–3879.

Yoshizawa S, Horiuchi T, Fujiki H, Yoshida T, Okuda T and Sugimura T (1987) Antitumor promoting activity of (–)-epigallocatechin gallate, the main constituent of 'tannin' in green tea. Phytother Res 1: 44–47.

Zhou YC and Zheng RL (1991) Phenolic compounds and an analog as superoxide anion scavengers and antioxidants. Biochem Pharmacol 42: 1177–1179.