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Dynamics of Vitamin E Action against LDL Oxidation

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Vitamin E acts as an important antioxidant against oxidative modification of low density lipoprotein (LDL) which is accepted as an initial event in the pathogenesis of atherosclerosis. In spite of the numerous studies and reports, the action and role of vitamin E have not been fully elucidated yet. In this brief overview, the dynamics of action of vitamin E as an antioxidant have been discussed and it is emphasized that the total antioxidant potency is determined by the relative importance of many competing reactions which is determined by the reactivities and concentrations of substrates, radicals and antioxidant and by physical factors of the environment.

Keywords: Lipid peroxidation, low density lipoprotein, antioxidant, vitamin E, spin label

INTRODUCTION

With increasing evidence of chemical, biological, clinical and epidemiological studies which shows the key role of oxidative modification of low density lipoprotein (LDL) in the pathogenesis of atherosclerosis, the role and action of antioxidants have received much attention (reviewed most extensively by Esterbauer).[1–4] Much studies have been carried out on the myriad of natural antioxidant compounds such as flavonoids and polyphenols as well as vitamin E, vitamin C and ubiquinol, while synthetic antioxidant drugs have also been explored. Nevertheless, there still remain many issues which are neither well understood nor elucidated yet. For example, the potency of vitamin E, the most abundant antioxidant in LDL particles, as a radical-scavenging antioxidant is still a matter of debate. For example, vitamin E is accepted as an important antioxidant which suppresses the oxidative modification of LDL,[3] but, on the other hand, it has been proposed that vitamin E acts as a prooxidant against LDL oxidation.[5] Probucol, which has radical-scavenging activity and prevents the oxidation of LDL *in vitro*, is used as a therapeutic drug for atherosclerosis.[6] One of the mysteries about probucol is that it acts as an effective drug for rabbits[7] but that it enhances atherosclerosis for mouse.[8] The reason for such adverse effect for different species is not known. In order to understand further the role of antioxidant,

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the dynamics and action of vitamin E against LDL oxidation will be briefly overviewed and discussed.

OUTLINE OF LDL OXIDATION AND ITS INHIBITION

It has been extensively studied and documented that LDL particle is oxidized by various oxidants and by different mechanisms to give modified LDL which has cytotoxic and complex biological activities. LDL lipids may be oxidized by (1) enzymes such as 15-lipoxygenases, (2) non-enzymatic, free radical-mediated mechanism, or (3) non-enzymatic, non-radical mechanism. Various oxidants are capable of inducing oxidation of lipids. Cholesteryl esters and phosphatidylcholines having polyunsaturated fatty acid moieties, major substrate in LDL particle, are readily oxidized to give corresponding hydroperoxides and their secondary oxidation products. For example, Esterbauer and his colleagues[9] have observed loss of arachidonate (20:4) and linoleate (18:2), the increase in conjugated diene due to lipid hydroperoxides and hydroxides, and the concomitant formation of 4-hydroxynonenal. Several kinds of aldehydes are known to be formed in the oxidation of LDL and have potent biological effects.[10,11] Free cholesterol is also oxidized to yield cytotoxic products.[12]

Apolipoprotein B-100 (apo B) is also oxidized and modified by various routes. It may be directly attacked by free radicals or modified by lipid oxidation products such as 4-hydroxynonenal, malonaldehyde and acrolein. It has been shown that acrolein is formed from both lipid and amino acid. Apo B has received less attention than lipids but its modification may well be more important than lipid peroxidation in the development of atherosclerosis in terms of recognition by scavenger receptor and hence should be studied more extensively.

Human LDL is protected against oxidative stress by a well-prepared defense system composed of versatile antioxidants with different functions: preventing antioxidants, radical-scavenging antioxidants and repair and *de-novo* antioxidant enzymes. Proteins such as transferrin and ceruloplasmin sequester iron and copper respectively, while glutathione peroxidases reduce hydroperoxides to alcohols, thus preventing metal-dependent formation of active radicals. Other enzymes may also contribute in the defense system by metabolizing lipid hydroperoxides: for example, lecithin–cholesterol acyltransferase (LCAT) converts phosphatidylcholine hydroperoxide (PCOOH) into cholesteryl ester hydroperoxide to suppress the accumulation of PCOOH. However, it is essential to inhibit the formation of lipid hydroperoxides not to overwhelm such enzyme capacity. The radical-scavenging antioxidants act as the second line of defense by breaking chain oxidation. Vitamin C and vitamin E are probably the most important hydrophilic and lipophilic radical-scavenging antioxidant, respectively. These and other radical-scavenging antioxidants function independently at their inherent localized site and also cooperatively or even synergistically.

A typical example of the oxidation of LDL is shown in Figure 1.[13] The oxidation of LDL *in vitro* can be followed by measuring oxygen uptake, loss of substrate lipids, formation of conjugated diene, lipid hydroperoxides and thiobarbituric acid reactive substances (TBARS), fluorescence, or modification of apo B such as degradation or increase in relative electrophoretic mobility. The continuous monitoring of the change of the 234 nm absorption due to conjugated diene first reported by Esterbauer[14] is very convenient and has been used quite frequently. In the free radical-mediated LDL oxidation, lag phase is often observed before the onset of rapid oxidation phase (which is often called propagation phase), which is then followed by a termination phase (Figure 2). The absorption at 234 nm does not increase monotonously but it remains constant or even decreases. This is because the lipid hydroperoxides undergo secondary reactions, but the oxidation still proceeds

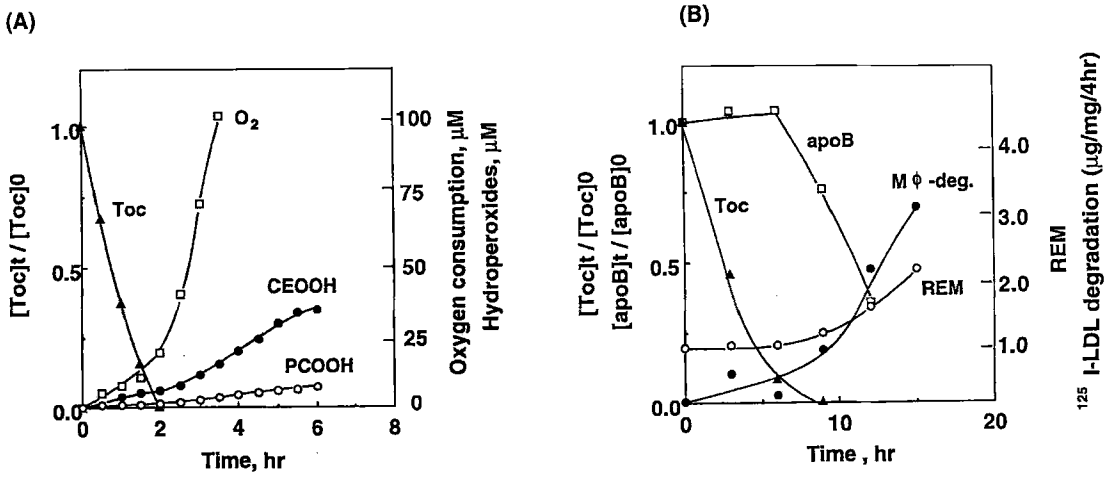

FIGURE 1 (A) Oxidation of human LDL (0.25 mg protein/ml PBS) induced by 2 μM $CuCl_2$ at 37°C in air. Oxygen uptake (□), consumption of α-tocopherol (▲), and formation of phosphatidylcholine hydroperoxide (○) and cholesteryl ester hydroperoxide (●) were followed. $[Toc]_t/[Toc]_0$ shows remaining fraction of α-tocopherol. (B) Oxidative modification of apolipoprotein B100 during the oxidation of human LDL induced by $CuCl_2$ at 37°C in air. □: intact apolipoprotein B100 as measured by SDS-PAGE; ○: REM; relative electrophoretic mobility; ●: Mϕ-deg; uptake of oxidized LDL by macrophage expressed in μg/mg/4 h.

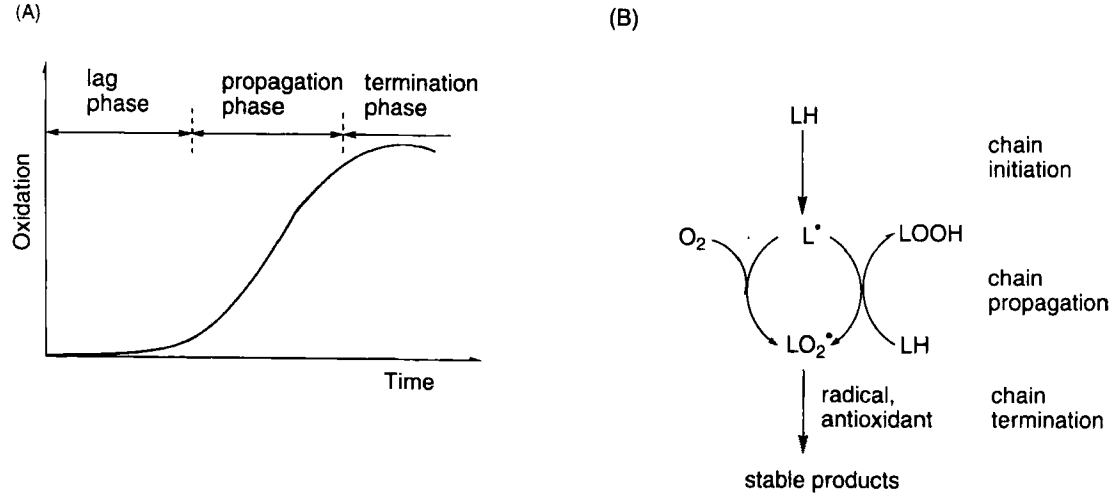

FIGURE 2 Lipid peroxidation pathway. Chart A shows the time course of lipid peroxidation. Little oxidation proceeds during the lag phase, which is followed by a propagation phase where rapid oxidation takes place. Then the rate of oxidation decreases to a very slow level in the termination phase. It should be noted that some parameter may decrease faster than the other. For example, it is often observed in the LDL oxidation that the absorption at 234 nm reaches plateau or even decreases, while the oxygen uptake continues to increase. Thus, care should be taken in following the oxidation and interpreting the data. Chart B shows the sequence of free radical chain oxidation which is composed of three steps: chain initiation, chain propagation and chain termination. In the chain initiation step, the radicals are formed, while in the chain propagation step lipids are oxidized continuously by a sequence shown. The active peroxyl radicals are destroyed by a reaction with another radical or antioxidant. All these three steps take place in every phase shown in (A). The chain propagation reaction may take place even during lag phase, but under certain conditions the radical formed in the chain initiation step may undergo to the chain termination reaction without any chain propagation.

and oxygen uptake is observed even during the "termination phase". It may also be noteworthy that the chain initiation, chain propagation and chain termination reactions which correspond to the steps of formation of free radicals, continuation of oxidation sequence and disappearance of radicals (Figure 2), all take place in the three phases.

Figure 1 shows that when vitamin E is completely exhausted under the conditions studied, a rapid oxidation proceeds: that is, oxygen uptake and formation of cholesteryl ester hydroperoxide and phosphatidylcholine hydroperoxide increase markedly and modification of apolipoprotein B100 and resulting uptake by macrophage become significant. Several other antioxidants are also contained in LDL, although in much lower concentrations, and they are consumed in the order of ubiquinol, α- and γ-tocopherol, and carotenoids such as β-carotene and lycopene.[14–16] Vitamin E is the most abundant antioxidant in LDL particle and its role and action against oxidation of LDL have been the subject of extensive studies and will be briefly overviewed in this article.

ACTION OF VITAMIN E AS A RADICAL-SCAVENGING ANTIOXIDANT WITHIN LDL PARTICLES

Although it has not been clearly shown what initiates LDL oxidation *in vivo*, many oxidants may contribute to the initiation of lipid peroxidation in LDL. Irrespective of how oxidation is initiated, the free radical-mediated lipid peroxidation proceeds by a chain mechanism in which lipid peroxyl radicals play an important role as a chain carrier. On the other hand, as described later, Bowry, Ingold and Stocker proposed that, in the presence of vitamin E and under certain circumstances, the oxidation is mediated by vitamin E radical in LDL particles.[5] Vitamin E scavenges peroxyl radical rapidly; about 10^4 times as rapidly as the peroxyl radicals attack polyunsaturated lipids in solution.[17,18] This is why only a small concentration of vitamin E compared with lipid can inhibit the lipid peroxidation so efficiently. On the other hand, the preferential scavenging of hydroxyl and alkoxyl radicals by vitamin E over substrate such as polyunsaturated lipids and proteins is not kinetically favored. However, it has been shown that the apparent rate of scavenging radicals by vitamin E is smaller in the membranes as compared in homogeneous solution.[19,20] In fact, it has been demonstrated experimentally by using a spin probe technique that the rate of scavenging peroxyl radicals by vitamin E becomes smaller as the radical goes deeper into the interior of the membranes from the surface.[21] It is important to appreciate that LDL is a large sphere composed of relatively thin outer monolayer of phospholipids and free cholesterol and large core containing cholesteryl esters and triglycerides. It was shown experimentally that the efficacy of scavenging radicals by vitamin E also decreases in LDL particle as the radical goes deeper into the monolayer[22] and it is anticipated that the efficacy of scavenging radicals in the core becomes even more difficult. The OH group of vitamin E (α-tocopherol), the active site for scavenging radicals, is accepted to reside predominantly at the water–lipid interface, while lipid peroxyl radicals are formed within LDL particles and their collision must be less efficient in LDL than in organic solution. The dipole moment (one of the indices for polarity) of the peroxyl moiety of the lipid peroxyl radical is 2.6 debye,[23] suggesting that lipid peroxyl radical becomes more polar than parent lipid, and it is most likely to float to the surface of LDL particle as first proposed by Barclay and Ingold[24] and react with α-tocopherol.

The phytyl side chain of vitamin E is essential for its incorporation and retainment of vitamin E in the membranes, but it reduces the mobility of vitamin E molecule within and between the membranes.[25] Interestingly, 2,2,5,7,8-pentamethyl-6-chromanol, a vitamin E

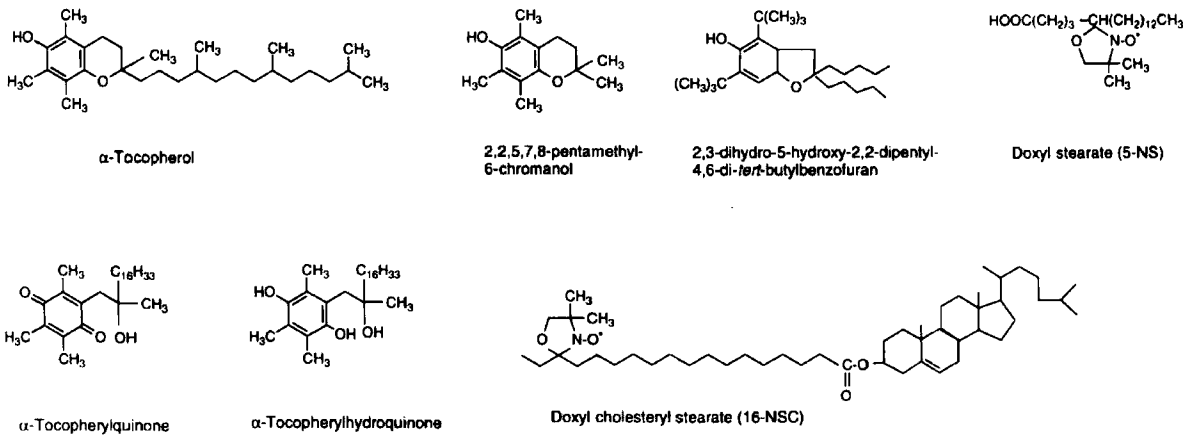

FIGURE 3 Structure of antioxidants and spin probes described in this article.

analogue without a long side chain, can scavenge radicals more efficiently than α-tocopherol within LDL,[22] although α-tocopherol and 2,2,5,7,8-pentamethyl-6-chromanol (Figure 3) exert the same reactivities toward peroxyl radicals and the same antioxidant activities against lipid peroxidation in organic solution. These and other results show clearly that the total antioxidant potency of radical-scavenging antioxidant is dependent very much on the medium where it works.

DUAL FUNCTIONS OF VITAMIN E

It has been observed that, under certain circumstances, the antioxidants such as vitamin E and vitamin C act as a prooxidant rather than as antioxidant. For example, Cillard[26] has observed the prooxidant action of vitamin E for lipids especially at its high concentration and its mechanism has been discussed later in some more detail.[27,28] It has also been well known that the combination of ascorbate and iron acts as a strong oxidant, and this mixture has been often used as an oxidant source in the *in vitro* study on oxidation. Such a prooxidant action of antioxidant may be explained by either of the following reactions, that is, (1) the antioxidant-derived radical attacks substrate and induces oxidation, (2) the antioxidant reduces metal ions such as Fe(III) and Cu(II) to give corresponding metal ions at lower valence state Fe(II) and Cu(I) respectively, which decompose hydroperoxides much faster than the metal ions at higher valence state, and (3) autoxidation of antioxidant to yield superoxide and hydrogen peroxide, which act as oxidant.

Vitamin E radical formed when vitamin E scavenges active radical may undergo several reactions. It reacts rapidly with another peroxyl radical to give a stable adduct.[29] However, when the concentration of peroxyl radicals is low, vitamin E radical may not encounter another radical but attack, although slowly, the substrate it is supposed to protect. The rate constant for hydrogen atom abstraction by various chromanoxyl radicals from lipids has been measured by Mukai and colleagues in solution.[30–32] Under these conditions, vitamin E continues the oxidative chain.

In 1992, Bowry, Ingold and Stocker[5] proposed that the peroxidation within LDL particle is propagated not by lipid peroxyl radicals but by reaction of α-tocopheroxyl radical with polyunsaturated fatty acid moieties in the lipid and that vitamin E acts as a prooxidant rather than as antioxidant. In the following numerous papers,

Stocker and his colleagues explained such prooxidant action of vitamin E named as tocopherol-mediated-peroxidation (TMP) by phase transfer action and chain transfer action.[33,34] They have repeatedly reported that such prooxidant action is important in the oxidation of LDL induced by copper, aqueous peroxyl radicals generated from hydrophilic azo initiator, hydroxyl radical, horseradish peroxidase and 15-lipoxygenase.[23,33–39] It is conceivable that vitamin E acts as a radical carrier and enhances the influx of aqueous radicals into the LDL particle (phase transfer action). Waldeck and Stocker[23] presented kinetic models for the lipid peroxidation in LDL particles and showed that the extended two-compartment model of TMP satisfactorily explains the experimentally observed results in the early stages of LDL lipid peroxidation under various oxidizing conditions. They propose that α-tocopheroxyl radical which is more lipophilic than α-tocopherol descends into the outer monolayer and even central core and attacks both phosphatidylcholine and cholesteryl ester. They estimated that α-tocopheroxyl radical moves at least 100–1000 times throughout the entire LDL particle before it undergoes reaction with the polyunsaturated lipids. Apparently, α-tocopheroxyl radical is less reactive (probably several hundred times) than peroxyl radical in the hydrogen atom abstraction from polyunsaturated lipids, but since α-tocopheroxyl radical is the most stable radical that can be formed and it cannot readily leave LDL particle, α-tocopheroxyl radical is assumed to have enough chance to attack lipid and continue chain reaction (chain transfer effect) before it encounters another lipid peroxyl radical or α-tocopheroxyl radical. Under these circumstances where phase transfer and chain transfer effects are important, α-tocopherol may act as a prooxidant. This is especially important when there is only one radical per LDL particle.[23] In any event, the LDL oxidation proceeds faster after complete depletion of vitamin E than during its presence, although it has been recently reported that depletion of vitamin E from LDL renders LDL resistant toward radical oxidants and that replenishment with vitamin E restores its oxidation susceptibility.[39] It may be noteworthy that similar pattern of oxidation is observed when LDL oxidation is initiated by lipophilic azo initiator which generates two radicals within LDL particle. It is also important to note that the relevant rate constants used in the simulation for LDL oxidation[23,40,41] have been measured mostly for homogeneous solution and those in LDL particles are not known, but should be considerably different.

As the second possibility, vitamin E may exert prooxidant activity by acting as a reductant of metal ions. The metal-dependent oxidation of lipids and LDL are accepted to be initiated by a radical formed in the decomposition of pre-formed lipid hydroperoxides by transition metal ions such as copper and iron.[42–47] Copper ion in ceruloplasmin may also initiate LDL oxidation. In fact, complete reduction of hydroperoxides to alcohol by triphenylphosphine or ebselen inhibits the metal-dependent lipid peroxidation.[48,49] It has been found that α-tocopherol in LDL directly reduces Cu(II) to Cu(I), with the concomitant formation of the α-tocopheroxyl radical and Cu(I).[50–52] Cu(I) decomposes hydroperoxides much faster than Cu(II) and therefore α-tocopherol acts as a prooxidant even if α-tocopheroxyl radical does not attack lipids.

Thus, it is possible that α-tocopherol acts as a prooxidant in the *in vitro* concocted experimental systems. However, it must be noted that this does not necessarily mean that similar prooxidant action takes place *in vivo*. Firstly, metal ions are sequestered by proteins *in vivo* and such sequestered metal ions are not readily reduced by α-tocopherol. LDL is often oxidized *in vitro* in the isolated system, however, LDL is not isolated *in vivo* but surrounded by such antioxidants that are capable of diminishing the prooxidant action of α-tocopherol as described below.

SYNERGISTIC INHIBITION OF OXIDATION BY VITAMIN E WITH OTHER ANTIOXIDANT

As mentioned at first, the antioxidant functions not only individually but also cooperatively or even synergistically with other antioxidants. The combination of vitamin E and vitamin C is a typical well-known example. Vitamin C reduces α-tocopheroxyl radical rapidly to regenerate and maintain α-tocopherol. This also inhibits the attack of α-tocopheroxyl radical upon substrate. It has been observed that the addition of vitamin C diminishes the prooxidant action of α-tocopherol against lipid peroxidation[27,28] and also LDL oxidation.[5,22,34,35,38]

It has been demonstrated experimentally that vitamin C present in the aqueous phase is capable of reducing α-tocopheroxyl radical localized in the membranes[53,54] and LDL.[55,56] However, the efficacy of reduction of radical by ascorbate decreases as the radical goes deeper into the interior of the membranes[21,57] and LDL particle.[22] An example is shown in Figure 4. A spin label, doxyl stearate having stable nitroxide radical along the stearic acid chain, is incorporated into the LDL particle and subjected to the reaction with ascorbate added into the aqueous phase. Ascorbate reduces nitroxide radical quite rapidly in the homogeneous solution. However, the rate of reduction of nitroxide radical by ascorbate decreases as the radical goes deeper into interior and the nitroxide radical attached to the cholesteryl stearate is not reduced. Interestingly, however, it is reduced at appreciable rate when 6-ascorbylpalmitate, a lipophilic analogue of ascorbate, is added into LDL suspensions. These results clearly show that ascorbate in the aqueous phase reduces radicals at or near the LDL surface efficiently, but that it cannot reduce radicals localized in the core of LDL particle. It may also be added that a novel antioxidant, 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (Figure 3) which is more lipophilic than α-tocopherol, is not spared by ascorbate during the oxidation of LDL, although its phenoxyl radical is rapidly reduced by ascorbate in solution.[58]

Ubiquinol is also a potent reducing agent for α-tocopheroxyl radical and spares α-tocopherol during oxidation.[59,60] Since ubiquinol is also lipophilic, it is in a better position than ascorbate

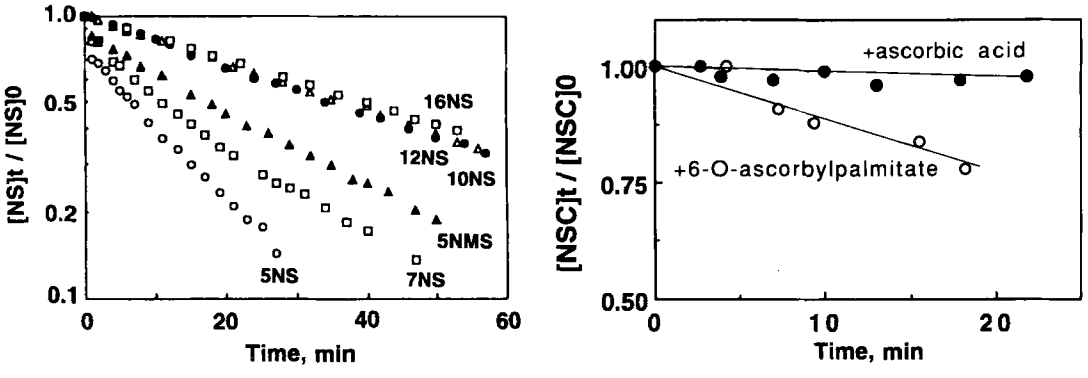

FIGURE 4 Reduction by ascorbate of nitroxide radical incorporated into LDL. (A) Doxyl stearic acid (NS) and its methyl ester (NMS) were incorporated into LDL particles and their reduction by ascorbate added into the aqueous phase was followed with an electron spin resonance. The numbers preceding NS show the carbon atom from the carboxyl group on which doxyl group is attached, that is, 5NS has doxyl group on the 5th carbon from the LDL surface. The results show that the rate of reduction of nitroxide radical by ascorbate becomes smaller as the radical goes deeper from the LDL surface. (B) Reduction of cholesteryl ester of 16-NS (NSC) by either ascorbate or 6-O-ascorbylpalmitate. The nitroxide radical attached on the cholesteryl stearate which is assumed to be present in the core of LDL particle is not reduced by ascorbate but it is reduced by its lipophilic ester, 6-O-ascorbylpalmitate. See Ref. [22] for details.

to reduce α-tocopheroxyl radical. It has been observed in the oxidation of phosphatidylcholine liposomal membranes inhibited by a combination of α-tocopherol, ascorbic acid and ubiquinol 10 that the antioxidants were consumed in the order of ascorbate > ubiquinol 10 > α-tocopherol when the oxidation was initiated by aqueous radicals, whereas the order was ubiquinol > ascorbate > α-tocopherol when the oxidation was initiated by radicals generated within the lipophilic domain of the membranes.[61] Thus, the competition between ascorbate and ubiquinol for sparing α-tocopherol depends on the conditions such as location of radicals and concentrations. The concentration of endogenous ubiquinol-10 in human LDL is quite small and the physiological role of ubiquinol as an antioxidant for LDL oxidative modification may not be so significant unless ubiquinone-10 is readily reduced to ubiquinol-10 in the sub-endothelial space.

α-Tocopheryl quinone, known as a metabolite and oxidation product of α-tocopherol,[62] may also play an important role. It is not a radical scavenger by itself but its reduced form, α-tocopheryl hydroquinone, is a potent antioxidant and capable of reducing α-tocopheroxyl radical to give α-tocopherol.[63–65] The concentrations

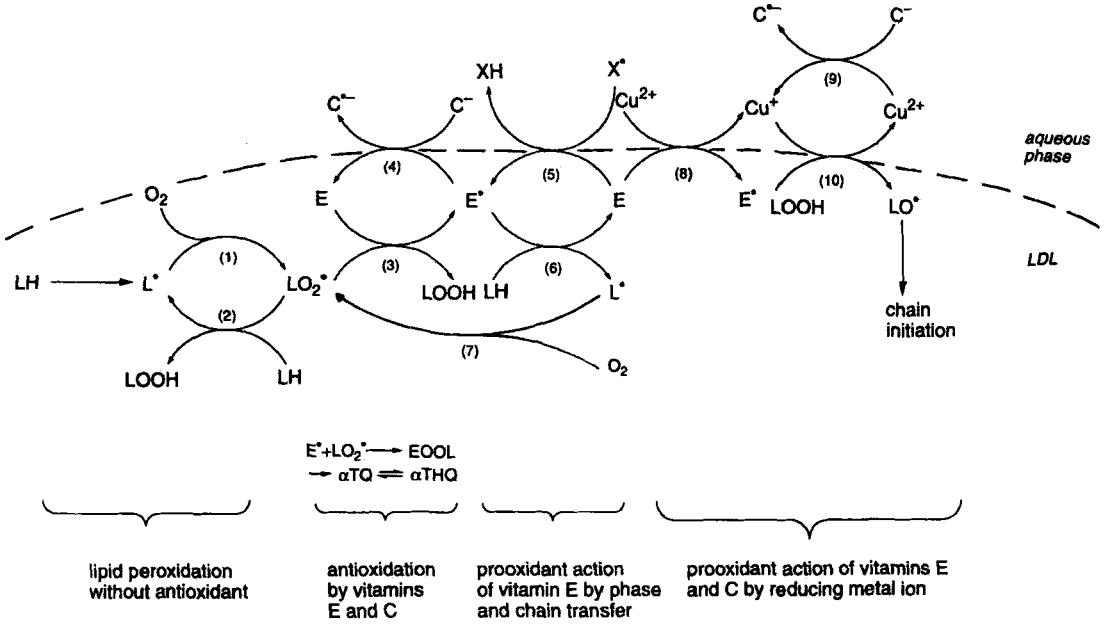

FIGURE 5 Action of vitamin E and vitamin C against lipid peroxidation (extension and modification of Ref. [68] and other papers cited herein). In the absence of antioxidant, lipid (LH) is oxidized by a chain propagation sequence of reactions (1) and (2) until the bimolecular chain termination of radicals (not shown) takes place. Vitamin E (E) scavenges peroxyl radical (reaction (3)) to break chain propagation and the resulting vitamin E radical ($E^{\bullet}$) is reduced by vitamin C (C^{-}) (reaction (4)) to regenerate vitamin E with concomitant formation of monodehydroascorbate ($C^{\bullet -}$). Vitamin E radical may scavenge another lipid peroxyl radical to give an adduct (EOOL), which gives α-tocopherylquinone (αTQ) and α-tocopherylhydroquinone (αTHQ). Vitamin E radical formed by reaction (3) or by reacting with other radical oxidant (reaction (5)) may attack lipid or lipid hydroperoxide to give lipid radical ($L^{\bullet}$) or lipid peroxyl radical respectively (reaction (6)), which initiates chain reaction by a sequence of (7)–(3)–(6) and results in the prooxidant action of vitamin E.[34] Under physiological conditions, the reaction (4) proceeds much faster than the reaction (6) and thus vitamin C inhibits prooxidant action of vitamin E. As described in the text, ubiquinol may exert similar effect (not shown). Vitamins E and C may exert prooxidant action by reducing copper(II) to copper(I) (reaction (8) and (9) respectively), which decomposes lipid hydroperoxide (reaction (10)) rapidly to give alkoxyl radical ($LO^{\bullet}$) and initiate chain reaction. It is important to appreciate that the relative importance of these reactions is determined by the reaction conditions and physical factors and that it determines the total potency of antioxidants.

of α-tocopheryl quinone and hydroquinone in human LDL are not known.

Recently, Bohm *et al.*[66] proposed that β-carotene reduces α-tocopheroxyl radical by electron transfer and the resulting β-carotene cation radical is reduced by ascorbate to regenerate β-carotene. This is interesting but not in accordance with some published data,[67] and such action of β-carotene should be clarified in the future studies.

The proposed scheme for the action of vitamin E as an antioxidant is summarized in Figure 5.

PHYSIOLOGICAL ROLE OF VITAMIN E

Numerous studies on vitamin E *in vitro* show that it acts as a potent antioxidant against oxidation of lipids and oxidative modification of LDL. A prooxidant action of vitamin E is observed under certain oxidation systems as stated above, but the studies in more biologically relevant medium suggest that such prooxidant function should not be important *in vivo*. In agreement with this, the epidemiological and intervention studies show that vitamin E is effective in reducing coronary heart disease.[69–72] It has been also reported that vitamin E has anticancer effects[73] and therapeutic effect for Alzheimer's disease,[74] and that it enhances immune response.[75]

Furthermore, the function of vitamin E beyond radical-scavenging antioxidant action has been proposed and received much attention.[76] For example, it has been suggested that α-tocopherol acts as a negative regulator of smooth muscle cell proliferation in a way independent of its antioxidant properties and suppresses atherosclerosis.[76,77] In this context, the role and function of vitamin E in endothelial cells, macrophages and smooth muscle cells as well as that in LDL particle may play an important protective role against the progression of atherosclerosis.

The role and action of vitamin E have to be clearly elucidated further by future studies and we would like to emphasize the importance of quantitative evaluation of its action taking seriously the effects of reaction medium and conditions into consideration, since the total efficacy of vitamin E as an antioxidant is determined by a relative importance of many competing reactions.

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