Review

Do free radicals play causal role in atherosclerosis? Low density lipoprotein oxidation and vitamin E revisited

Etsuo Niki[1,2,*]

[1]Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan
[2]National Institute of Advanced Industrial Science & Technology, Health Research Institute, Ikeda, Osaka 563-8577, Japan

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Lipid peroxidation induced by free radicals has been implicated in the pathogenesis of various diseases. Numerous *in vitro* and animal studies show that oxidative modification of low density lipoprotein (LDL) is an important initial event of atherosclerosis. Vitamin E and other antioxidants inhibit low density lipoprotein oxidation efficiently *in vitro*, however, human clinical trials with vitamin E have not yielded positive results. The mixed results for vitamin E effect may be ascribed primarily to the two factors. Firstly low density lipoprotein oxidation proceeds by multiple pathways mediated not only by free radicals but also by other non-radical oxidants and vitamin E is effective only against free radical mediated oxidation. Secondly, in contrast to animal experiments, vitamin E is given at the latter stage where oxidation is no more important. Free radicals must play causal role in pathogenesis of atherosclerosis and vitamin E should be effective if given at right time to right subjects.

***Key Words*:** **atherosclerosis, biomarker, free radical, lipid peroxidation, vitamin E**

It is now widely accepted that free radicals and related reactive oxygen and nitrogen species, ROS/RNS, play an important role in the pathogenesis of various disorders and diseases, although, like oxygen molecule, they are double edged sword and under certain circumstances they exert important physiological functions to protect the host from foreign compounds and to maintain homeostasis. Many lines of evidence suggest involvement of free radicals in the pathogenesis of various diseases and in fact numerous observations have been reported which suggest the correlation between the increase in free radical-mediated oxidation products and the progression of diseases. One of such examples is the oxidation of low density lipoprotein (LDL) and atherosclerosis. Since the first proposal of the LDL oxidation hypothesis for atherosclerosis by Steinberg and his colleagues in 1989,[1] ample evidence has been presented supporting the hypothesis that oxidative modification of LDL is the key initial event for the progression of atherosclerosis.[2] Oxidized LDL stimulates endothelial cells to produce inflammatory markers, is involved in foam cell formation, has cytotoxic effects on endothelial cells, inhibits the motility of tissue macrophages, and inhibits nitric oxide-induced vasodilatation. It is now clear that oxidation of LDL lipids and apolipoprotein B 100 renders LDL pro-atherogenic[3] and furthermore high density lipoprotein (HDL) oxidation impairs its inherent anti-atherogenic properties.[4] Thus, it is generally accepted that oxidative modification of LDL followed by uptake of the modified LDL by macrophages and formation of cholesterol laden foam cells is important initial event of atherosclerosis leading to vascular diseases.

The above evidence suggests that the antioxidants which inhibit oxidation of LDL and HDL should be effective for prevention of atherosclerosis and related diseases and numerous studies have been performed to examine the beneficial effect of antioxidants. It has been shown that the antioxidants which inhibit LDL oxidation *in vitro* are in general, if not always, effective for prevention of atherosclerosis in animal models. However, the results of large scale human intervention studies have been inconsistent and disappointing. Among the antioxidants, vitamin E has been studied most extensively, but the results did not show consistently positive effects as summarized in Table 1. These results have casted doubts on the oxidation hypothesis and also the role of antioxidants. Considering the facts that the diseases related to atherosclerosis such as cardio- and cerebral vascular diseases are one of the major causes of death today, the free radicals, lipoprotein oxidation, and vitamin E are important issues that should be addressed in more detail.

This brief review focuses on the LDL lipid oxidation and effect of vitamin E as an antioxidant against LDL oxidation atherosclerosis and discusses possible complications related to them.

LDL Oxidation

LDL particle contains several hundred molecules each of phospholipids, cholesteryl esters, and triglycerides together with free cholesterol. LDL oxidation proceeds by multiple mechanisms induced by different oxidants.[3] LDL may be oxidized within artery wall and also in peripheral sites of inflammation. Lipids, above all polyunsaturated fatty acids (PUFAs) are quite vulnerable to free radical attack and readily oxidized to give versatile products including aldehydes, which react with lysines and tyrosines in apo-lipoprotein B-100 resulting in their modification and functional loss. A minimally oxidized LDL, which is still recognized by LDL receptors but not by a ligand for scavenger receptors, exerts pro-atherogenic effects.[5] The oxidation of LDL lipids has been studied extensively and the products of phospholipids and cholesteryl esters have been studied in detail for their levels and roles in atherosclerosis.[6] Polycyclic ring and side chain of cholesterol are also an important substrate and oxidized to give various products named oxysterols.[7] One important issue is that lipids are oxidized by several types of oxidants by distinct mechanisms and the capacity of antioxidants depends on the oxidants. Therefore, it is important to analyze lipid oxidation products from which the oxidants can be specified and antioxidant efficacy may be assessed.

*To whom correspondence should be addressed.
E-mail: etsuo_niki@yahoo.co.jp

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Table 1. Results of human clinical trials on the effects of vitamin E against atherosclerosis and related diseases

YES	NO
CHAOS (1996)	MVP (1997)
CLAS (1996)	ATBC (1997, 1998)
IEISS (1996)	GISSI (1999)
ASAP (2000)	HOPE (2000)
SPACE (2000)	PPP (2000)
TAAS (2002)	HPS (2002)
WACS (2007)	VEAPS (2002)
ICARE (2008)	SUVIMAX (2004)
	HATS (2004)
	WAVE (2006)
	PHS II (2008)

ASAP: Antioxidant supplementation in atherosclerosis prevention study; ATBC: The alpha-tocopherol, beta carotene cancer prevention study; CHAOS: Cambridge heart antioxidant study; CLAS: Cholesterol lowering atherosclerosis study; Fang *et al.*;[33] GISSI: Gruppo Italiano per lo studio della sopravvivenz nell infarto miocardico; HATS: HDL atherosclerosis treatment study; HOPE: The heart outcomes prevention evaluation study; HPS: Heart protection study; ICARE: Israel cardiovascular events reduction with vitamin E study; IEISS: Indian experiment of infarct survival-3; MVP: Multivitamin prevention study; PHS II: Physicians' Health Study II; PPP: Primary prevention project; SPACE: Secondary prevention with antioxidants of cardiovascular disease in endstage renal disease; SUVIMAX: SUpplémentation en VItamines et Minéraux AntioXydants Study; TAAS: Transplant associated arteriosclerosis study; VEAPS: Vitamin E atherosclerosis prevention study; WACS: Women's antioxidant cardiovascular study; WAVE: Women's angiographic vitamin and estrogen trial.

Table 2. Hydroxyeicosatetraenoic acid (HETE) isomers from oxidation of arachidonic acid: *5,8,11,14-eicosatetraenoic acid*

	Isomers		
	Regio	*Stereo*	*Enantio*
Free radical	5, 8, 9, 11, 12, 15	*ZE*, *EE*	*R* = *S*
Lipoxygenase	5, 12, 15	*ZE*	*R* or *S*
Cytochrome *P450*	5, 8, 9, 11, 12, 15, 19, 20	*ZE*	*R* or *S*
Singlet oxygen	5, 6, 8, 9, 11, 12, 14, 15	*ZE*	

Arachidonates, esters of 5,8,11,14-eicosatetraenoic acid, are one of the major PUFAs *in vivo* and their oxidation gives hydroxyeicosatetraenoates, HETE, as major lipid oxidation product found in human plasma. Various isomers of HETE are produced by different oxidants as summarized in Table 2, which shows that 5-, 12-, and 15-HETE are formed by either free radicals, lipoxygenase, cytochrome P450 or singlet oxygen. α-Tocopherol, a major isoform of vitamin E *in vivo*, is effective only against free radical-mediated oxidation and it is not a potent antioxidant against oxidation induced by lipoxygenase, cytochrome P450, nor singlet oxygen.

The oxidants responsible for LDL oxidation *in vivo* have been the subject of extensive studies and arguments. Various compounds have been shown to induce LDL oxidative modification to such a form observed in atherosclerotic lesions. Metals such as iron and copper may be a potent candidate, although they are sequestered by proteins *in vivo*. Hemoglobin (Hb) and myoglobin are known to induce LDL oxidation. Hemoglobin may be leaked from damaged erythrocytes, especially in the vascular regions with turbulent flow, or in hemodialysis patients. Hemoglobin is sequestered by haptoglobin (Hp) which exists as a homodimer, whose monomer could be one of the allelic variants, Hp1 or Hp2. Hp2 variant is less effective in preventing oxidation than Hp1 and interestingly subjects with the Hp2-2 phenotype have higher risk for CVD than those with Hp1-1 or Hp1-2 variants.[8] The patients of Wilson disease have abnormally low levels of ceruloplasmin which bind copper and copper accumulated in the liver may cause oxidative stress.[9] The redox active transition metal ions such as iron and copper contribute to the formation of free radicals and in the initiation of LDL oxidation.

It has been observed that LDL is oxidized when incubated with several types of cells such as endothelial cells, smooth muscle cells, neutrophils, and macrophages. Various enzymes may contribute in the oxidation such as myeloperoxidase, lipoxygenase, NADPH oxidases and nitric oxide synthases.

Myeloperoxidase (MPO), a heme enzyme released by activated neutrophils and monocyte/macrophages at sites of inflammation, has been implicated in the oxidation of LDL and HDL.[4] MPO together with hydrogen peroxide and chloride ion produces hypochlorite, which is a strong oxidant and oxidizes proteins and lipids by radical and non-radical mechanisms. Chlorohydrins are specific product of hypochlorite mediated non-radical oxidation and it was found that lysophosphatidylcholine chlorohydrins were elevated over 60 fold in human atherosclerotic lesions,[10] although hypochlorite reacts with protein components more rapidly than with lipids.[11] There is now ample evidence showing that MPO modifies both LDL and HDL into pro-atherosclerotic forms. Importantly, vitamin E is not a potent inhibitor of hypochlorite mediated oxidation.

Lipoxygenase, LOX, is another important oxidant. Above all, 15-LOX is capable oxidizing arachidonate and linoleate of phopholipids and cholesteryl esters within LDL particles directly to give 15-hydroperoxy-eicosatetraenoic acid (15-HpETE) and 13-hydroperoxy-octadecadienoic acid (13-HpODE) respectively. In contrast to free radical oxidation which gives random, racemic products, the oxidation by 15-LOX gives regio-, stereo-, and enantio-specific products, which are in fact found in human atherosclerotic lesions. Kuhn and his colleagues found that in young human lesions the hydroxyl-linoleic acid/linoleic acid ratio was much smaller than that in advanced lesions and that the ratio of S/R of 13- hydroxy-octadecadienoic acid (13-HODE) was significantly higher than 1, suggesting a contribution of 15-LOX especially for early atherogenesis.[12]

As described above, LDL may be oxidized *in vivo* by different oxidants by either free radical or non-radical mechanisms. Specific products of lipid oxidation for different oxidants are summarized in Table 3. Cholesterol is oxidized also by different oxidants to give specific products. The trans, trans form of HODE and HETE are specific products of free radical oxidation and 9- and 13-trans,trans-HODE are convenient marker for free radical oxidation of linoleates. F_2-isoprostanes and F_4-neuroprostanes formed by free radial oxidation of arachidonates and docosahexanoates respectively are now accepted as most reliable biomarker of lipid oxidation *in vivo*. 7β-hydroxycholesterol and 7-ketocholesterol are formed primarily by free radical oxidation of cholesterol.

An increase in lipid oxidation products in atherosclerosis patients and experimental animals has been observed in many studies[13] and references cited therein. It is known that the oxidative modification of LDL increases electronegativity of LDL particles. We have confirmed that the levels of trans,trans-HODE, 8-isoprostane(8-isoP), and 7β-hydroxycholesterol, specific products of free radical oxidation, increased with an increase in LDL electronegativity of human LDL, both in absolute concentration and in the ratio relative to respective parent substrate.[14]

HDL Oxidation

HDL is inherently capable of exerting anti-atherogenic effect by metabolizing and transporting lipid oxidation products as well as cholesterol from the cells to the liver by virtue of paraoxonase-1 (PON-1), lecithin-cholesterol acyltransferase (LCAT),

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Table 3. Specific oxidation products of linoleates, arachidonates, and cholesterol for different oxidants

	Linoleate	Arachidonate	Cholesterol
Free racdical ($LO_2^{\cdot}$)	9-,13-*EE*-H(p)ODE	F_2-isoprostane	7α-OHCh, 7-KCh
NO_2	nitro-linoleates		NO_2-Ch
$^1O_2$	10-,12-*ZE*-H(p)ODE		5-OOHCh, secosterol
Ozone			secosterol
LOX	13(S)-H(p)ODE, S/R>>1	5-,12,15-H(p)ETE	
COX		PGH_2, PGD, PGE, PGF, TX	
CYP450		19,20-HETE	4α-,24(S)-,27-OHCh
MPO, HOCl	chlorohydrin		chlorohydrins, secosterol

COX: cyclooxygenase; HETE: hydroxy-eicosatetraenoic acid; H(p)ETE: hydroperoxy-eicosatetraenoic acid; H(p)ODE: hydroperoxy-octadecadienoic acid; KCh: ketocholesterol; LOX: Lipoxygenase; MPO: Myeloperoxidase; PGD: prostaglandin D; PGE: prostaglandin E; PGF: prostaglandin F; PGH2: prostaglandin H2; TX: thromboxane; OOHCh: hydroperoxycholesterol; OHCh: hydroxycholesterol

Table 4. Biomarkers of oxidative stress

Lipid	Protein	DNA
Ethane and pentane	Protein carbonyl	Comet assay
in exhaled gas	Hydroperoxide	Thymine glycol
TBARS	Nitro-, chloro-, bromo-amino acid	2-, 8-Hydroxyadenine
Conjugated diene	Disulfide -SS-	8-Hydroxyguanine
Hydroperoxide	-SOH, -SOOH, -SOOOH	8-Nitro-, chloro-, bromo-guanine
Aldehydes	Aldehyde-modified protein	Lipid peroxidation product-modified
Ketone	Hydroperoxide-modified protein	DNA bases
Isoprostane	Crosslinked protein	
Neuroprostane	Dityrosine	
Isofuran	Albumin dimer	
Neurofuran	Advanced oxidation products	
HODE	Creatol	
HETE	Myeloperoxidase	
Lyso PC	Lipofuscin	
Nitrofatty acids	Cleavage products	
Chlorohydrins		
Oxidized LDL		
Oxysterols		

HETE: hydroxy-eicosatetraenoic acid; HODE: hydroxy-octadecadienoic acid; LysoPC: lysophosphatidylcholine; Oxidized LDL: oxidized low density lipoprotein; TBARS: thiobarbituric acid reactive substances

phospholipase A_2, and platelet-activating factor acetylhydrolase (PAF-AH).[15,16] The oxidative modification of HDL impairs the "reverse cholesterol transport" ability of apoA-I and also anti-inflammatory function of HDL.[17] It is assumed that HDL is oxidized by substantially the same oxidants and mechanisms as LDL. It has been found that HDL is a major carrier of lipid hydroperoxides[18] and isoprostanes.[19] The higher lipid oxidation products in HDL than in LDL may be due to higher activity of PAF-AH in LDL which hydrolyses esterified oxidation products from phospholipids.

Effect of Vitamin E against LDL Oxidation and Atherosclerosis

Numerous studies show that vitamin E and related radical scavenging antioxidants inhibit LDL oxidation efficiently *in vitro*, especially together with vitamin C. The dynamics of antioxidant action of vitamin E in LDL particle has been studied in detail.[20] Furthermore, vitamin E and related synthetic antioxidant such as 2,3-dihydro-5-hydroxy-2,2-dipentyl-4,6-di-tert-butylbenzofuran (BO-653) suppressed atherosclerosis development in animal models.[21] These results strongly suggest that antioxidants should exert protective and therapeutic effects against atherosclerosis and related diseases and supplementation of antioxidants, particularly vitamin E, received much attention.

Effects of Vitamin E in Humans

Many large scale epidemiologic studies such as WHO/MONICA study,[22] Health Professionals' follow-up study,[23] Nurses' Health Study,[24] and EVA study[25] supported the beneficial effect of vitamin E against atherosclerosis and cardiovascular diseases. However, in contrast to this expectation, the results of large scale randomized clinical intervention studies of vitamin E were disappointing. Moreover, some meta-analyses showed that vitamin E supplementation may increase mortality. These results have raised much arguments and criticisms.[26–28] The choice of proper subjects, antioxidant, and dose and duration of supplementation has been argued as important factors that determine the outcome. The importance of biomarker measurement, patient compliance, and data interpretation has been pointed out also.

Two points should be emphasized here as critical factors which

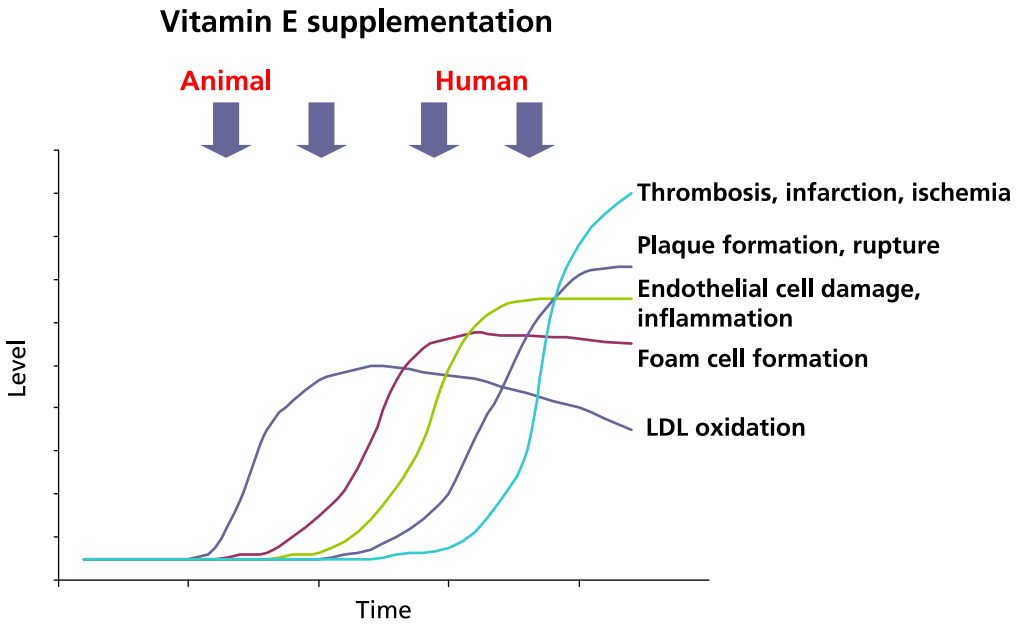

Fig. 1. Progress of atherosclerosis and vitamin E supplementation (see text).

determine the efficacy of vitamin E. Firstly, as mentioned above, oxidative modification of LDL and HDL proceeds by different oxidants by different mechanisms, and vitamin E is effective against free radical mediated oxidation, but not against oxidation induced by lipoxygenase and hypohalite. An increase in the levels of oxidation products produced not only by free radical oxidation but also by lipoxygenase and hypochlorite has been observed and the relative importance of these different mechanisms may vary, suggesting that the relative importance of vitamin E may also vary according to time and circumstance.

Secondly, atherosclerosis is a chronic disease and its onset, that is, the oxidative modification of LDL may have taken place decades before the symptoms appear. The major difference between human trials and animal experiments is that, in general, human trials start often after the onset of oxidative modification of LDL at the age of 50 or above, whereas in animal studies vitamin E or other antioxidant is given usually at the same time as the onset of oxidative stress (Fig. 1). It may be unrealistic to expect that a few years of antioxidant supplementation can turn around the effects of oxidative stress on endothelium which lasted more than 30 years. In contrast to human trials, the animal studies are performed under the same lifestyle with the same diet, which may yield more consistent outcome than human trials.

Biomarkers

It has been pointed out that the measurement of biomarkers for oxidative stress and antioxidant level in the subjects is important for the evaluation of antioxidant supplementation. Various biomarkers have been used for assessment of oxidative stress *in vivo* (Table 4). The levels and products of antioxidants have been used also as a biomarker of oxidative stress.[29] Plasma and urine have been used often as a convenient, non-invasive specimen, but these are global measurements and it is difficult to specify where the oxidation takes place: the oxidation may be occurring at sites relating to atherogenesis or anywhere in the body. It should be considered also that the biomarkers listed in Table 4 are surrogate markers which may give different effect from that based on clinically relevant outcome.

It is important to develop new tools and methods to prove if vitamin E is effective in the prevention of atherosclerosis in humans. Non-invasive imaging techniques appear to be promising. There is increasing evidence that (18)F-fluorodeoxyglucose (FDG)—positron emission tomography (PET) imaging can be useful for non-invasive measurement of atherosclerotic plaque inflammation in humans.[30] Radio isotope labeled anti-lectin like oxidized LDL receptor 1 (LOX-1) monoclonal IgG has been used as potential agent for imaging atherosclerotic.[31] Furthermore, CT/PET imaging using radioisotope-labeled monoclonal antibody against oxidized LDL or related oxidation products can be a powerful tool for monitoring progression of atherosclerosis and assessment of the antioxidant effects.[32]

Conclusion

Free radicals must play important causal role in the pathogenesis of some diseases and antioxidants such as vitamin E must be beneficial for prevention and/or treatment of such diseases when used at right time and to right subjects under oxidative stress.

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