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Reviews

Antioxidants and atherogenesis

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The clinical sequelae of atherosclerosis, coronary heart disease, stroke, and peripheral vascular disease are the leading causes of morbidity and mortality in the United States. A collection of "risk factors" explains only part of the variation in atherosclerosis between individuals, suggesting that other yet to be determined factors contribute to the risk of atherosclerosis and cardiovascular disease. Several lines of evidence suggest that oxidation of lipoproteins plays a role in atherogenesis. Population-based studies and studies in experimental animals suggest that antioxidants reduce atherosclerosis and cardiovascular disease. Basic biochemical studies offer a number of mechanism(s) by which antioxidants might provide this protection. However, the few data from clinical trials of supplementation of the diet with isolated nutrient antioxidants do not consistently support an inhibitory role for antioxidants in atherogenesis and cardiovascular disease. Thus, appropriately designed clinical trials are needed before recommendations for dietary intake of specific antioxidants to reduce risk of atherosclerosis and cardiovascular disease can be developed. Such trials should be designed based on a critical evaluation of the existing body of basic and clinical data for the inhibitory effects of antioxidants on atherogenesis. Attention should be specifically paid to the body of data showing interaction of various antioxidants. The issue of antioxidant needs for optimal prevention of atherosclerosis as a function of pathological, physiological, dietary, and behavioral characteristics of individuals should be addressed. Execution of properly designed clinical trials would be facilitated by identification of biomarker(s) that accurately reflect the extent to which an antioxidant intervention inhibits atherosclerosis. Despite uncertainty regarding the health benefits of isolated antioxidant(s), all available evidence continues to support the health benefits of increased consumption of a wide variety of fruits and vegetables rich in antioxidants. (J. Nutr. Biochem. 9:424–445, 1998) © *Elsevier Science Inc. 1998*

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Pathogenesis of atherosclerosis

In the United States, cardiovascular disease is the leading cause of morbidity and mortality.[1] Cardiovascular disease, including coronary heart disease, stroke, and peripheral vascular disease, is the clinical expression of advanced atherosclerosis.[1] Variation in atherosclerosis between individuals can be partially explained by a collection of "risk factors," with plasma cholesterol concentration, distribution of cholesterol among lipoproteins, blood pressure, and smoking status contributing significantly to risk of atherosclerosis-related diseases.[2–5]

Atherosclerosis is characterized by the focal development of atherosclerotic lesions in large arteries. Atherosclerotic lesions are thought to be initiated by accumulation of lipoproteins within the intima, adhesion of monocytes to the arterial endothelium, emigration of monocytes into the arterial intima, possibly in response to chemotactic stimuli provided either directly or indirectly by oxidized lipoproteins, and accumulation of cholesterol within macrophages.[6–8] Growth factors, cytokines, and other vasoactive substances secreted by macrophages, smooth muscle cells,

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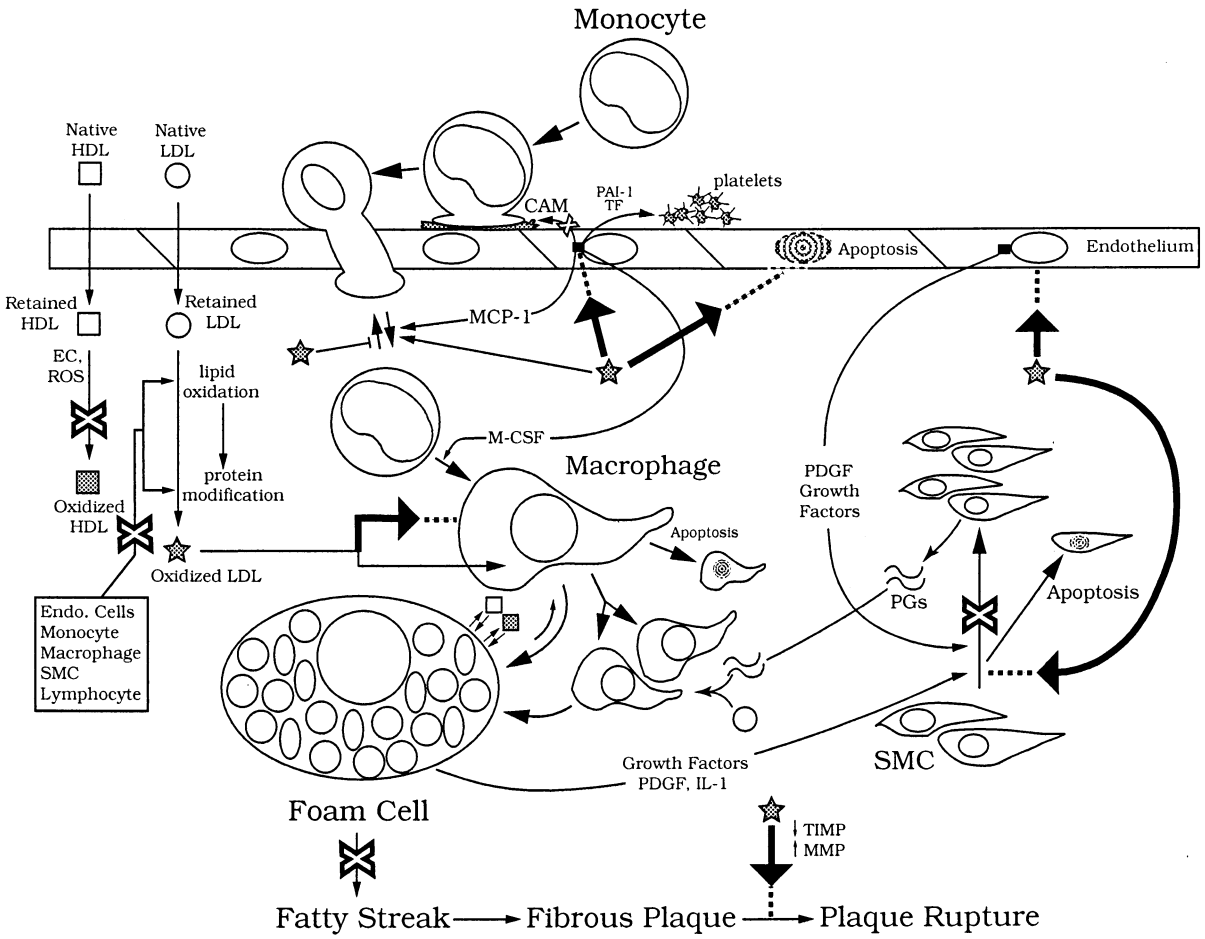

Figure 1 Schematic representation of the role that oxidation of LDL might play in the development of atherosclerosis. Thick arrows and dotted lines indicate direct effects of oxidized LDL. LDL, low density lipoprotein; HDL, high density lipoprotein; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; CAM, cell adhesion molecule; MCP-1, monocyte chemotactic protein-1; M-CSF, monocyte colony stimulating factor; PDGF, platelet-derived growth factor; IL-1, interleukin-1; PGs, proteoglycans; SMC, smooth muscle cell; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; EC, endothelial cell; ROS, reactive oxygen species. Unfilled X, processes that are inhibited by antioxidants.

and endothelial cells influence the further progression of atherosclerosis.[6,7]

At later stages of atherosclerosis, smooth muscle cells migrate from the arterial media into the intima, proliferate, and accumulate cholesterol.[6,7,9] The composition of the extracellular connective tissue proteins are altered, and the amount of extracellular connective tissue proteins is increased,[6,7,10] potentially facilitating retention of lipoproteins.[11,12] In advanced atherosclerotic lesions (atherosclerotic plaques), cholesterol is present extracellularly as cholesterol crystals, and evidence of calcification, necrosis, and hemorrhage can be found.[10] The most common cause of an acute heart attack or stroke is sudden blockage of a coronary or cerebral artery, respectively, due to thrombosis at sites of rupture of an atherosclerotic plaque. Most such thrombotic events occur in association with atherosclerotic plaques that are relatively small (occluding less than 50% of the arterial lumen) but with altered composition characterized by increased numbers of cholesterol-loaded macrophages and reduced amounts of connective tissue proteins.[7,13]

Mechanisms by which oxidation might promote and antioxidants might inhibit atherosclerosis

The oxidation hypothesis, supported primarily by experiments in vitro, suggests multiple mechanism(s) by which oxidation of low density lipoprotein (LDL) might promote atherogenesis (see *Figure 1*): Lipoproteins, including LDL and high density lipoprotein (HDL), are transported into the artery.[14,15] LDL retained within the artery can be oxidized by a number of cell types present within arteries, including endothelial cells,[16] smooth muscle cells,[17] monocytes,[18] and macrophages,[19] and by lymphocytes,[20] a cell type that is present in atherosclerotic lesions.[21] Macrophage foam cells isolated from atherosclerotic lesions of rabbits also oxidize LDL in vitro.[22] HDL can also be oxidized by endothelial cells and by chemical means.[23–27] Oxidation of these lipoproteins can be blocked by antioxidants.[26,28,29]

Lysophosphatidylcholine, a product generated by the phospholipase A_2 activity of apoprotein B during oxidation of LDL,[30] attracts monocytes and inhibits the mobility of tissue macrophages and, thus, potentially could contribute

to monocyte recruitment into arteries.[8,31] In vitro, oxidized LDL enhances production of chemotactic factors such as monocyte chemotactic protein-1 (MCP-1) by endothelial cells,[32] and enhances endothelial expression of intercellular cell adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1),[33] which could facilitate monocyte recruitment.[34]

In vivo, supplementation of hypercholesterolemic rabbits with probucol reduced arterial expression of VCAM-1,[35] reduced monocyte adhesion,[36] and prevented the development of macrophage-rich atherosclerotic lesions.[35] Oxidized LDL could also promote macrophage proliferation by inducing endothelial cells to secrete macrophage-colony stimulating factor (M-CSF).[37] Oxidation converts LDL to a form that is rapidly taken up and degraded by macrophages in vitro,[16] converting macrophages into foam cells.[38] In an in vitro perfusion system, arterial uptake of LDL could be blocked by either acetylated LDL or vitamin E,[39] suggesting that part of the uptake of LDL occurred via scavenger and/or oxidized LDL receptors[40] subsequent to intra-arterial oxidation. Other studies suggest that oxidized LDL may also increase macrophage expression of scavenger receptors.[41] Studies in vivo suggest that antioxidants reduce atherosclerosis by inhibiting metabolism of LDL in atherosclerotic lesions secondary to blocking oxidation of LDL within the atherosclerotic lesions.[42]

Studies in vitro indicate that oxidized LDL can induce smooth muscle cell migration[43] and induce both proliferation[44–46] and apoptosis[44,47–51] in endothelial cells,[48,50] smooth muscle cells,[44,46,49,51] and macrophages.[44,47] Several studies[44,52,53] suggest that the degree of oxidation determines whether proliferation, apoptosis, or both, will occur. Smooth muscle cells exposed to oxidized LDL express increased numbers of surface receptors for platelet-derived growth factor (PDGF)[54] potentially enhancing the mitogenic effect of PDGF secreted by foam cells and endothelial cells and by the smooth muscle cells themselves. In addition, oxidized LDL stimulates interleukin-1 production by macrophage foam cells, which could further promote smooth muscle cell proliferation.[55] These studies do not allow one to predict how oxidation of LDL in vivo might influence cellular proliferation or apoptosis in atherosclerotic lesions. However, atherosclerotic lesions show evidence of both cellular proliferation[56,57] and apoptosis.[49,56,58]

Oxidized LDL also has other potentially atherogenic effects, stimulating endothelial cells to release plasminogen activator inhibitor-1 (PAI-1)[59] and tissue factor activity,[60] which could promote platelet aggregation, and possibly foam cell formation.[61] Other data[62] suggest that the proliferation of smooth muscle cells induced by oxidized LDL might cause these cells to synthesize proteoglycans with increased affinity for unoxidized LDL. This could increase macrophage degradation of unoxidized LDL in complexes with proteoglycan and promote the formation of foam cells.[63–66] Also, the interleukin-1 released from macrophage foam cells exposed to oxidized LDL[67] could increase smooth muscle cell expression of matrix metalloproteinases (MMP),[68,69] and possibly alter the balance between MMP and tissue inhibitors of metalloproteinases (TIMP) in ways that would promote rupture of atherosclerotic plaques.[70,71]

Other data suggest that antioxidants may influence atherosclerosis independent of inhibition of oxidation of LDL. For example, the endothelial expression of VCAM-1 induced by exposure to oxidized LDL is partially blocked by pretreatment of endothelial cells in vitro with antioxidants such as probucol or vitamin E.[33] In vitro, vitamin E blocked the stimulation of smooth muscle cell proliferation induced by oxidized LDL,[72] lysophosphatidylcholine,[46] and by PDGF.[73] Tocopherol and tocotrienols also reduced smooth muscle cell proliferation induced by serum.[73–75] Also, antioxidants reduce toxicity of oxidized LDL to endothelial cells,[76] smooth muscle cells,[53,77] and macrophages.[78] Those studies did not consider the mechanism by which oxidized LDL induced cell death. However, other work indicates that several antioxidants prevent apoptosis induced in endothelial cells by lipopolysaccharides.[79] Thus, it is possible that antioxidants also inhibit apoptosis mediated by oxidized LDL.

From in vitro studies, it is not possible to predict how antioxidants might influence the balance between cellular proliferation and cell death by apoptosis and other means that determine cellular accumulation in atherosclerotic lesions in vivo. In animals in which atherosclerosis was induced by hypercholesterolemia, the antioxidant probucol reduced the macrophage content of atherosclerotic lesions.[80–82] In comparison, vitamin E decreased smooth muscle cell proliferation[73] after arterial injury, and both probucol[83] and vitamin E[72,73] reduced intimal smooth muscle cell accumulation after arterial injury. This suggests that, in vivo, any inhibition of smooth muscle or macrophage apoptosis (or necrosis) by these antioxidants was more than counterbalanced by inhibition of proliferation of these cells. Potentially, these effects may be related to effects of antioxidants on monocyte-endothelial cell interactions and production of cytokines or growth factors,[84] or response of smooth muscle cells to such factors.

In tissue culture, the proliferative state of smooth muscle cells modulates the synthesis of proteoglycan[85] and collagen.[86] These studies suggest that the inhibition of smooth muscle cell proliferation by antioxidants might alter synthesis of extracellular matrix components. Such alterations in extracellular matrix components might influence atherosclerosis secondary to effects on binding of LDL to extracellular matrix and subsequent rapid uptake and cholesterol accumulation in macrophages.[63–66] Antioxidants might also influence cardiovascular disease by means independent of effects on atherosclerosis, such as by reducing oxidative degradation of nitric oxide,[87,88] both limiting vasoconstriction[89] and reducing blood pressure.[90]

The processes contributing to atherogenesis may vary as atherosclerotic lesions develop, and antioxidants may have multiple effects. Thus, intervention with antioxidants might well have disparate effects at different stages of atherosclerosis. For example, reduction of monocyte infiltration and foam cell formation by antioxidant treatment would be expected to delay initiation of atherosclerosis, consistent with results in experimental animals.[42,91–97] However, it is possible that the inhibition of smooth muscle cell proliferation out of proportion to the reduction of apoptosis by antioxidants could be harmful when atherosclerotic plaques are present: reduced smooth muscle cell proliferation in fibrous caps might reduce the thickness of such fibrous caps and predispose atherosclerotic plaques to rupture.

Evidence for and against the oxidation hypothesis of atherosclerosis

Evidence of several types suggests that oxidation promotes atherosclerosis. Several studies have reported on the presence of products of cholesterol oxidation in atherosclerotic lesions.[98,99] Other work showed that LDL carefully extracted from atherosclerotic lesions shared many characteristics with oxidized LDL.[100,101] Other evidence is provided by the presence of oxidized epitopes in atherosclerotic lesions.[102–104] However, as shown by another study,[105] the presence of oxidized epitopes in atherosclerotic arteries may reflect oxidation of other proteins, rather than, or addition to, LDL protein. Furthermore, the presence of oxidized epitopes within atherosclerotic lesions does not establish that oxidation plays a causative role in atherosclerosis.

A number of studies have shown that treatment of animals with pharmacologic antioxidants, including probucol,[42,91–95,106,107] butylated hydroxytoluene (BHT),[96] and *N*,*N*′-diphenyl-phenylenediamine,[97] inhibits atherosclerosis. While such data are consistent with the oxidation hypothesis, there are other possible interpretations, particularly with respect to probucol. Two studies reported that the inhibition of atherosclerosis by probucol treatment was not associated with reduction in arterial staining for oxidized epitopes,[80,82] contrary to what might be expected if probucol reduced atherosclerosis by inhibiting oxidation of LDL. Probucol has a number of effects on cells in vitro[77,108–110] and alters intravascular metabolism and composition of lipoproteins in vivo[111,112] in ways that would be expected to influence atherosclerosis. It is possible that the multiple effects of probucol may explain why it effectively inhibits atherosclerosis in rabbits,[42,91–95,106] but exacerbates atherosclerosis in mice genetically altered to lack apoprotein E.[113] For example, probucol increases the activity of cholesterol ester-exchange protein,[111,114] an important player in the intravascular metabolism of lipoproteins in human beings, primates, and rabbits, but which is functionally absent in mice.[115–118] Probucol treatment also enhances the ability of HDL to promote cholesterol efflux.[119]

In addition, in primates, probucol inhibits atherosclerosis in some, but not other, arterial sites,[107] suggesting that the processes that promote atherosclerosis and that are inhibited by probucol differ among regions of artery. Interestingly, the muscular abdominal and iliac arteries of monkeys were unaffected by probucol treatment,[107] and this is consistent with the lack of effect of probucol (when added to treatment with diet and cholestyramine) on atherosclerosis in the muscular femoral arteries of humans.[120] Thus, although inhibition of atherosclerosis by pharmacologic antioxidants is consistent with the oxidation hypothesis, the multiple effects of probucol, and possibly other antioxidants, diminish the support that such studies can provide for the oxidation hypothesis.

While oxidized LDL has a number of effects that could promote atherosclerosis as described above, other observations are inconsistent with the idea that oxidation of LDL promotes atherosclerosis. One difficulty with the oxidation hypothesis relates to the prediction that it would make regarding the role of dietary fat saturation in atherosclerosis. Compared with monounsaturated fat, high dietary intake of polyunsaturated fat increases the susceptibility of isolated LDL to in vitro lipid peroxidation[121–124] while susceptibility of LDL to in vitro lipid peroxidation is similar for diets high in monounsaturated or saturated fat.[121] If one assumes that susceptibility to in vitro lipid peroxidation might predict potential for intra-arterial oxidation, one might predict that polyunsaturated fat would increase atherosclerosis compared with monounsaturated or saturated fat. However, clinical trials have shown that morbidity and mortality due to cardiovascular disease can be decreased by reducing the proportion of dietary fat that is saturated.[2,125,127]

No controlled clinical trial has directly compared the influence of monounsaturated and polyunsaturated fat on total or cardiovascular mortality. Results from epidemiological studies conducted within[128,129] and across cultures[130,131] and a case-control study[132] have provided inconsistent results for the relative effects of polyunsaturated and monounsaturated fat on several indices of atherosclerosis and cardiovascular disease. Four studies suggested that polyunsaturated fat was either protective[129,132] or neutral,[130,132] whereas one suggested polyunsaturated fat was harmful.[128] Three studies suggested that monounsaturated fat was either protective[130,131] or neutral,[132] with one study suggesting that monounsaturated fat was harmful.[129] Of importance is a recent study in nonhuman primates showing that feeding diets enriched in polyunsaturated fat reduced atherosclerosis compared with diets enriched in monounsaturated fat or saturated fat, with no difference in atherosclerosis between animals fed monounsaturated or saturated fat.[133] Differences in mortality due to cardiovascular disease associated with differences in type and amount of dietary fat appear to be mediated in part by effects on plasma[2,131] and LDL[134] cholesterol concentrations. Thus, it is of significance that in the controlled study in primates, the protection from atherosclerosis by polyunsaturated fat compared with monounsaturated fat was observed despite similar concentrations of LDL cholesterol.

Thus, while a large body of data would be consistent with the promotion of atherosclerosis by oxidation of LDL, that hypothesis in its current form cannot account for the influence of dietary fat saturation on atherosclerosis. However, it should be noted that extrapolation from effects of dietary fat saturation on susceptibility of LDL to in vitro lipid peroxidation to effects of dietary fat saturation on atherosclerosis ignores other important elements of the equation: effects of dietary fat saturation on arterial cells and the influence of other lipoproteins.

It seems likely that changes in cellular function[135] related to alteration in cellular membrane fatty acid composition toward the composition of the dietary fat[136] may contribute to the modulation of atherosclerosis by dietary fat saturation. One study reported that cellular enrichment with long-chain n-3 fatty acids enhanced cellular oxidation of LDL.[137] Very few studies have directly compared the effects of cellular enrichment with saturated, monounsaturated, and polyunsaturated fatty acids on cellular function. On study showed that cholesterol efflux mediated by a subfraction of HDL (HDL_3) did not differ among endothelial cells enriched with oleic or linoleic acid but was increased for endothelial cells enriched with palmitic acid.[138] Two studies reported opposite effects of cellular

enrichment with eicosapentaenoic acid on cholesterol efflux mediated by HDL_3.[138,139] Potentially, enrichment of membranes of arterial cells with polyunsaturated fatty acids might alter other aspects of cellular function such as phagocytosis and receptor-mediated uptake that might reduce cellular uptake and degradation of oxidized LDL, thus reducing the impact of increased intra-arterial oxidation of LDL on cellular cholesterol accumulation and atherogenesis.

Retention of LDL within the artery in association with components of the extracellular matrix is thought to promote atherosclerosis secondary to enhancing cellular uptake of LDL complexed with components of the extracellular matrix such as proteoglycans.[11,12,140] However, oxidized lipids reduces smooth muscle cell synthesis of proteoglycans,[141] and oxidation of LDL reduces affinity of LDL for proteoglycans.[142] Also, linoleic acid reduces cellular production of proteoglycans.[143] Furthermore, other studies found that LDL isolated from animals fed either polyunsaturated fat rich in n-6 fatty acids[144] or n-3 fatty acids[145] had lower affinity for arterial proteoglycans than LDL isolated from animals fed monounsaturated fat. The reduced binding to proteoglycans for LDL enriched in polyunsaturated fatty acids could limit intra-arterial oxidation of LDL because work in vitro showed that binding of LDL to proteoglycans promoted oxidation of LDL.[66] The studies required to investigate the combined effect of enrichment of both cell membranes and lipoproteins with polyunsaturated fatty acids (compared with monounsaturated and saturated fatty acids) on cell-mediated oxidation of LDL, and cellular uptake and degradation of oxidized LDL, remain to be conducted.

Cellular cholesterol accumulation is the net result of processes contributing cholesterol to cells and processes removing cholesterol from arterial cells. Potentially, atherosclerosis could be reduced even if delivery of cholesterol to arterial cells were increased as long as there was a greater increase in removal processes. HDL is thought to remove cholesterol from arterial cells by reverse cholesterol transport.[146] Also, oxidized LDL promotes secretion of apoprotein E by macrophages,[147] an effect that would be expected to increase cholesterol efflux to HDL_3.[148] In addition, lysophosphatidylcholine, a lipid that is increased in oxidized LDL, promotes cholesterol efflux from macrophages.[149]

Several studies have investigated how oxidation or fatty acid composition of HDL influence its ability to promote cholesterol efflux in vitro.[23–27,150–152] Studies in which HDL was oxidized by copper consistently show reduced ability of oxidized HDL to promote cholesterol efflux.[25–27] However, when HDL is oxidized by myeloperoxidase, an enzyme present within atherosclerotic lesions,[153] ability of oxidized HDL to promote cholesterol efflux is enhanced.[23,24] Other data suggest that compared with monounsaturated fatty acids, enrichment with polyunsaturated fatty acids increases susceptibility of HDL to oxidation.[151] One study suggested that fatty acid saturation of HDL phospholipids did not influence ability of HDL to promote cholesterol efflux from fibroblasts.[152] Later the same group reported that, compared with sunflower oil, feeding olive oil resulted in HDL_3 with enhanced ability to remove cholesterol from fibroblasts.[150]

However, another study with cultivers of sunflower oil that differed only in fatty acid composition showed that HDL_3 isolated after a polyunsaturated fat-rich diet promoted efflux of cholesterol from fibroblasts and macrophages equally as well as HDL isolated after a monounsaturated fat-rich diet.[151] The combined influence of fatty acid saturation and oxidation on the ability of HDL to promote cholesterol efflux, and effects of cellular fatty acid composition on this process, remains to be elucidated. For this reason, and because the mechanism(s) by which oxidation occurs in vivo remain to be determined, it is not clear how fatty acid composition and oxidation of HDL might affect reverse cholesterol transport in vivo.

Case-control studies of the role of antioxidants in atherosclerosis-related diseases in human individuals

Case-control studies have suggested that increased dietary intake,[154] serum levels,[155] and adipose tissue levels[156] of several dietary antioxidants including vitamin E, vitamin C, and β-carotene are associated with reduced risk of cardiovascular disease.[154,156,157] Two of these studies reported that the benefit of β-carotene was limited to smokers.[155,156] In comparison, a recent study reported that low serum levels of several carotenoids, but not β-carotene, were associated with increased risk of early carotid artery atherosclerosis, but that such relationships were no longer evident after adjustment for several risk factors including smoking.[158] One study reported that reduced serum levels of α-tocopherol were associated with increased risk of myocardial infarction only in hyperlipidemic individuals.[155] Another study showed absolute levels of plasma and LDL vitamin E to be similar for survivors of myocardial infarction and age-matched controls while lipid standardized levels of vitamin E were reduced in the survivors of myocardial infarction.[159] Of interest is the finding that LDL vitamin E (whether standardized by lipid or protein) was inversely correlated with global coronary stenosis score.[159] In comparison, another study reported that severity of coronary atherosclerosis was not related to plasma concentrations of vitamins E or C but was inversely related to levels of vitamin E in the wall of the internal mammary artery.[160]

Cohort studies of the role of antioxidants in atherosclerosis-related diseases in human individuals

A number of prospective cohort studies have reported on the association of dietary intake of vitamin E, vitamin C, and β-carotene with various measures of cardiovascular disease. Several such studies report a reduction in relative risk of clinical cardiovascular disease,[161,162] cardiovascular disease mortality,[163–166] angiographically assessed progression of coronary[167] or carotid[168] atherosclerosis, or early carotid atherosclerosis as assessed by ultrasound,[169] in association with increased intake of vitamin E from food or supplements. Most of the studies found protection only with the level of vitamin E that could be obtained from supplements.[161,162,164,167,168] Among those studies, there is general agreement that the minimum level of supplementation

associated with benefit is about 100 IU.[161,162,167,168] However, two studies reported that dietary vitamin E greater than or equal to 9.64 IU[165] or 7.1 IU[163] for women and 8.9 IU for men[163] were associated with reduced coronary mortality compared with lower levels of vitamin E intake. Two studies reported that the association of increased intake of vitamin E from supplements with reduced risk of coronary heart disease was strongest for those who had taken the supplements for more than 2 years,[161,162] and another study reported similar results.[166] This is consistent with a causative role for vitamin E in the inhibition of atherosclerosis. Of note is one cross-cultural study showing that mortality due to ischemic heart disease was predicted better by plasma vitamin E standardized by lipids than by more widely recognized risk factors for cardiovascular disease, such as plasma cholesterol.[170]

Other studies reported reduced risk of fatal myocardial infarction,[171] total cardiovascular mortality,[163,171] and other cardiovascular endpoints[161] with increased dietary intake of carotenoids from fruits and vegetables. However, for one of these studies this was true only for women.[163] One of these studies[161] reported that the benefit of increased carotene consumption was limited to those who smoked. In contrast, another study reported reduced risk of coronary heart disease and all cause mortality with higher values for a combined index of dietary intake of vitamin C and β-carotene,[172] but found the protection associated with higher intake of these nutrients to be reduced for those who smoked. Also, a study that investigated the relationship of serum carotenoids with coronary events, including nonfatal myocardial infarction and cardiovascular deaths, reported that the protection associated with increased serum carotenoids was strongest for men who had never smoked and was not evident for current smokers.[173] In addition, several studies have failed to find an association between dietary carotenoids and early atherosclerosis[169] or death from coronary heart disease.[165]

Several studies reported that high levels of vitamin C did not influence progression of atherosclerosis[167,168] or risk of coronary heart disease[161] or mortality due to coronary heart disease.[165] In comparison, a study in another older (>65 years) population with lower overall intake of vitamin C found relatively low levels of vitamin C intake to be associated with increased mortality from stroke but not be to associated with death due to coronary heart disease.[174] Another study reported that vitamin C deficiency as assessed by serum vitamin C levels was associated with increased risk of acute myocardial infarction.[175] Another group, which studied a population in which vitamin C intake was intermediate between those studies showing no benefit of high levels of vitamin C,[161,165,167,168] and those suggesting increased risk due to low levels of vitamin C,[174,175] reported that early atherosclerosis assessed by ultrasound was inversely related to dietary intake of vitamin C only for men and women older than age 55.[169] One study found supplemental vitamin C and supplemental vitamin E combined to be associated with relatively greater reduction in risk of coronary disease mortality than supplemental vitamin E alone, even though no benefit was associated with supplemental vitamin C alone.[166] This would be consistent with the idea that vitamin C enhances the protective effect of vitamin E, as suggested by in vitro data (see below).

Overall, data from population-based studies suggest that levels of vitamin E that can only be obtained from supplements may reduce risk of cardiovascular disease. Data for β-carotene is inconsistent, particularly with regard to differences in efficacy between smokers and nonsmokers. It appears that while marginal vitamin C status may increase risk of some cardiovascular endpoints, particularly in older people, there is little evidence that high levels of vitamin C will be protective. The relatively little data for the potential benefit of selenium is inconsistent.[170,176–178]

Nature of evidence necessary to prove that antioxidants influence atherosclerosis-related diseases

Both case-control and cohort studies of antioxidant nutrients suggest that such nutrients are associated with protection from atherosclerosis or from thrombotic events that precipitate clinical cardiovascular disease. However, both of these types of studies are subject to potential confounding effects.

First, neither type of study is randomized, and thus could be subject to bias on that basis. Second, many of the studies that assessed intake of selected antioxidant nutrients obtained only qualitative data for dietary intake[154,171] or intake from supplements.[166,169] Other data suggest that plasma or serum levels of nutrients measured in other studies[155,173,175] may poorly reflect dietary intake.[179] Third, several studies have investigated the association of risk of cardiovascular mortality with both dietary intake of fruits and vegetables and calculated dietary intake of selected individual nutrients.[163,171] These studies found protection associated with increased consumption of fruits and vegetables to be equal or greater than that associated with calculated intake of the individual nutrients.[163,171] Thus, it is possible that the individual nutrients selected for study might only be present in the same food as the active nutrients. Alternatively, it may be that antioxidant nutrients most effectively inhibit atherosclerosis when present in combination (see below). Fourth, the association of reduced risk of cardiovascular disease with increased intake of nutrient antioxidants via the diet or supplements could be associated with other health-promoting behaviors. For example, a study of older individuals showed that, compared with those who did not take a vitamin supplement, those who did were more physically active and were more likely to practice other healthful behaviors such as avoiding too much fat, cholesterol, salt, sugar, and caffeine and to emphasize consumption of fiber-rich foods.[180] Furthermore, case-control studies are limited by the possibility that presence of disease alters dietary intake or metabolic processes that determine plasma levels of nutrients in question.

Controlled, blinded, randomized clinical trials are needed to establish the efficacy and safety of nutrient antioxidants for prevention or inhibition of atherosclerosis and atherosclerosis-related diseases. Studies in experimental animals provide valuable insight because compliance to treatment can be assured, the diet and physical environment can be rigorously controlled, and extensive invasive inves-

tigation of atherosclerosis endpoints is possible. Further information regarding mechanisms of action of antioxidant nutrients will facilitate design and interpretation of clinical trials by providing insight into dosages that maximize benefits while minimizing adverse effects as well as predicting the nature of potential harmful effects. Understanding the mechanisms of actions of antioxidant nutrients will also facilitate identification of the physiologic, metabolic, dietary, and behavioral characteristics of individuals that might influence optimal levels and potentially could provide useful biomarkers for assessing the adequacy of treatment.

Potential biomarkers of oxidative stress/antioxidant efficacy

A number of measures have been used as biomarkers of oxidative stress, including plasma or tissue levels of malondialdehyde (MDA),[181–183] lipid peroxides,[184–186] isoprostanes,[187,188] oxidized forms of cholesterol such as 7-ketocholesterol,[96,181] and 7β-hydroxycholesterol,[189] redox status of ubiquinol-10,[190–192] and autoantibody titer to a model of oxidized LDL (LDL modified by MDA, MDA-LDL).[193–197] Relative susceptibility of LDL to in vitro lipid peroxidation[95,106,107,120,198–205] has been used as a surrogate that may reflect relative susceptibility of LDL to oxidation in vivo. Potentially, all of these measures may have utility for assessing efficacy of antioxidant treatment as they may decrease if oxidation in vivo were, in fact, reduced.

In addition, a biomarker should (a) increase with severity of disease, (b) be decreased by treatments that reduce atherosclerosis by antioxidant mechanism(s), and (c) accurately indicate the level of intervention needed for inhibition of atherosclerosis. Ideally, the decrease in the biomarker should occur rapidly and be directly related to the therapeutic efficacy of the antioxidant intervention. Only some of the criteria cited here have been investigated for the potential biomarkers described above. Several of the putative biomarkers have been evaluated for association with severity of atherosclerosis or cardiovascular disease.

MDA in the LDL fraction, assessed as thiobarbituric acid-reactive substances (TBAR), was positively correlated with progression of carotid atherosclerosis in one study.[189] In comparison, another study found that plasma TBAR bore no relationship to carotid artery intimal thickness.[178] Plasma TBAR has been variously reported to be elevated in men with carotid atherosclerosis[178] but not altered in men with coronary artery disease.[206] This inconsistency with respect to the association of plasma TBAR or MDA with atherosclerosis may relate to the recently reported day-to-day variation in plasma MDA within subjects.[207] One study found plasma concentrations of fluorescent products of lipid peroxidation not to be related to severity of coronary atherosclerosis.[160] Another study showed that redox status of ubiquinol-10 was reduced in patients with coronary artery disease compared with controls, although no attempt was made to relate redox status of ubiquinol-10 to severity of coronary artery disease.[208] Plasma levels of isoprostanes have not been related to severity of atherosclerosis. One study reported serum levels of 7β-hydroxycholesterol to be positively associated with progression of coronary atherosclerosis.[189] In contrast, another study found serum concentrations of 7-ketocholesterol to be lower for individuals with peripheral vascular disease compared with control subjects.[181]

In one study the autoantibody titer to MDA-LDL was associated with progression of carotid atherosclerosis.[193] Another study found high autoantibody titer to MDA-LDL to predict risk of myocardial infarction in hyperlipidemic individuals during the subsequent 5 years.[197] In contrast, another study showed that the autoantibody titer to another model of oxidized LDL (LDL oxidized by copper) did not predict the development of cardiovascular death, myocardial infarction, stroke, any cardiovascular event, or increase in intimal thickness during 10 years of follow-up in non-insulin-dependent diabetes mellitus.[209] Also, some,[194,195,210] but not other,[196,206] studies reported higher autoantibody titers to MDA-LDL for individuals with cardiovascular disease or peripheral vascular disease compared with control subjects. The reasons for these differences in results are not clear. However, as described below, it seems possible that the difference may relate to the interactive contributions of lipoprotein concentrations and autoantibody titer to MDA-LDL to atherosclerosis.

We have preliminary data that in rabbits fed a high-fat diet, the autoantibody titer to MDA-LDL is a significant predictor of atherosclerosis. We used a stepwise multivariate analysis including values for plasma concentrations of lipids, lipoproteins, antioxidants, and autoantibody titer to MDA-LDL to identify the factors predicting atherosclerosis. That analysis indicated that concentrations of intermediate density lipoprotein (IDL) and the autoantibody titer to MDA-LDL together accounted for 75.5% of the variability in atherosclerosis in the abdominal aorta. The contribution of the autoantibody titer to MDA-LDL was significant, accounting for a third of the variation in atherosclerosis that could be explained by IDL and the autoantibody titer to MDA-LDL combined. Of importance is the finding that the association of the autoantibody titer to MDA-LDL with atherosclerosis was not apparent until after accounting for the potent influence of IDL on atherosclerosis (Schwenke D.C. and Behr S.R., unpublished data). Susceptibility of LDL[203] or LDL combined with less dense lipoprotein fractions[189] to in vitro lipid peroxidation has been reported to correlate positively with progression of atherosclerosis in coronary[203] and carotid[189] arteries. However, other studies did not find susceptibility of LDL to in vitro lipid peroxidation to differ between control subjects and those with coronary[186] or carotid atherosclerosis.[194]

A smaller number of studies with atherosclerosis endpoints have investigated whether antioxidant treatment reduced biomarkers of oxidative stress. Treatment with vitamin E and selenium[211] or selenium alone[211] reduced both atherosclerosis and plasma levels of MDA assessed as TBAR. However, only some studies that showed reduction in plasma MDA by vitamin E[182,183,211] also showed vitamin E treatment to significantly reduce atherosclerosis.[182,183] A recent study of a low level of vitamin E (24 IU/rabbit) reported that vitamin E neither influenced atherosclerosis nor had any effect on lipid peroxides in LDL.[202] The ability of antioxidants to reduce plasma levels of isoprostanes and

Table 1 Inhibition of aortic atherosclerosis in rabbits by treatment with vitamin E alone

Reference	Daily Dose of Vitamin E, IU[2]	Percent change in atherosclerosis in vitamin E-treated group[1]			
		Lesion area		Aortic cholesterol	
		Uncorrected[3]	Corrected[4]	Uncorrected[3]	Corrected[4]
(201)	13.2	+15	+10	NR[5]	—
(202)	24	−13	+0	NR	—
(199)	25	−17	−16	NR	—
(198)	25	−7	−14	NR	—
(211)	38	−25	−1	NR	—
(200)	112–168	−16	−10	NR	—
(218)	146	−35	−21	−33	−18
(182)	129–207	−74[6]	−79	NR	—
(214)	660	−32[6]	+21	−33	+20

[1]Values for the influence of vitamin E supplementation on atherosclerosis are for the average of effect on multiple aortic regions if multiple areas of aorta were evaluated.
[2]Daily doses of vitamin E supplement were computed from the concentrations of the vitamin E supplement in the diet and food consumption. In cases where the form of vitamin E supplement was not indicated, the maximum and minimum doses were calculated assuming that the vitamin E supplement was supplied as *d*-α-tocopherol and *dl*-α-tocopherol acetate, respectively.
[3]Percent differences in atherosclerosis in rabbits supplemented with vitamin E compared with that in unsupplemented control rabbits without accounting for plasma cholesterol concentrations as reported in the references given at the left.
[4]Percent differences in atherosclerosis in rabbits supplemented with vitamin E compared with that in unsupplemented control rabbits after correction for differences (trends or significant differences) in plasma cholesterol concentrations.
[5]NR, not reported.
[6]Significant effect of vitamin E supplementation.

redox status of ubiquinonol-10 has not been investigated in studies of antioxidants with atherosclerosis endpoints.

One study with rabbits showed that treatment with BHT was associated with both reduced extent of atherosclerosis and reduced plasma levels of 7-ketocholesterol.[96] Other studies reported that treatment of rabbits with vitamins E and C[212] or probucol[94] was associated with reduced plasma levels of a number of oxidized forms of cholesterol, including both 7β-hydroxycholesterol and 7-ketocholesterol, and reduced extent of atherosclerosis.[94,212] No published studies have investigated the influence of antioxidants on autoantibody titers to MDA-LDL. However, we have preliminary data that in rabbits fed a high-fat diet, autoantibody titer to MDA-LDL is inversely related to plasma α-tocopherol and plasma α-tocopherol standardized by plasma cholesterol ($r = -0.30$, $P < 0.02$; $r = -0.33$, $P < 0.01$, respectively), (Schwenke D.C., and Behr S.R., unpublished data) and as described above, the autoantibody titer to MDA-LDL was positively associated with atherosclerosis.

A number of studies have reported on the effect of antioxidants on susceptibility of LDL to in vitro lipid peroxidation and atherosclerosis. Several studies showed that probucol[92,107,213] and other nondietary antioxidants[97,205,213] reduced both atherosclerosis and the susceptibility of LDL to in vitro lipid peroxidation. One of these studies showed that for untreated nonhuman primates and those treated with probucol combined, susceptibility of LDL to in vitro lipid peroxidation was positively associated with atherosclerosis.[107] However, another study showed that the dose response for the reduction in susceptibility of LDL to in vitro lipid peroxidation by a probucol analog differed from the dose response for inhibition of atherosclerosis by the probucol analog.[213]

A third study showed there was no relationship between the extent of atherosclerosis and susceptibility of LDL to in vitro lipid peroxidation for untreated rabbits and those given dietary antioxidants.[202] Furthermore, five studies with vitamin E,[198–202] a study with vitamins E and C,[106] a study with an antioxidant structurally related to probucol,[95] and two studies with probucol, one in human beings[120] and another in rabbits,[199] reported that these interventions reduced the susceptibility of LDL to in vitro lipid peroxidation without reducing atherosclerosis. Such data support the suggestion that inhibition of oxidation of LDL must reach some threshold before atherosclerosis is inhibited.[95] If this is indeed the case, the use of susceptibility of LDL to in vitro lipid peroxidation as a biomarker of inhibition of atherosclerosis by antioxidants is problematic. Another difficulty with the assay of susceptibility of LDL to in vitro lipid peroxidation as a biomarker for evaluation of interventions to inhibit atherosclerosis relates to the influence of dietary fat as described above.

Thus, in contrast to a recent suggestion,[204] it seems that susceptibility of LDL to in vitro lipid peroxidation is not likely to be a good index of whether an antioxidant intervention is likely to inhibit atherosclerosis. Further work will be needed to identify biomarkers that will reliably indicate the efficacy of antioxidant treatments to inhibit atherosclerosis and cardiovascular disease.

Studies of antioxidant intervention on atherosclerosis in experimental animals

Inhibition of atherosclerosis by supplementation with individual nutrient antioxidants

A number of studies have investigated in a quantitative manner the inhibition of atherosclerosis by vitamin E in hypercholesterolemic animals.[182,183,198–202,211,214–218] Most of these studies have been done in rabbits.[182,198–202,211,214,218]

Table 2 Inhibition of aortic atherosclerosis in rabbits by treatment with vitamin E compared with vitamin E in combination with other antioxidant nutrients

Reference	Daily Dose[1]	Atherosclerosis in vitamin E-treated group as a percentage of the control[2]			
		Lesion area		Aortic cholesterol	
		Uncorrected[3]	Corrected[4]	Uncorrected[3]	Corrected[4]
(222)	89 IU vitamin E + 31, 400 IU vitamin A	−63[5]	−57	−40	−30
(93)	35–56 IU vitamin E + 35 mg vitamin C	−46[5]	−58	−44	−57
(106)	1100 IU vitamin E + 60 mg vitamin C	−34	−40	NR[6]	—
(211)	38 IU vitamin E	−25	−1	NR	—
(211)	38 IU vitamin E + 17.5 μg Se	−63[5]	−40	NR	—
(218)	146 IU vitamin E	−35	−21	−33	−18
(218)	146 IU vitamin E + 22 μg Se	−68[5]	−54	−63[5]	−50[5]

[1]Daily doses of vitamin E supplement were computed from the concentrations of the vitamin E supplement in the diet and food consumption. In cases where the form of vitamin E supplement was not indicated, the maximum and minimum doses were calculated assuming that the vitamin E supplement was supplied as *d*-α-tocopherol and *dl*-α-tocopherol acetate, respectively.
[2]Values for the influence of vitamin E supplementation on atherosclerosis are for the average of effect on multiple aortic regions if multiple areas of aorta were evaluated.
[3]Percent differences in atherosclerosis in rabbits supplemented with vitamin E compared with that in unsupplemented control rabbits without accounting for plasma cholesterol concentrations as reported in the references given at the left.
[4]Percent differences in atherosclerosis in rabbits supplemented with vitamin E compared with that in unsupplemented control rabbits after correction for differences (trends or significant differences) in plasma cholesterol concentrations.
[5]Significant effect of vitamin supplementation.
[6]NR, not reported.

Five studies, which used levels of supplementation between 13–38 IU/rabbit/day, observed no effect on atherosclerosis (Table 1) and no effect on plasma cholesterol concentrations.[198,199,201,202,211] Two[182,214] of four published studies[182,200,214,218] that used daily doses of vitamin E between 112–660 IU/rabbit reported significant inhibition of atherosclerosis, whereas two other studies[200,218] showed a tendency toward reduction of atherosclerosis by vitamin E. For one of the studies reporting inhibition of atherosclerosis by vitamin E,[214] the benefit of vitamin E was consistent with the reduction in plasma cholesterol that occurred in vitamin E-treated rabbits in that experiment[214] and was eliminated when differences in atherosclerosis between groups were corrected for difference in plasma cholesterol concentrations (Table 1). In contrast, inhibition of atherosclerosis by vitamin E in the other study[182] was 74%, despite a slight but not significant increase in plasma cholesterol concentration in the vitamin E-treated group. Overall, these studies suggest that supplementation with vitamin E alone modestly decreases atherosclerosis induced by hypercholesterolemia in rabbits and that the efficacy may be greatest at an intermediate dose.

Arterial injury occurs in conjunction with percutaneous transluminal angioplasty (PCTA), a treatment used in some cases of occlusive atherosclerosis, and thus the influence of vitamin E on the arterial response to such injury is of interest. One semiquantitative study of a relatively small number of animals reported that a higher dose of vitamin E (1394 IU/rabbit/day) had adverse effects on intimal thickening after balloon injury.[219] A more recent report described a more carefully conducted study that included about four times as many rabbits per treatment group and in which arteries were fixed by perfusion.[72] In that study, α-tocopherol supplementation was observed to reduce intimal proliferation and thickening after balloon injury of previously injured artery.[72] In addition to the more careful characterization of the arterial response to α-tocopherol intervention, the later study of reinjury of a previous injury[72] is a better model of the restenosis following PCTA that occurs in human individuals than is the earlier study.[219] The single study in nonhuman primates[215] provided inconsistent results; one measure of atherosclerosis was significantly inhibited by vitamin E treatment, whereas other measures of atherosclerosis were not affected by treatment.

Previous work in rabbits showed that dietary treatment with vitamin C reduced both intimal thickness[220,221] and aortic cholesterol concentrations.[221] Effects of vitamin C on surface areas of atherosclerotic lesions are inconsistent.[220,221]

One study investigated the influence of selenium on atherosclerosis in rabbits and reported that atherosclerosis was reduced 49% by selenium.[211] Another study considered the effect of supplementation with low levels of either *cis* or *trans* isomers of β-carotene on atherosclerosis in rabbits.[201] That study reported inhibition of atherosclerosis by the *trans* isomer, with no effect of the *cis* isomer. Another study,[202] which supplemented rabbits with only one-fifth as much *trans* β-carotene (1.6 mg/day/rabbit), failed to find any effect of *trans* β-carotene on atherosclerosis.

Inhibition of atherosclerosis in animals by combinations of nutrient antioxidants

Only a few studies have investigated in a quantitative manner inhibition of atherosclerosis by combinations of nutrient antioxidants (Table 2). One study reported vitamins E and C combined to inhibit atherosclerosis in rabbits.[93] In comparison, another study in rabbits, which used about a 20-fold higher dose of vitamin E and about twice the amount of vitamin C, observed a nonsignificant trend toward inhibition of atherosclerosis by vitamins E and C combined.[106] A recent third study provided only qualitative data for inhibition of atherosclerosis by vitamin E and C

Table 3 Comparison of the two completed randomized trials of vitamin E supplementation on cardiovascular endpoints

Trial	Daily Dose, IU	Percent change in risk associated with treatment: Nonfatal MI	CVD or CHD death	Total mortality
ATBC[1,2]	50	−38[1]*	+33[1]	+2[2]
CHAOS[3]	400–800	−77*	+18	+25

MI, myocardial infarction; CVD, cardiovascular disease; CHD, coronary heart disease.
[1](Ref. 225)
[2](Ref. 224)
[3](Ref. 226)
*Significant effect. All other differences not significant. In comparison, a meta-analysis of 34 trials of cholesterol reduction in individuals with preexisting coronary heart diseases indicated a significant reduction in cardiovascular mortality and a significant (13%) reduction in total mortality.[227]

combined.[212] Another study demonstrated inhibition of atherosclerosis in rabbits by combined treatment with vitamins E and A.[222] None of these studies compared the combined treatment with that by each supplement separately.

One study reported that atherosclerosis was reduced 49% by selenium, 63% by vitamin E combined with selenium, and to be slightly (25%) but not significantly reduced by vitamin E.[211] In a recent study we found 68% and 35% reduction in the same measure of atherosclerosis by vitamin E and selenium combined and by vitamin E alone, respectively.[218] In that study we also showed that selenium and vitamin E were effective in reducing another measure of atherosclerosis, aortic cholesterol concentrations. Of importance was the finding that additional inhibitory action of selenium on aortic cholesterol accumulation was in part independent of effects on plasma cholesterol concentrations.[218] The enhanced inhibition of atherosclerosis by combinations of antioxidants (Table 2) emphasizes the importance of considering combinations of antioxidants that might interact to inhibit atherosclerosis better (see below).

Clinical trials of antioxidant interventions in human individuals

Two large, randomized, double-blind placebo-controlled clinical trials have investigated the influence of vitamin E on cardiovascular disease.[223–226] The Cambridge Heart Antioxidant Study (CHAOS) reported that for individuals with angiographically proven coronary atherosclerosis, α-tocopherol treatment reduced risk of nonfatal myocardial infarction by 77%[226] (Table 3). In comparison, a recent report from the Alpha Tocopherol, Beta Carotene Cancer Prevention Study (ATBC Study) found that in individuals with previous myocardial infarction, α-tocopherol decreased risk of nonfatal myocardial infarction by 38%.[225] The dose of α-tocopherol used in the ATBC Study was equivalent to 50 IU,[225] a level that most of the cohort studies described above would have suggested would have little or no benefit, and only 1/8 to 1/16 of the dose used in CHAOS.[226] Consistent with that difference, serum concentrations of α-tocopherol for the α-tocopherol group were higher in CHAOS than in the ATBC Study.[225,226] Thus, it is not surprising that the reduction of nonfatal myocardial infarction was greater in CHAOS than in the ATBC Study.

The ATBC Study also reported that α-tocopherol treatment slightly, but not significantly, increased risk of fatal coronary heart disease.[225] Similarly, CHAOS reported that α-tocopherol treatment slightly, but not significantly, increased risk of cardiovascular death, an endpoint that included not only fatal coronary heart disease but also death from other cardiovascular diseases.[226] While these differences were not significant, if anything, the data would be consistent with less increase in risk of fatal coronary heart disease or cardiovascular disease with higher levels of α-tocopherol supplements. This is because the magnitudes of the nonsignificant increases in risk were 33% in the ATBC Study[225] compared with 18% in CHAOS,[226] which used a higher α-tocopherol supplement (Table 3). In CHAOS, most of the deaths due to cardiovascular disease occurred in the first 200 days of treatment.[226] In comparison, in the ATBC Study,[225] deaths due to coronary heart disease occurred uniformly during the treatment period. Individuals in the ATBC Study were treated on average for 5.3 years,[225] compared with less than 1.5 years for CHAOS.[226]

In addition to the differences in amounts of α-tocopherol supplements, there are a number of other differences between these studies that may have influenced the results. All of the participants in the ATBC Study were smokers,[225] whereas only a small fraction of the participants in CHAOS were smokers.[226] After angiography establishing the presence of coronary atherosclerosis, participants in CHAOS were randomized to treatment group based on several known risk factors for cardiovascular disease and on planned therapy, which was left to each participant's personal physician.[226] More than half of the participants in CHAOS (approximately equal numbers in each of the α-tocopherol and placebo groups) were consuming aspirin.[226] None of the participants in the ATBC Study were taking anticoagulants, and no other information on medications is provided.[225] In contrast, more than two-thirds of the participants in CHAOS were being treated with calcium antagonists, more than half with nitrate, and a third with β-blockers (slightly higher for the α-tocopherol-supplemented group).[226] In addition, the intended therapy for about 65% of the participants in CHAOS was either coronary artery bypass grafting or percutaneous transluminal coronary angioplasty.[226]

CHAOS was not designed to consider whether the α-tocopherol intervention had different effects on cardiovascular outcomes in participants receiving different forms of medical or surgical management of coronary atherosclerosis. Further investigations will be needed to determine whether there may be some medical or surgical treatments for coronary atherosclerosis that are incompatible with supplementation with antioxidants such as α-tocopherol, and whether this might explain the increase in cardiovascular mortality observed during the first 200 days of treatment with α-tocopherol in CHAOS.[226] Other differences between the ATBC Study and CHAOS are the use of different forms of α-tocopherol, synthetic *dl*-α-tocopherol acetate in the

ATBC Study,[225] and free 2R, 4′R, 8′R-α-tocopherol from natural sources in CHAOS.[226]

Finally, it is likely[228] that vitamin C status of smokers in the ATBC Study may have been reduced compared with that in the participants of CHAOS, and, as discussed below, this may have reduced the effectiveness of the α-tocopherol supplement. Overall, given the many differences between CHAOS and the ATBC Study, results of these studies appear remarkably consistent with regard to the effects of α-tocopherol on nonfatal myocardial infarction and fatal coronary heart disease/fatal cardiovascular disease.

The ATBC Study also commented on other cardiovascular endpoints, reporting a 10% reduction in risk of development of angina pectoris in asymptomatic men treated with α-tocopherol.[223] The same study suggested that α-tocopherol may have slightly increased risk of death from hemorrhagic stroke while slightly reducing risk of death from ischemic heart disease and ischemic stroke, with no effect on total mortality.[224] Several factors might explain the differing results for different cardiovascular endpoints assessed in the ATBC Study.

First, the populations considered for the different cardiovascular disease outcomes differed. For the angina pectoris outcome, those ATBC participants initially free of coronary heart disease were considered.[223] The report on risk of death from hemorrhagic stroke, ischemic heart disease, and ischemic stroke considered the entire ATBC Study group,[224] of whom 76% were considered to be free of coronary heart disease.[223] In comparison, the effect of α-tocopherol on nonfatal myocardial infarction and on fatal coronary heart disease discussed above was evaluated in the 6.3% of the ATBC Study group that had previous myocardial infarction.[225] These results suggest that within a population of smokers, α-tocopherol might have differential effects on different cardiovascular endpoints.

Taken together, the results of these studies provide some support for the potential for vitamin E to inhibit atherosclerosis and cardiovascular disease in individuals with preexisting cardiovascular disease. However, many questions remain to be addressed, namely: What is the appropriate dose of vitamin E to achieve maximal inhibition of atherosclerosis/cardiovascular disease? Is there a difference in efficacy of natural compared with synthetic forms of vitamin E? What accounted for the nonsignificant increase in coronary heart disease/cardiovascular disease mortality that was observed in both the ATBC Study and in CHAOS? What will be the effect of a longer interval of supplementation? Does vitamin E supplementation differentially affect prevention of clinically relevant cardiovascular disease in individuals with and without preexisting coronary atherosclerosis? How does vitamin E interact with other medical and surgical treatments used in individuals with cardiovascular disease? Does the effect of vitamin E differ among coronary, carotid, and other peripheral arteries? Are there risks associated with supplementation with high levels of α-tocopherol for certain population groups such as smokers? Does vitamin E supplementation reduce risk of atherosclerosis/cardiovascular disease similarly in men and women? Accordingly, additional clinical trials will be needed.

Recently, a small trial with a combination of 30,000 IU of β-carotene, 500 mg of vitamin C, and 700 IU of vitamin E showed that that intervention had no effect on restenosis 6 months after angioplasty.[229] However, that study and another small trial[230] found probucol to be effective in limiting restenosis after angioplasty. In addition, another study provided preliminary results for a decrease in ischemic events for individuals treated with vitamin E and aspirin compared with aspirin alone.[231]

Four large, randomized, double-blind placebo-controlled clinical trials have investigated the influence of treatment with β-carotene alone[223–225,232,233] or β-carotene in combination with vitamin E[223–225] or vitamin A[234] on several cardiovascular disease endpoints. Two studies found supplementary β-carotene to have no influence on death from cardiovascular disease or death from any cause.[232,233] One study reported β-carotene supplementation to increase risk of fatal coronary heart disease[225] and all-cause mortality.[224] Another study reported β-carotene and vitamin A supplementation to increase all-cause mortality and to slightly increase risk of cardiovascular death.[234]

Supplements of β-carotene were similar in the studies that found no effect of β-carotene on cardiovascular deaths[232,233] (50 mg on alternate days and 50 mg per day, respectively) and in the studies that observed increases in cardiovascular deaths[225,234] (20 mg/day and 30 mg/day, respectively). Studies that showed no influence of β-carotene on cardiovascular disease included relatively low percentages of smokers.[232,233] In contrast, the two studies that observed adverse cardiovascular effects of β-carotene were conducted in populations that were either smokers[225] or comprised smokers and others at risk for lung cancer.[234] The interval of follow-up was relatively longer in the studies that found no effect of β-carotene on cardiovascular deaths[232,233] (12 and 8.2 years, respectively) compared with the studies that observed increases in cardiovascular deaths[225,234] (5.3 and 4 years, respectively).

Results of these studies suggest that β-carotene supplements are unlikely to reduce atherosclerosis/cardiovascular disease and may cause harm to certain groups such as smokers and former smokers. Interestingly, one of the studies that found supplemental β-carotene to have no influence on cardiovascular deaths and deaths from all causes also observed that basal plasma β-carotene concentrations were inversely related to death from cardiovascular diseases and death from all causes.[233] However, β-carotene supplementation did not reduce mortality in individuals with low initial plasma concentrations of β-carotene.[233] Such data would be consistent with the idea that plasma levels of β-carotene are a biomarker of other factors associated with reduced risk of death due to cardiovascular diseases and all causes. Alternatively, it may be that other complementary antioxidants, or appropriate balance of antioxidants, are needed for β-carotene to provide protection from cardiovascular disease. Results of the ATBC Study suggest that α-tocopherol at the level of 50 IU per day was not able to negate the adverse effects on cardiovascular mortality conferred by supplementary β-carotene[225] but could block the increased risk of development of angina pectoris conferred by supplementary β-carotene.[223]

The Linxian Nutrition Intervention Trial investigated a combined antioxidant cocktail in a population diagnosed

with esophageal dysplasia and likely to be of marginal nutritional status.[235] The antioxidant supplement included 60 IU of *dl*-α-tocopherol acetate, 15 mg β-carotene, and 180 mg vitamin C in a multivitamin including 20–700% of the U.S. recommended dietary allowance of other vitamins and minerals, including those known to be cofactors of antioxidant enzymes.[236] In this population, the combined supplement slightly but nonsignificantly reduced overall mortality, with a greater effect on deaths due to cerebrovascular disease.[235] In men, the supplement reduced risk of cerebrovascular disease 58% while in women the reduction in risk of cerebrovascular disease was not significant.[235] This difference may relate to the greater decrease in blood pressure in men than in women given the antioxidant supplement.[235]

Further work will be needed to determine whether a similar antioxidant cocktail would reduce risk of coronary heart disease and other cardiovascular diseases and to elucidate gender differences. However, it is worth emphasizing that the reduction in stroke mortality for men in the Linxian Trial[235] was observed with a balanced antioxidant supplement that included 15 mg β-carotene and 60 IU vitamin E. These are values only 25% lower and 20% higher, respectively, than the β-carotene and α-tocopherol supplements used in the ATBC Study,[224] which found β-carotene and α-tocopherol to increase mortality due to stroke.[224,235] One explanation for this disparity is the differences in the antioxidant supplement. The relatively greater benefit of the balanced antioxidant cocktail used in the Linxian Trial[235] than the α-tocopherol and/or β-carotene supplement used in the ATBC Study[224] is consistent with other data (below) for the interaction of antioxidants. Alternatively or additionally, it may be that differences in the populations studied contributed to the differences that were observed: participants in the Linxian Trial had been diagnosed with esophageal dysplasia, and although they were likely to be of marginal nutritional status, relatively few were smokers.[235] In comparison, participants in the ATBC Study were all smokers,[224] who as discussed above may have marginal vitamin C status.[228]

Ongoing placebo-controlled double-blind clinical trials of antioxidants

Several randomized placebo-controlled double-blind trials are in progress. Ongoing trials in women are the Women's Antioxidant and Cardiovascular Study (WACS) and the Women's Health Study.[237] WACS is a secondary prevention trial of vitamins E and C and β-carotene in a 2 × 2 × 2 factorial design in women with preexisting cardiovascular disease. This trial will be complementary to the Women's Health Study, a primary prevention study that will assess the effects of vitamin E, β-carotene, and aspirin.[237] Amounts of supplements used in WACS will be β-carotene, 50 mg; natural vitamin E, 600 IU; and vitamin C, 500 mg. Antioxidant supplements will be consumed every second day; participants will be followed for 4 years.[237]

The Heart Outcomes Prevention Evaluation Study (HOPE) will be conducted in both men and women. This is a randomized trial of angiotensin-converting inhibitor (ramipril) and vitamin E for prevention of myocardial infarction, stroke, or cardiovascular death.[238] The study is designed as a 2 × 2 factorial with 400 IU natural vitamin E or 10 mg ramipril or the respective placebos. Participants will be followed for 4 years.[238] Features that distinguish HOPE from most other clinical trials of antioxidant supplementation are the study of a population at high risk of cardiovascular disease, due to previous cardiovascular disease or risk factors for cardiovascular disease such as diabetes, and the inclusion of women.[238]

In addition, a number of substudies will provide mechanistic information to complement the main study. One of these will be the Study to Evaluate Carotid Ultrasound Changes in Patients Treated with Ramipril and Vitamin E (SECURE), which will evaluate progression of atherosclerosis by ultrasound.[239] This study will investigate in a 3 × 2 factorial design a 400-IU supplement of natural vitamin E and two doses of ramipril, a low dose that has little influence on blood pressure and a higher dose (the same as that used in HOPE).[239] This study will indicate how effects of the treatments on clinical events are related to progression of atherosclerosis. Enrollment in this substudy is complete; the 4-year period of scheduled follow-up will conclude by the end of 1998.[239] Another substudy of HOPE, MICRO-HOPE, will investigate the influence of the vitamin E/ramipril intervention on progression of renal and cardiovascular disease in individuals with diabetes.[240]

The Heart Protection Study will evaluate the influence of vitamins E and C, β-carotene, and the cholesterol-lowering agent simvastatin in cardiovascular disease. This is a trial of secondary prevention of preexisting cardiovascular disease and prevention of cardiovascular disease in individuals with diabetes.[241] The *Supplementation en Vitamines et Mineraux Antioxydants Trial* will investigate the influence of β-carotene, α-tocopherol, vitamin C, selenium, and zinc in the prevention of cardiovascular disease in normal men and women.[241]

Patient-specific factors to be considered in evaluating individual requirements for dietary antioxidants

As described above, some population-based studies suggested that protection from atherosclerosis by vitamin E may be limited to hyperlipidemic individuals.[155] A potential mechanistic explanation for that result is the idea that hyperlipidemic individuals experience an increase in oxidative stress. In support of this, a recent study reported that despite normal plasma concentrations of vitamin E, erythrocyte vitamin E was reduced in asymptomatic hypercholesterolemic men compared with normocholesterolemic men.[242] This reduction in erythrocyte vitamin E was associated with increased susceptibility of erythrocytes to oxidative stress.[242] Thus, it may be that hypercholesterolemic individuals require higher plasma concentrations of vitamin E in order to maintain adequate cellular levels of vitamin E.

Also, another study showed that erythrocyte vitamin E was inversely associated with thickness of the carotid artery, an early measure of atherosclerosis.[178] Interestingly, the agent used to control hypercholesterolemia may influence

antioxidants needs. For example, lovastatin treatment has been variously associated with decreased[243] and increased[244] susceptibility of LDL to in vitro lipid peroxidation. Other studies indicate that susceptibility of LDL to in vitro lipid peroxidation is reduced by treatment with pravastatin,[245] simvastatin,[246] and fluvastatin[247] and strikingly reduced by probucol.[120] Furthermore, other data suggest that simvastatin and pravastatin inhibit macrophage growth induced by oxidized LDL.[248]

Several other medical conditions are both associated with increased risk of cardiovascular disease and increased measures of oxidative stress, including hypertension,[249,250] diabetes,[88,184,251] and renal failure requiring chronic hemodialysis.[185] Some[252] but not other[253] data suggest that hyperhomocysteinemia may also induce increased oxidative stress. Potentially, individuals afflicted with such conditions may have a greater need for antioxidants compared with individuals not affected.

Other studies have shown that estrogen regenerates α-tocopherol from the tocopheryl radical in vitro[254] and preserves tissue vitamin E in vivo without influencing plasma concentrations of vitamin E or vitamin C.[255] This suggests that estrogen status of women will influence needs for vitamin E. Dietary factors can influence antioxidant needs. For example, increased dietary unsaturated fat induces oxidative stress that increases vitamin E requirements.[256] A recent study reported that a single pretreatment with 800 IU vitamin E in combination with 1 g of vitamin C prevented the impairment of flow-mediated brachial artery vasodilation that was associated with a high-fat meal.[257] This suggests that not only fat saturation but also fat quantity may influence needs for antioxidants. Other studies with methionine loading in rabbits[258] suggest that dietary protein quality or quantity may also influence oxidative stress and, thus, a need for antioxidants. Behavioral characteristics such as smoking habit increase oxidative stress[160,188] and requirements for vitamin C[228] and possibly other antioxidants. Exposure to environmental smoke and, perhaps, exercise might also influence antioxidant requirements.

Consistent with the idea that hyperlipidemia, hypertension, and diabetes increase requirements for antioxidants, studies of antioxidant supplementation of individuals with these conditions have shown reduction in measures of oxidative stress. Several studies showed vitamin C supplementation to improve endothelium-dependent vascular reactivity of forearm resistance vessels[87,88] selectively in hypercholesterolemic[87] and diabetic[88] individuals without influence on the control subjects. Another study showed that supplementary vitamin C improved endothelium-dependent vascular reactivity of epicardial coronary arteries of hypertensive individuals.[250] Thus, it seems clear that antioxidant needs will differ for different individuals. Future studies will be needed to clarify further the increased antioxidant needs in different conditions thought to be associated with increased oxidative stress.

Interaction of dietary antioxidants

Vitamin E, a lipophilic antioxidant, is believed to be the major chain-breaking antioxidant in cellular membranes and lipoproteins.[259,260] In vitro, vitamin C regenerates or otherwise preserves vitamin E levels.[261–264] Glutathione decreases the amount of vitamin E required to inhibit peroxidation of microsomal lipids by preserving the microsomal content of vitamin E.[265,266] Either vitamin C or glutathione blocks the oxidation of platelet tocopherol.[263,264] Glutathione is thought to react with dehydroascorbic acid to regenerate ascorbate.[267,268] If such interactions also occur in vivo, then in circumstances when concentrations of vitamin E are limiting, vitamin C and/or glutathione may function to increase vitamin E levels and, thus, further reduce oxidative stress. Therefore, minimal vitamin E requirements to reduce oxidative stress to a given level may depend on vitamin C status and/or activity of glutathione reductase, the enzyme that maintains concentrations of reduced glutathione.[236]

If vitamin E levels are not sufficient to prevent lipid peroxidation, there are several potential backup mechanisms. Selenium-dependent phospholipid hydroperoxide glutathione peroxidase can reduce phospholipid hydroperoxides in liver mitochondria, and that activity accounts for most of the reduction of these hydroperoxides in liver mitochondria.[269] This same enzyme can reduce not only phospholipid hydroperoxides, but also cholesterol ester hydroperoxides in oxidized low density and high density lipoproteins to the corresponding alcohols.[270] Such activity would prevent decomposition of the hydroperoxides into harmful free radicals,[271] thus reducing oxidation. Selenium may also enhance antioxidant defense in its role as a cofactor of glutathione peroxidase.[272]

Other enzymes such as superoxide dismutase and catalase also play important roles in reducing levels of active oxygen with potential to initiate lipid peroxidation.[236] Cytosolic superoxide dismutase, dependent on copper and zinc, and mitochondrial superoxide dismutase, dependent on manganese, convert superoxide anion into hydrogen peroxide, while catalase, dependent on iron, converts hydrogen peroxide into water.[236] β-carotene may have a role by functioning to quench singlet oxygen and interact synergistically with vitamin E to inhibit lipid peroxidation.[236] Other phenolic constituents of foods, such as flavonoids, may also play a role in reducing oxidative stress. Thus, various nutrient antioxidants contribute to antioxidant activity in membranes, cytosol, and other cellular and fluid compartments of the body. Therefore, a combination of antioxidants with different sites and mechanisms of action may provide more effective inhibition of oxidation than a single antioxidant and thus be a more effective inhibitor of atherosclerosis.

Future directions

Studies in experimental animals and the few randomized placebo-controlled double-blind clinical trials of antioxidant supplementation that have been completed to date are sufficiently encouraging to justify further exploration of the potential of antioxidants to inhibit atherosclerosis. Much of the interest in the potential of antioxidants to inhibit or prevent atherosclerosis is based on the premise that oxidation of LDL contributes to atherosclerosis. This assumption is only partially consistent with available data as described above. The mechanism(s) by which oxidation occurs in vivo

remains to be established, and the interactive effects of fatty acid composition of lipoproteins and arterial cells on oxidation of LDL and cellular sequelae of such oxidation are yet to be elucidated. Much remains to be learned about the mechanism(s) by which oxidation of LDL influences atherosclerosis.

Careful consideration will need to be given to the nature of the antioxidant supplement(s). Multiple nutrients have antioxidant activity, but not all nutrients have been tested for antioxidant activity. The factor(s) associated with foods rich in β-carotene that might account for the apparent protection from cardiovascular disease observed in population-based studies remains to be determined. Also, among forms of vitamin E, α-tocopherol is thought to be the most effective antioxidant and to have the highest biological activity.[273] However, γ-tocopherol is more abundant than α-tocopherol in several food oils[273] and may account for some of the protection from cardiovascular disease risk associated with dietary vitamin E in some cohort studies.[163,165] Interestingly, one study reported that levels of γ-tocopherol but not α-tocopherol were reduced in individuals with coronary heart disease.[274] A potential mechanistic explanation for that observation is the finding that in vitro, compared with α-tocopherol, γ-tocopherol more effectively detoxifies nitrogen dioxide,[275] a potential precursor of nitric oxide that can react with superoxide to form peroxynitrite, a potent oxidizing agent.[101] The potential for γ-tocopherol to contribute significantly to any inhibition of atherosclerosis by vitamin E deserves further study. Additional work on the potential interaction of nutrient antioxidants, possibly including flavonoids,[276] to influence atherosclerosis and cardiovascular disease is needed.

Antioxidants have multiple effects, some of which are independent of effects on oxidation of LDL. However, the mechanism by which antioxidants might inhibit atherosclerosis needs to be fully clarified. Much remains to be learned from carefully controlled studies in isolated in vitro systems. Once the mechanisms by which LDL is oxidized in vivo are known, and processes by which oxidized LDL and antioxidants influence atherosclerosis are further clarified, it will be possible to design better antioxidant interventions to inhibit atherosclerosis. The interactive effect of nutrient antioxidants and dietary fat saturation on atherosclerosis and cardiovascular disease is unknown. However, data suggest that increased intake of unsaturated fat reduces both total and cardiovascular mortality.[127] Thus, it may be that a particularly effective intervention would include polyunsaturated fat together with an antioxidant cocktail.

Predictions based on studies in isolated systems will need to be tested first in animal models. Ultimately, those interventions found to be effective in animal models will have to be tested in clinical trials. Patient-specific characteristics such as the presence of hyperlipidemia, hypertension, and diabetes and gender may influence the response to antioxidant supplementation. Ideally, clinical trials would include adequate numbers of individuals with these characteristics so as to provide data on which to make recommendations for such individuals.

Finally, it should be noted that the only intervention that has been shown to reduce risk of cardiovascular disease morbidity and mortality in multiple randomized placebo-control clinical trials is dietary or pharmacologic treatment to lower blood cholesterol. Clinical trials of intensive cholesterol lowering have shown marked reduction in clinical events despite only modest reduction in atherosclerosis as determined by angiography.[227,277–280] The reduction in clinical events may reflect "stabilization" of atherosclerotic plaques so they are less likely to rupture.[278,279] Thus, cholesterol reduction should still be the first line of defense in any treatment program to reduce atherosclerosis-related diseases. Other data suggest that cholesterol reduction and antioxidant treatment combined may improve endothelial function in hypercholesterolemic individuals.[281] Therefore, for established atherosclerosis, antioxidant(s) combined with a cholesterol-lowering agent may reduce risk of clinical cardiovascular disease relatively more than intervention with antioxidant(s) alone. Such a combination is in fact being investigated in an ongoing clinical trial.[241]

Summary

It is clear that the agent (or agents) accounting for the apparent protection from cardiovascular disease associated with increased intake of fruits and vegetables has not been identified. Overall, results from correlative studies of vitamin E are relatively consistent with those of the few clinical trials. However, results for correlative studies of β-carotene are at odds with the few clinical trials. It is likely that those apparent inconsistencies will be explained once more is learned regarding the biological effects of β-carotene, other carotenoids, and other nutrients in foods rich in β-carotene. The inhibition of atherosclerosis and cardiovascular disease by isolated antioxidant(s) remains to be established by ongoing and future clinical trials. However, in the meantime, results of population-based studies continue to support the health benefits of increased consumption of a wide variety of antioxidant-rich fruits and vegetables. Those epidemiological data are supported by biochemical studies showing interactive effects of antioxidants.

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