Antioxidants in the Prevention of Human Atherosclerosis

Daniel Steinberg, MD, PhD, and Workshop Participants*

Summary of the Proceedings of a National Heart, Lung, and Blood Institute Workshop: September 5–6, 1991, Bethesda, Maryland

*See appendix of workshop participants.
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The evidence that oxidative modification of low density lipoprotein (LDL) may play an important causative role in atherosclerosis has been increasing rapidly over the past several years. Some investigators have been sufficiently impressed by the already available epidemiological, biochemical, and experimental animal data to propose clinical intervention trials to test the oxidative modification hypothesis. Ultimately, such tests will be necessary. However, in view of the complexity and cost of clinical trials, one would like to be reasonably sure that any protocol proposed rests on a solid experimental base of information to maximize the chances of a positive result if the hypothesis is correct. Negative results in a badly designed trial might considerably reduce the chances of obtaining the interest and monies necessary for a second chance. For these reasons, it was proposed to the National Heart, Lung, and Blood Institute in September 1990 that a group of experts on various aspects of this problem be convened to assess the available evidence and discuss the possible design of clinical trials.

Dr. Daniel Steinberg, Professor of Medicine at the University of California San Diego, was asked to organize and chair the meeting. The participants (listed in the "Appendix") brought to the conference table expertise covering a broad range of topics relevant to the oxidative modification hypothesis and to the design of clinical trials. They met in Bethesda on September 5 and 6, 1991. The format of the meeting was framed in terms of a series of questions to be discussed in the context of all of the available data. The summary of the proceedings presented here will follow that format.

How Strong Is the Evidence in Experimental Animals That Oxidative Modification of LDL Occurs In Vivo?

The evidence on this was felt to be compelling and included the isolation from arterial lesions of lipoproteins with all of the properties of oxidized LDL, the demonstration in plasma (in particular, the plasma of diabetic rats) of oxidized LDL, the immunochemical identification of epitopes of oxidized LDL in arterial lesions and in the lipoprotein extracts from arterial lesions, the demonstration in both human and rabbit plasmas of autoantibodies reactive with oxidized LDL, and, most important, the demonstration in at least four studies that antioxidants (probucol or butylated hydroxytoluene) inhibit the progression of atherosclerosis in LDL receptor-deficient and cholesterol-fed rabbits. In two of these studies, the antiatherogenic effect was independent of any cholesterol-lowering effects. One negative study in cholesterol-fed rabbits has been reported.

What Are the Mechanisms by Which LDL Is Oxidized and Becomes Atherogenic? What Interventions Are Suggested by These Mechanisms?

It is not known how LDL oxidation is initiated. Regardless of the source of first-formed radicals, lipid peroxidation can occur, beginning with a free radical-mediated hydrogen atom abstraction from a polyunsaturated fatty acid group present in the LDL and followed by a rapid reaction of the carbon-centered radical product with molecular oxygen to form a peroxyl radical. In the absence of antioxidants, the peroxy radical will abstract a hydrogen atom from another polyunsaturated fatty acid group to form a fatty acid hydroperoxide and another fatty acid carbon-centered radical, thereby setting in motion an uncontrolled chain reaction (propagation phase). Furthermore, the reaction may accelerate because the hydroperoxides products themselves are a potential source of further radicals via transition metal ion-catalyzed decomposition (e.g., Fe, Cu). This peroxidation mechanism is analogous to that occurring in simpler systems in which fats undergo oxidative damage (rancidification). LDL carries within it a number of natural antioxidants that can trap free radicals and prevent the chain reaction from starting or limit its extent. These include, notably, vitamin E, β-carotene, lycopene (and a variety of other carotenoids), ubiquinol, and probably a number of other minor components as well. When LDL is subject to pro-oxidative conditions, these antioxidants are themselves oxidized before any extensive oxidation of the polyunsaturated fatty acids or sterols can occur. This "lag phase" (the number of minutes before the polyunsaturated fatty acids begin to be oxidized at a maximum

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rate as measured by diene conjugation or related methods) can serve as a measure of the extent to which LDL is "protected" against oxidative damage. Thus, the lag phase for a particular lipoprotein preparation can be increased by increasing its vitamin E content. However, comparison of the results of in vitro oxidation of LDL preparations from different individuals shows very poor correlation with vitamin E content, presumably because a large number of additional factors are relevant to the susceptibility of LDL to oxidative damage. Nevertheless, increasing the antioxidant content of the LDL would be one way to interfere and possibly prevent the undesirable consequences of LDL oxidation.

It was stressed that oxidative modification of LDL is extremely complex. There is a continuum of changes from the mildest (MM-LDL, representing LDL that has undergone very limited oxidative damage) through a spectrum of LDL particles that are more extensively oxidized with progressively more extensive degradation of fatty acids, sterols, and the apolipoprotein B itself. The panel urged that investigators specify very carefully not only the conditions under which their LDL preparations are oxidized but also the relevant properties of the product because even with the most careful control of starting conditions, the extent of oxidation can vary widely. This may reflect differences in the number of hydroperoxides present in the LDL particle before oxidative modification, but many additional factors may be relevant. It was stressed that the term "oxidized LDL" should always be further defined along these lines. The system of interest to the investigator will probably determine his or her choice of definition. For example, if he or she is interested primarily in the potential cytotoxicity of oxidized LDL, he or she will characterize it in terms of damage to cells in culture; the investigator interested in the generation of foam cells will probably characterize it in terms of its binding to scavenger receptors.

Over the past decade, it has become clear that oxidized LDL is potentially more atherogenic than native LDL in many different ways, including its recognition and rapid uptake via specific macrophage receptors, giving rise to foam cells; cytotoxicity; chemotactic properties for monocytes; inhibition of the motility of tissue macrophages; ability to stimulate the release of monocyte chemoattractant protein-1 from endothelial cells; ability to stimulate the release of macrophage colony-stimulating factor from endothelial cells; ability to stimulate the release of interleukin-1 and other cytokines from macrophages; ability to induce the formation of autoantibodies; and ability to inhibit the relaxation of arteries in response to agents working through endothelium-dependent relaxation factor.

It is important to keep in mind that even if a proposed intervention does not totally prevent oxidative modification, it may have value. It might be a mistake, then, to discard potential interventions simply because they do not completely prevent oxidative modification. On the other hand, the greater the antioxidant "power" of the agent used, presumably the greater would be the chances of successful intervention, always bearing in mind the possibility of adverse effects at very high dosage levels.

How Strong Is the Evidence in Experimental Animals That Oxidative Modification Plays a Significant Role in Atherogenesis?

The evidence on this issue was felt to be reasonably strong but by no means totally conclusive.

The evidence that oxidative modification occurs is very strong, but the evidence that this oxidative modification is important in pathogenesis is less compelling. The first focused in vivo tests of the oxidative modification hypothesis were done in LDL receptor–deficient rabbits and used probucol as the antioxidant. Earlier studies showed an antiatherogenic effect of probucol, but it was possible that the cholesterol lowering alone could account for the result. The choice of probucol was based on earlier research showing that in both animals and humans, probucol, in addition to lowering levels of LDL, protects it very effectively against oxidative modification. The hypothesis underlying these studies was that prevention of oxidative modification of LDL by probucol would be antiatherogenic. In two independent studies, the results were clear-cut. However, more recent studies suggest additional mechanisms that may have contributed to the observed antiatherogenic effect of probucol. In addition to inhibiting oxidative modification of LDL, probucol inhibits the release of interleukin-1, increases plasma levels of cholesteryl ester transfer protein and its activity, and facilitates the selective transfer of cholesterol esters from HDL into the liver, thus facilitating reverse cholesterol transport.

In view of these findings, it would be a mistake to conclude that the effectiveness of probucol in the animal studies was entirely due to its ability to reside in and protect the LDL from oxidative modification. Furthermore, recent studies show that probucol can enter cells and inhibit the ability of those cells to oxidatively modify LDL at least in vitro. If the same is true in vivo, the effectiveness of the probucol would still be due to its ability to prevent LDL oxidation, but it would be by a two-pronged mechanism: direct inhibition of oxidation by virtue of its presence in the LDL particles as an antioxidant, and indirect inhibition, by virtue of its presence in cells; thus, limiting their ability to oxidize LDL in the surrounding interstitial fluid would be limited. The importance of this is that it may not be legitimate to evaluate antioxidant agents simply by testing the extent to which they directly protect LDL. Compounds that enter the cell and protect indirectly as well as directly may be considerably more effective.

Another difficulty with the interpretation of some of the animal studies is that probucol lowers LDL levels and high density lipoprotein (HDL) levels. In some studies, attempts have been made to correct for this by manipulating the diet or drug treatment of the control group, but such attempts have not always been totally successful.

Three studies have been reported in cholesterol-fed rabbits. It should be noted first that the major lipoprotein accumulating in cholesterol-fed animals is β-very low density lipoprotein (β-VLDL), rather than LDL. β-VLDL is taken up rapidly by macrophages even without prior oxidative modification. The uptake is attributable to a unique high-affinity binding of β-VLDL to the LDL receptor of macrophages. The essential point is that oxidative modification may not be
as important in the case of β-VLDL because even without oxidation it can be taken up readily by macrophages and generate foam cells, whereas native LDL cannot do so unless it is oxidized. In any case, one group of investigators has reported a significant inhibition of atherogenesis in cholesterol-fed rabbits using probucol, whereas a second group failed to find a significant effect. Recently, it has been reported that a different but structurally related antioxidant—butylated hydroxytoluene (BHT)—was effective in protecting cholesterol-fed rabbits from fatty streak formation. Because BHT actually raised plasma cholesterol levels slightly, the confounding effect due to changes in cholesterol level appears not to be an issue in that study.

In summary, the strength of the evidence in experimental animals for an important role of oxidative modification is moderately strong but not conclusive. More animal studies are needed, using other animal species and using other antioxidants.

How Strong Are the Epidemiological Correlations Between ‘Antioxidant Status’ and Risk of Coronary Heart Disease (Vitamin E, β-Carotene, Vitamin C, Selenium, and Others)?

Epidemiological studies suggest that populations with high plasma levels of vitamin E have a lower risk of coronary heart disease (CHD), even after corrections have been made for the other known risk factors. This is best observed using populations with plasma cholesterol levels clustered around the same mean. The significance is even greater when the observed vitamin E levels are normalized according to plasma lipid levels. Occasional reports have suggested similar negative correlations with selenium intake or selenium levels in plasma, but the strength of these correlations was weaker. At the workshop, new data from the Nurses’ Health Survey were presented by Drs. JoAnn Manson and Charles H. Hennekens. Intake of vitamin E and β-carotene was calculated from diet questionnaires. Approximately 87,000 women were involved in this study, which was begun in 1980, and the data show an inverse correlation between calculated vitamin E intake and CHD risk. Similar results were obtained with regard to β-carotene intake. CHD risk was reduced 30–40% in those with high calculated intakes of vitamin E or β-carotene. New prospective cohort data also were presented by Dr. J. Michael Gaziano and Dr. Hennekens based on a group of 1,271 elderly people. They found an inverse relation between β-carotene consumption and cardiovascular mortality, even after adjustment for available coronary risk factors. These may be the first detailed prospective studies on the relation between dietary antioxidant intake and CHD risk.

In summary, the consensus was that the epidemiological evidence with respect to selenium and vitamin C was weak, the evidence for a role for β-carotene was somewhat stronger, and the data for correlations with vitamin E were moderately strong. Needless to say, statistical association can never fully establish pathogenetic significance. Nevertheless, the epidemiological data are suggestive.

What Is the Strength of the Evidence at a Clinical Level That Antioxidant Supplementation May Reduce the Risk of Coronary Heart Disease?

There are no published double-blind, carefully controlled, large-scale studies to establish the value of antioxidant supplementation. All we have so far is a preliminary report of results on a subset of men from the Harvard Physicians’ Health Study. This study was originally designed to test the hypothesis that β-carotene might be valuable in preventing cancer. In view of the increasing level of interest in the possibility that oxidative modification might be relevant to CHD, the investigators examined the results in a subset of 333 men who at the time of randomization approximately 7 years ago had had no prior myocardial infarction, stroke, or transient ischemic episodes but had chronic stable angina or had coronary revascularization. Within this subset, those receiving β-carotene experienced a statistically significant 50% reduction in combined end points (myocardial infarction, revascularization, stroke, or coronary death). This randomized trial is continuing with the much larger group of more than 20,000 physicians originally randomized, and data should become available in approximately 4 years with regard to primary prevention. Other than this preliminary report, the literature contains mostly anecdotal or poorly controlled observational studies.

If We Need Further In Vitro and/or Animal Studies Before a Clinical Trial Is Warranted, Which Questions Need to Be Addressed?

There was a consensus that we simply do not have enough information to determine which antioxidant (or combination of antioxidants) offers the best hope or how much of each should be administered. It was pointed out that in any given population, there is a wide range of blood levels of antioxidants, so the effects of supplementation will vary considerably. Even more important, the level of antioxidant needed to protect against different oxidation-driven processes may vary. For example, experimental studies show that the level of vitamin E needed to prevent hemolysis of red blood cells in rats is much lower than the level needed to maximize immunocompetence. We are just beginning to explore directly the levels of vitamin E needed to prevent oxidation of LDL. Another difficulty is that there are interactions among the various antioxidant nutrients, and one cannot simply “add them up.” It has been reported that vitamin C “spares” vitamin E, i.e., reduces the dietary vitamin E requirement since it can reduce oxidized vitamin E, thus making it available for a second “round” of antioxidant protection (although there are recent data questioning this in some species). In vitro studies show that vitamin C protects isolated LDL from oxidative modification. Whether increments in vitamin C intake would prolong the lag phase is not known with certainty.

It was pointed out that β-carotene can trap singlet oxygen but that under some circumstances, it is not a very effective antioxidant in the conventional sense. It was also noted that in β-carotene, as with other antioxidants, there is a balance between antioxidant and pro-oxidant character. A pro-oxidant effect is possible in vivo because β-carotene readily undergoes spontaneous oxi-
dation in vitro. Unfortunately, studies in experimental animals using β-carotene are going to be very difficult. For example, rabbits do not even absorb β-carotene. Conflicting results have been obtained on the addition of β-carotene in vitro to LDL, possibly reflecting only the difficulty of getting β-carotene into solution.

More studies are needed on this issue. Studies in two centers have shown, surprisingly, that the addition of β-carotene to the diet of human subjects in amounts sufficient to significantly raise the β-carotene content of LDL fail to confer any protection when that LDL was isolated from the plasma and tested in vitro for susceptibility to oxidative modification. Clearly, more studies are needed in human subjects taking known doses of this and other antioxidants if we want to select the best combination. Studies are in progress in several laboratories along these lines, and results should be available soon.

It has been shown in animals and human volunteers that LDL enriched in monounsaturated fatty acids is less susceptible to oxidation, but it is not known whether diets enriched in monounsaturated fats will reduce the rate of progression of atherosclerosis. Because dietary substitution of monounsaturated fat for saturated fat is easy and totally safe (and lowers LDL levels as effectively as substituting polyunsaturated fats), studies to determine whether such substitution is antiatherogenic should be pursued.

Another area deserving further study is the dietary intake of oxidized lipids and their contributions. Should more precautions be taken to prevent oxidation of foodstuffs?

In summary, there are still many gaps in our knowledge of antioxidants, how they work, and whether they work effectively to prevent LDL oxidation in vivo. It was agreed that further studies of antioxidants such as probucol need to be done in animal species other than the rabbit. Some primate studies are underway, but perhaps more need to be initiated. Different antioxidants need to be tested because of the possibility that probucol works in other ways, as outlined above. Further in vitro and experimental studies of other candidate antioxidants need to be encouraged so we can more intelligently select a protocol for testing the validity of the hypothesis in humans. Some of the questions of relevance are not going to be readily answered by in vitro or experimental studies, and additional clinical investigation is called for. In fact, some aspects may be approachable only by carrying out empirical clinical intervention trials.

If We Are Satisfied That an Intervention Trial Is Warranted, Which Intervention Would Be Most Likely to Yield a Clear Answer (e.g., Probucol, β-Carotene, Other Carotenoids, Vitamin E, Vitamin C, Other Natural Antioxidants, Synthetic Antioxidants, Diets Rich in Oleic Acid, Drugs That Inhibit Superoxide Anion Formation or Increase its Destruction, Lipoxygenase Inhibitors, or Combinations of One or More of the Above)?

Probucol and BHT, which are closely related in chemical structure, are the only antioxidants that have been shown thus far to slow the progression of experimental atherosclerosis. Furthermore, probucol has proved to be an even more effective antioxidant than vitamin E or other natural antioxidants in several in vitro test systems. These considerations would suggest it as a logical prime candidate to be tested clinically. However, probucol lowers both LDL and HDL cholesterol levels significantly, confounding the experiment and making it difficult, even if results are positive, to be certain that it is working as an antioxidant. Because it has a number of additional potentially relevant effects, the mechanism of action would remain uncertain after a trial of probucol. Also, although it is remarkably safe in clinical use, it has some side effects, like every other drug. Therefore, it was felt that further studies of probucol should be deferred at least until the QRST Study, which is currently under way in Sweden to test the efficacy of probucol in slowing the progression of femoral atherosclerosis, is completed (around the end of 1992). The feeling was that the first clinical trials should use natural antioxidants so there would be less concern about side effects.

Supplementation with vitamin E has been shown to confer protection on LDL ex vivo, but the magnitude of the effect is quite variable. Plasma levels can be increased only approximately twofold by dietary supplementation. It was felt that a trial ought to include other antioxidants in addition to vitamin E. Because vitamin C may regenerate and spare vitamin E, the protection afforded by the two given together should be additive. In vitro experiments have shown that vitamin C at low levels can, paradoxically, act as a pro-oxidant but that at high levels it is an effective antioxidant. After a great deal of discussion, it was concluded that the first clinical trials should probably not be undertaken with a single agent but with an antioxidant “cocktail.” By using a factorial design, it should be possible in a sufficiently large study to test the effects of the individual agents and the effects of all of them combined. The consensus was that a trial of vitamin C, vitamin E, and β-carotene in a 2×2×2 factorial design would be the best approach at this time. Because these micronutrients, used at reasonable doses, have no known toxic side effects, the trial could be done on a large scale with minimum surveillance and therefore at a reasonable cost.

If a Clinical Trial Is Warranted, at What Stage of the Disease Should the Study Be Done? Will Antioxidant Therapy Be Expected to Have an Effect on Advanced Lesions, and Will Angiography Therefore be a Suitable End Point? Or Will It Be Necessary to Start Earlier and Use Other End Points?

The oxidative modification hypothesis was put forward initially to account for the generation of foam cells in fatty streak lesions. Therefore, the question was raised as to whether antioxidant therapy would be effective only in inhibiting the very early stages of atherogenesis, i.e., the generation of fatty streak lesions. It was pointed out that a number of additional effects of oxidized LDL have now been described, and some of these could contribute to late lesion progression and clinical events. For example, oxidatively modified LDL is cytotoxic and could play a role in endothelial damage. Damage to the endothelium could lead to any and all of the phenomena embraced by the original endothelial
injury hypothesis. For example, loss of endothelial cells could lead to platelet aggregation with release of platelet-derived growth factor and contribute to smooth muscle cell replication and the progression of the stenotic lesion. Damage to endothelial cells could destroy their antithrombotic status and lead to fatal thrombosis. It was also pointed out that even in advanced lesions, one continues to see new foam cells, particularly at the leading edges of the lesions. The fissuring that almost always precedes a fatal thrombosis also occurs at the leading edges of lesions, i.e., at domains where there is still ongoing inflammatory activity with ongoing recruitment of macrophages.

Finally, the preliminary results from the Harvard Physicians’ Health Survey show a beneficial effect of β-carotene on clinical events in men who had had symptomatic CHD when they entered the study.

In summary, although we cannot say with any certainty the extent to which antioxidant treatment will affect the development and clinical expression of later lesions, there is at least some reason to believe that effects can be obtained. At the very least, however, studies should probably cover a long enough time to optimize the chances of detecting effects on late and/or early lesions.

What Would Be a Sound Trial Design? Should the Study Be Done in Very-High-Risk Individuals, or Should It Be Done on a Broader Population With, Presumably, Earlier Stages of Disease?

The only prospective trial that has been reported, albeit in preliminary form, was carried out in men who had established coronary heart disease (chronic stable angina or a prior revascularization procedure). This, together with the experimental studies, the epidemiological correlations, and the recent prospective observational studies described above, provide the basis for concluding that a trial in the presence of established CHD may be warranted at this time.

A trial is in process at the University of California, San Francisco, that tests whether the addition of probucol to a potent three-drug cholesterol-lowering regimen will confer additional protection as evidenced by coronary angiography. The PROST Trial in Sweden will test whether vascular lesions progress more slowly on probucol. However, even if a positive result is obtained, it will be difficult to decide whether the probucol has worked because of its antioxidant activity, its cholesterol-lowering activity, a combination of the two, or one of the alternative mechanisms discussed previously.

The β-carotene arm of the Harvard Physicians’ Health Survey is continuing, and results on a large number of individuals with respect to primary prevention should become available in approximately 4 years. Also, a large randomized trial of vitamin E and β-carotene (factored with aspirin in a 2×2×2 factorial design) in 40,000 women has been recently funded by the National Cancer Institute and the National Heart, Lung, and Blood Institute. Thus, there are at least two large-scale randomized trials in primary prevention of cardiovascular disease and cancer that will evaluate antioxidant therapy. Trials like these involving the general population are particularly valuable if we are interested in answering the question of whether supplementation with antioxidants should be recommended to the public.

It was also felt that trials in patients with early clinical CHD are warranted since these patients have so much to gain and shorter-term studies would be sufficient to test the hypothesis in them.

Several members of the panel stressed that with the proper design, studies can be done in large numbers of subjects yet at very reasonable budget levels. This becomes possible when the treatment modality is without potential toxicity and no elaborate patient surveillance is necessary. The Physicians’ Health Study is an example of such a large-scale, randomized trial that confers great power by virtue of large numbers without entailing the expense involved in studies in which the subjects are carefully stratified and followed closely in clinics. It was also suggested that trials of antioxidants could be piggybacked onto trials attempting to answer other questions of interest (e.g., anticoagulants in secondary prevention).

With regard to the timing of a clinical trial, it was pointed out that a very high percentage of the US population is already taking vitamin supplements. Fortunately, the level of such supplementation is currently quite low, and it would be possible to carry out a supplementation with high levels of antioxidant micronutrients at this time. Delay, on the other hand, could make it more difficult to carry out an effective study.

As discussed, there are good reasons to propose the use of a multifactorial trial studying the effects of vitamin E, β-carotene, and vitamin C, alone and in combination.

In summary, we are dealing with a situation that may require a departure from our usual criteria when considering interventions. Certainly we need more information about the effects of micronutrient antioxidants in patients, and it is hoped that further studies will be carried out and that the information will become available. The unique situation here is that we are dealing with natural antioxidant compounds that do not have any known toxic side effects at reasonable dosages and can be administered without the need for a great deal of continuous surveillance and concern for safety. That makes it possible to study very large numbers of subjects and to do so inexpensively. We know that the biological effectiveness of supplements increases only up to a certain intake level. For example, it is doubtful that much is gained by increasing intake of vitamin E above 400 to 800 units/day; the intake of vitamin C above 1 g has little or no additional effect on plasma levels of vitamin C; and β-carotene, aside from causing skin pigmentation, has no known toxic side effects and therefore can be administered in maximum “nonyellowing” doses. Therefore, it is reasonable to propose proceeding with high (but safe) doses of these antioxidant vitamins. We will never be able to do more than that unless and until we are willing to add antioxidant drugs.

The consensus of the panel was that a multifactorial trial of supplementation with vitamin E, vitamin C, and β-carotene in a 2×2×2 design was justified at this time and that studies with antioxidant drugs should be deferred.
Summary Recommendations

More Basic Research Is Needed on the Mechanisms Involved in the Oxidative Modification of LDL and on the Ways in Which Various Antioxidants Influence It

It became clear from the discussion that some fundamental and, on the surface, rather simple questions have, surprisingly, not been satisfactorily answered. For example, the fact that β-carotene is progressively destroyed as a product of oxidative modification of LDL has been taken to imply that it serves an antioxidant function. Yet attempts in some laboratories to demonstrate protection of LDL by incorporating β-carotene into it in vitro have yielded negative results. Furthermore, LDL isolated from human volunteers taking large doses of β-carotene—doses large enough to increase the β-carotene content of LDL by as much as 20-fold—was not protected against in vitro oxidation by cells or by copper ions in the absence of cells. These LDL preparations showed a paradoxical increase in extent of oxidation as judged by a number of criteria. Dr. Burton pointed out that the disappearance of β-carotene means that it is being modified but does not necessarily mean that it is acting as a true antioxidant.

Another example is that in studies carefully comparing LDL preparations from different patients, it was found that susceptibility to oxidation (assessed by measurement of the lag phase before the rapid increase in diene conjugation, i.e., absorption at 234 nm) does not correlate at all well with the vitamin E content of the LDL preparations. In other words, even though vitamin E is clearly an important antioxidant in LDL, other factors override it in determining overall susceptibility of LDL to oxidative modification. What those factors are remains to be determined. When all of the different known antioxidants are taken into account, there is a somewhat better correlation, but it is still far from complete. Factors that have not been adequately studied include the fatty acid composition of the phospholipids and cholesteryl esters, the content of minor lipids, the size of the LDL particle, the contribution of apolipoprotein B, and the initial “seeding” of the LDL as isolated with lipoperoxides.

More Animal Studies Are Needed to Establish Firmly That Protection of LDL Against Oxidative Modification Does Influence the Progression of Lesions

Currently, the only antioxidant that has been extensively studied in this way in experimental animals is probucol. However, probucol has a number of additional modes of action that could account for its antiatherosclerotic effect in addition to or independent of its effect on oxidative modification of LDL. The literature with regard to the effects of vitamin E on experimental atherosclerosis is confusing and inconsistent. A recent study using BHT tends to support the interpretation that probucol works because it is an antioxidant, but BHT has not been tested to see if it has additional effects like those demonstrated for probucol.

Important confirmation of the oxidative modification hypothesis could come from interventions that instead of protecting the LDL against oxidation in a direct fashion, inhibit the ability of tissues to catalyze the oxidative modification of LDL. For example, there is evidence to suggest that lipoxygenases may be involved in the cell-induced oxidation of LDL. If it could be shown that inhibitors of lipoxygenase slow the progression of lesions and slow the incorporation of LDL into macrophage-rich areas of lesions, that would substantially the hypothesis in an independent manner.

Another option would be the use of diets rich in oleic acid. Oleic acid (one double bond) is much more resistant to oxidation than linoleic acid (two double bonds); therefore, LDL enriched in oleic acid should be less atherogenic if the hypothesis is correct. If oleic acid substitution could be accomplished without affecting the plasma LDL concentrations (or somehow adjusting the LDL concentrations in the two groups to be equal) and if that slowed the progression of atherosclerosis, it would again provide an independent verification of the hypothesis.

More Research Is Needed on the Possible Effects of Antioxidants on the Later Stages of Atherosclerosis and on Thrombosis

In view of the multiple mechanisms triggered by oxidized LDL, it could affect not only fatty streak formation but also the evolution of fatty streaks to fibrous plaques and complicated lesions. If this were demonstrated, it would obviously affect our choices of intervention approaches and design of intervention trials. There is a need for a suitable animal model of arterial thrombosis to test whether antioxidants influence the process.

It Was the Consensus That the Evidence Available Justifies a Clinical Trial of Natural Antioxidants (Associated With No Increase in Risk) But That Clinical Trials With Antioxidant Drugs (Which Might Carry Deleterious Side Effects) Should Be Deferred Until More Knowledge Is Available

As discussed above, there are significant gaps in our understanding of oxidative modification of LDL and the effects of antioxidants, both in vitro and in vivo. On the other hand, the summation of the experimental and animal evidence implicating oxidative modification, the population data showing negative correlations between CHD risk and dietary intake and/or plasma levels of antioxidant vitamins, and the preliminary report from the Harvard Physicians’ Health Study with regard to the protective effect of β-carotene makes a strong case for a clinical trial of antioxidants. Further clinical studies will allow us to “fine-tune” our understanding of these natural antioxidants—β-carotene, vitamin E, and vitamin C—but trials using high but safe doses could be undertaken in the near future. Close surveillance and repeated physician visits would be unnecessary because there are few concerns about safety. In the absence of the kind of detailed information that would inform a specific, highly focused study and in view of the known interactions between vitamin C and vitamin E, it was felt that a trial involving all three should be undertaken. Such a trial could be of multifactorial design so that the effects of individual agents also could be evaluated.
Appendix

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**KEY WORDS**
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