Is the Oxidative Modification Hypothesis Relevant to Human Atherosclerosis?

Do the Antioxidant Trials Conducted to Date Refute the Hypothesis?

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Several large-scale, double-blind, placebo-controlled trials have shown convincingly that neither β-carotene nor vitamin E, alone or in combination with other antioxidant vitamins, reduces the risk of fatal or nonfatal infarction (or other hard clinical end points) in an unselected population of people with established coronary heart disease (CHD) or at high risk of CHD. Two end point trials, much smaller trials that used vitamin E, have reported positive results, and one trial, which used ultrasound, showed that a combination of vitamins E and C slowed the progression of carotid artery lesions. However, these are far outweighed by the negative results in the other, much larger trials. Certainly there is no basis for recommending vitamin E supplementation to patients with CHD, especially because it may blunt the effectiveness of hypolipidemic therapy with statins and niacin. A surprisingly large fraction of cardiologists (≈40%) have been recommending such regimens despite warnings that this use was premature.

At first glance, it might seem that these negative results close the book and that additional clinical trials of any antioxidants would be pointless. Closer examination, we believe, will show that such a conclusion would be premature and inappropriate. The hypothesis that oxidative modification of LDL plays a significant role in atherogenesis in humans is not necessarily disproved by the failure of these particular clinical trials any more than a negative trial of an ineffectual antibiotic in Pneumococcal pneumonia would prove that pneumonia is not a bacterial disease. The oxidative modification hypothesis is not that vitamin E will ameliorate the human disease but that oxidative modification of LDL and/or other oxidative events play a significant role in human atherogenesis as it does in animal models of atherogenesis. A corollary of the hypothesis is that some appropriate antioxidant intervention, at some appropriate dosage, in appropriately selected patients over an appropriate time interval has the potential to improve prognosis. Otherwise, of course, the role of oxidation would remain of academic interest only. In the present report, we put the currently available information into context by briefly reviewing the origins of the LDL modification hypothesis and explaining why the trials to date have not adequately tested the basic hypothesis, as pointed out by a number of authors. It would be a mistake to jettison as irrelevant to humans a hypothesis that is so strongly supported by many epidemiological studies and by so many positive results in several animal models, including nonhuman primates, and with the use of several different antioxidant compounds. Instead, perhaps we should be reexamining the science underlying the hypothesis and asking what additional basic information we need to design trials that will appropriately test the hypothesis.

Origins of the Oxidative Modification Hypothesis

The concept that circulating LDL must undergo some kind of structural modification before it becomes fully proatherogenic was put forward originally by the Brown and Goldstein laboratory. They discovered that the macrophage, the precursor of the cholesterol-loaded foam cell, took up native LDL at a rate insufficient to load the cell with cholesterol. They also pointed out that patients totally lacking the native LDL receptor nevertheless accumulate large amounts of cholesterol in their macrophages. Therefore, they postulated that modifications of LDL must occur, leading to uptake of the modified forms through receptors other than the classic LDL receptor, which they termed “scavenger receptors.” Goldstein et al identified the first of these scavenger receptors, the acetyl LDL receptor, which was later cloned in Krieger’s laboratory and renamed scavenger receptor A. Subsequently, several other scavenger receptors have been identified. The fundamental correctness of this concept of LDL modification is now supported by many lines of evidence. Any scheme for the pathogenesis of atherosclerosis must include one or more modified forms of LDL and macrophage receptor(s) for them.

Oxidative stress may contribute to atherogenesis by mechanisms that are not necessarily linked to LDL oxidation. For example, free radical oxygen species such as superoxide anion can rapidly react with and inactivate nitric oxide, enhancing proatherogenic mechanisms (eg, leukocyte adherence to endothelium, impaired vasorelaxation, platelet aggregation). As pointed out by Landmesser and Harrison, vitamin E would be an inappropriate antioxidant in such a

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system because it reacts very slowly with superoxide. Oxidized LDL (OxLDL) itself can inactivate nitric oxide and induce the same proatherogenic processes, but OxLDL may not be an obligatory intermediate.

**Modifications of LDL That Might Be Involved**

Several different modifications of LDL have been described that convert it to a form recognized by one or more macrophage scavenger receptors. Modifications that can favor foam cell formation in vitro include oxidation, aggregation, enzymatic modification, complexing with immunoglobulins, and possibly others. The best studied of these and the only one for which there is good in vivo data is oxidative modification. As reviewed elsewhere, six different antioxidant compounds (probucol, probucol analogues, vitamin E, coenzyme Q, diphenylphenylenediamine, and butylated hydroxytoluene) have been studied in four different animal models of atherosclerosis (rabbits, mice, hamsters, and monkeys) and most of the results have been strikingly positive. A number of important ancillary lines of evidence are consonant with the hypothesis, including the fact that oxidation of LDL has been shown to occur in vivo and that OxLDL is demonstrable in lesions; that autoantibodies are generated against OxLDL and that the titers are correlated with the extent of atherosclerosis; that knocking out scavenger receptors (either scavenger receptor A or CD36) ameliorates atherosclerosis (establishing that some protective role for dietary antioxidants and because of the fact that the design of current clinical trials has not been approved for testing the hypothesis.

** Have the Clinical Trials Been Done With the Right Antioxidants at the Right Dosages?**

Because of a large body of epidemiological data showing a protective role for dietary antioxidants and because of the impressive data from experimental studies, a workshop was convened by the National Heart, Blood and Lung Institute in 1991 to review all of the available evidence about the oxidative modification hypothesis. The panel of experts concluded that the evidence was sufficiently strong to justify initiating clinical intervention trials. At the time, the data from trials that used antioxidants in experimental animal models were already quite persuasive, but the field was relatively new and there were many unanswered questions. Just to cite one important example, although OxLDL had been demonstrated in the atherosclerotic lesions of animals and humans, the mechanisms leading to such oxidation were not known and in fact remain unknown to this day. Nevertheless, the 1991 conference thought that the use of naturally occurring antioxidants would be safe and that one could therefore proceed even without requiring the kind of in-depth evidence regarding mechanisms that would have been expected if the trials were to be done with drugs. Hence, the recommendations of the committee were to start trials with vitamin E, vitamin C, or β-carotene. Yet, there was at that time no experimental evidence in animal models that any of these natural antioxidants would have an effect on atherosclerosis. The animal trials available at that time had been carried out mostly with probucol or probucol analogues, and only one study each with diphenylphenylenediamine and butylated hydroxytoluene. What the expert committee was saying, even if not explicitly, was that antioxidants could be looked on as a class of compounds sharing certain common properties and that they would be functionally more or less interchangeable. There may be some justification for such “lumping” when dealing with simple in vitro redox systems, although there are some striking differences. For example, β-carotene is an excellent trapper of singlet oxygen but much less effective at terminating free radical chain reactions; the reverse is true of vitamin E. In the context of a complex biological system, such “lumping” becomes indefensible. For example, vitamin C is water-soluble, readily absorbed, and transported in the aqueous phase of the plasma; vitamin E is lipid-soluble, poorly absorbed, and transported in lipoproteins. The pharmacodynamics of the various antioxidants differ greatly, and until we know where and how LDL is oxidatively modified in vivo, we have no way to predict which antioxidant, at what dosage and administered by what route, would be most effective.

Vitamin E is the antioxidant used in most of the clinical trials to date. In mouse models of atherosclerosis it has been effective alone or in combination with other antioxidants, but most of the studies in rabbits have been negative. Moreover, when administered to humans, vitamin E has been shown to have only a modest inhibitory effect on LDL oxidation ex vivo (delaying copper-induced oxidation by 15 to 20 minutes) but nowhere near the almost complete protection afforded by such a potent antioxidant as probucol (which can delay oxidation for as much as 20 hours). A recent report by Meagher et al is highly relevant to this discussion. They fed normal subjects doses of vitamin E ranging from 200 to 2000 mg/d for 8 weeks. The highest dose increased plasma vitamin E levels 5-fold, but urinary excretion of isoprostanes and 4-hydroxynonenal (breakdown products of fatty acid auto-oxidation) was unaffected. The results suggest that in normally nourished subjects, additional vitamin E will not necessarily confer any additional antioxidant protection. Earlier studies in cigarette smokers, in contrast, did show a vitamin E effect on plasma isoprostane levels, suggesting that only in subjects under some oxidative stress will a vitamin E effect be obtained. The protective effect of vitamin E against coronary events in the Boaz study may reflect the fact that the subjects were under the oxidant stress known to accompany hemodialysis. Moreover, it should be noted that in the absence of an appropriate antioxidant such as vitamin C, vitamin E can, paradoxically, act as a prooxidant. In any case, the available data suggest that vitamin E is not an appropriate antioxidant with which to test the hypothesis in otherwise healthy humans.

Observational data over the years have shown rather consistently that β-carotene intake is negatively correlated...
with risk of CHD. However, as mentioned above, \( \beta \)-carotene is not very effective as a chain-breaking antioxidant, compared with vitamin E. Moreover, \( \beta \)-carotene, even at very high doses, fails to protect circulating LDL against ex vivo oxidation and even fails to protect it in vitro. Consequently, the trials using \( \beta \)-carotene are in no sense meaningful tests of the oxidative modification hypothesis.

Clearly, we need more potent antioxidants, possibly with different pharmacodynamic properties. We have much to learn about the very different available antioxidant compounds, how they work, and how they are metabolized.

**The Need for Markers to Assess Whether or Not Oxidation of LDL Is Being Successfully Inhibited During a Clinical Trial**

It is relatively easy to test whether a given antioxidant at a given dosage reaches concentrations sufficient to protect the circulating LDL against oxidation ex vivo. In general, the compounds that have been effective in inhibiting atherosclerosis in animal studies have done so, but the correlation has been far from perfect and so even this most widely used test cannot be accepted as a satisfactory marker. Other approaches, such as the measurement of urinary or plasma levels of isoprostanes or of hydroperoxides or of OxLDL itself have been suggested. As discussed above, Pratico et al. showed that vitamin E treatment of apolipoprotein E–deficient mice inhibited atherogenesis. The plasma levels of vitamin E correlated inversely with the extent of lesions and inversely with the urinary excretion, plasma levels, and arterial levels of isoprostanes. Similarly, the titer of autoantibodies to OxLDL correlated directly with the extent of lesions in both LDL receptor-negative and apolipoprotein E–negative mice. Recently it was shown that the ability of vitamin C to improve the vasodilatory response to acetylcholine could identify patients more likely to have a CHD event. If further studies show that these correlations obtain at graded intakes of vitamin E (or other antioxidants), they could become examples of the badly needed markers for oxidative stress. So far, none of the candidate markers has been tested in a sufficiently systematic way in animal models to allow them to be used with any confidence as surrogate markers in a clinical trial. Therefore, even if the teams that designed the first generation of human trials had wanted to use a marker, that is, something that would tell them whether or not the antioxidant was “working,” they would not have found a tested, reliable method. In fact, with the exception of the Swedish probucol study, in which efficacy was shown with respect to inhibition of LDL oxidation ex vivo, none of the reported human trials to date has attempted to assess the efficacy of their antioxidant regimen. Thus, there is no way for us to know whether there was any reason to expect a positive result—there was no independent measure of efficacy. By analogy, it is as if a cholesterol-lowering drug were being tested for efficacy in preventing CHD events but without measurements of plasma cholesterol as part of the protocol. If for no other reason, the results of all of these trials are moot. But there are a number of additional reasons why these trials, however rigorously conducted, have not ruled out the role of oxidative processes in the pathogenesis of human atherosclerosis.

**Identification of Patients Likely to Benefit From Antioxidant Intervention Because of Increased Oxidative Stress**

In designing clinical trials of hypolipidemic therapy, it was assumed that patients with more marked degrees of hypercholesterolemia would be more likely to show the maximum benefit; therefore, patients with severe hypercholesterolemia were selected. Plasma cholesterol levels were monitored during therapy to document the effectiveness of the given hypolipidemic agent, and the impact on CHD events was correlated to the drop in cholesterol level. However, as noted above, we have no analogous marker to identify patients at high risk because of oxidative stress, that is, patients who would theoretically be expected to benefit most from antioxidant intervention. Equally frustrating is the fact that we have no reliable way to know whether a given antioxidant intervention, whether in patients under oxidative stress or not, effectively reduces the level of oxidant stress. It would seem reasonable that a population under high oxidative stress would stand to benefit the most from antioxidant intervention, and the results of the recently reported Secondary Prevention with Antioxidants of Cardiovascular disease in Endstage renal disease (SPACE) Trial provide some evidence in support of that idea. Patients with end-stage kidney disease who were undergoing hemodialysis were randomly assigned to 800 mg/d vitamin E or placebo. End points were myocardial infarction, ischemic stroke, peripheral vascular disease, or unstable angina. This population was chosen because it is well established that patients undergoing chronic hemodialysis are exposed to increased oxidative stress induced by the membranes used in dialysis. Events were reduced by 54% (\( P=0.014 \)) and myocardial infarction by 70% (\( P=0.016 \)). The study was small (\( n=196 \)), but the results are suggestive. Will other patients under increased oxidative stress, such as diabetics, also constitute a population more likely to benefit from antioxidants? Obviously, biomarkers are urgently needed to identify such high-risk populations and to assess whether therapy effectively lowers the oxidative burden.

**Have the Trials Been Started Early Enough and Have They Lasted Long Enough?**

The outstanding successes of clinical trials with lipid-lowering regimens led to the almost universal adoption of the canonical 5-year trial. It was natural to settle initially on 5 years as the appropriate duration for trials of antioxidants and, for the same reason, to choose fatal or nonfatal myocardial infarction as primary end points. Yet, the mechanisms by which cholesterol lowering reduces risk could be quite different from the mechanisms by which antioxidants work. For example, the unexpectedly early drop in clinical event rates with intensive cholesterol lowering\(^6\) may be caused by mechanisms not shared at all by antioxidants. Furthermore, the animal model studies on which the clinical trials are based do not deal with lesions that cause plaque rupture. Conceivably, the antioxidants might be effective in inhibiting the initial stages of human atherosclerosis, as they are in animals,
and yet ineffective or much less effective in reducing plaque instability and rupture. If this were the case, it might be necessary to find some way to assess early stages of lesion development (eg, high resolution ultrasound or MRI) rather than relying on the usual late clinical end points. Of course if the development of early lesions were successfully inhibited, there should eventually be a decrease in the frequency of clinical events, but in that case the trials might need to extend beyond the conventional 5 years.

**Are There Species Differences Such that the Results in Animal Models Do Not Extrapolate to Humans?**

Most of the animal model studies demonstrating antioxidant inhibition of atherosclerosis have been done in small animals—rabbits, hamsters, or mice. Only one systematic study has been done in nonhuman primates.49 Is the pathogenesis of oxidation13,53 by mechanisms still unclear, and the degree of sensitivity or even totally insensitive to treatment with antioxidants in small animals may always be generating OxLDL at a higher rate than relying on the usual late clinical end points. Of course if some threshold value of ROS generation is needed to trigger OxLDL production at a rate that enhances atherogenesis and if ROS generation is below that threshold in humans, then atherosclerosis may develop in normal humans in a manner less sensitive or even totally insensitive to treatment with antioxidants. In addition, hypercholesterolemia per se promotes oxidation13,53 by mechanisms still unclear, and the degree of hypercholesterolemia in animal models far exceeds that seen in human subjects. Still, we know that oxidation of LDL does occur at some rate in humans. Inhibition of that oxidation might prove effective if maintained over a sufficient period of time.

**Summary**

The lipid hypothesis, despite an overwhelming body of experimental evidence in animal models and epidemiological evidence in humans, was only definitively proved after the development of effective hypolipidemic agents. Similarly, there is now a large body of experimental evidence in animal models and epidemiological data in humans to support the oxidation hypothesis, but effective antioxidant regimens have yet to be developed. With the benefit of hindsight, the decision of the 1991 National Heart, Lung, and Blood Institute workshop to give a green light to trials, even trials that use safe, naturally occurring antioxidants, may have been premature. Not knowing how LDL is oxidized in vivo, we cannot be certain which antioxidants are likely to be most effective. We lack markers that would let us evaluate the efficacy of any given antioxidant intervention, and we lack criteria for rational selection of patients under high oxidative stress. Until we have such basic information, we should put a hold on further clinical trials. Instead, we should concentrate on developing the scientific base that will enable us to design an appropriate trial to test the oxidation hypothesis.

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