Oxidative Stress and Cardiovascular Injury
Part II: Animal and Human Studies
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This review so far has considered the role of vascular cells in the generation of reactive oxygen species (ROS) and novel approaches to their detection in integrated systems such as animal models of vascular disease and humans. We now turn attention to evidence consistent with a role for ROS in models of human disease and in discrete patient populations. We also briefly comment on the outcomes of clinical trials of antioxidants. Mainly, these have been disappointing. However, it is premature to conclude that the oxidation hypothesis of disease causality has been adequately tested.

ROS and Vascular Disease: Animal Studies
Animal studies, for the most part, support a fundamental role for ROS in cardiovascular disease. Any controversy could in part reflect the use of ineffective antioxidants and the selection of models in which ROS generation is of marginal relevance to the measured outcome. Both of these issues require a quantitatively accurate measurement of drug effect (ie, antioxidant capacity) before hypotheses relating to the role of oxidant stress can be addressed rationally. These issues pertain to clinical trials also, as we will discuss. However, animal studies permit administration of much more powerful antioxidants (eg, rate constant for interaction of superoxide dismutase (SOD) with O$_2^-$) than is possible in humans (eg, rate constant for vitamin E ≈0.59 mol/L$^{-1}$·s$^{-1}$) or, like in the case of vitamin E, doses of the antioxidant in excess of those usually applied in clinical research.

Atherosclerosis
Animal models of atherosclerosis have documented that all the constituents of the plaque produce and use ROS. Lesion formation is associated with the accumulation of lipid peroxidation products$^{1,2}$ and induction of inflammatory genes,$^3$ inactivation of NO resulting in endothelial dysfunction,$^4,5$ activation of matrix metalloproteinases,$^6$ and increased smooth muscle cell growth.$^7$

Atherogenesis in rodents is accompanied by increasing lipid peroxidation in vivo. Thus, levels of the F$_2$ isoprostane (iP), iPF$_{3,4}$-IV, are increased in both urine and aortic tissue as atherosclerosis evolves in both apolipoprotein E (ApoE)– and low-density lipoprotein (LDL) receptor–knockout mice.$^8,9$ Evidence exists for and against the efficacy of vitamin E in limiting atherogenesis.$^{10–12}$ However, a rational basis for selection of the interventional regimens, or indeed the possibility of comparing treatment regimens across models and species by anything other than weight-corrected dosing, does not exist, pointing to the need for a much more rigorous approach to the development, application, and testing of antioxidant therapies. If a dosing regimen of vitamin E is selected on the basis of its ability to suppress elevated urinary iPF$_{3,4}$-VI in atherosclerotic ApoE mice, vitamin E will retard atherogenesis by ≈50% while leaving hypercholesterolemia unaffected.$^8$ However, administration of much lower doses of vitamin E to the same model failed to detect an impact on atherogenesis.$^{10}$ It is noteworthy that the doses of vitamin E necessary to suppress lipid peroxidation in this model$^8$ greatly exceed those typically administered in clinical trials.

The role of macrophage-derived ROS in atherogenesis has been investigated in animals carrying homozygous deletion of either CD36 (a receptor for oxidized LDL) or the 12/15-lipoxygenase (one of the enzymes that initiate lipid peroxidation). When these animals are crossed with animals deficient in ApoE, and atherosclerosis is initiated by feeding a high-fat diet, lesion formation is dramatically inhibited.$^{13,14}$ These studies would seem to strengthen the argument for macrophage-derived ROS playing an important role in atherogenesis. However, 2 recent studies using homozygous disruption of p47phox or gp91phox to inactivate the phagocytic NADPH oxidase in ApoE mice showed no effect on lesion development in the ascending aorta, although a later study found marked reduction in lesions in the descending aortas of p47phox/ApoE double-knockout mice.$^{15–17}$ Clearly, further experiments are required to resolve these discrepancies.

Additional animal studies have addressed the potential importance of the generation of intracellular ROS in the cells that normally comprise the vessel wall. In rabbits fed cholesterol for a period of weeks, $O_2^-$ is increased in the aorta,$^{18}$ leading to impaired endothelial-dependent relaxation that can be reversed by treatment with polyethylene-glycolated SOD$^{19}$ or probucol.$^{20}$ Long-term administration of a high-cholesterol...
diet leads to persistent elevation of O$_2^-$ and continued inactivation of NO. 19 Warnholtz et al20 showed that vascular NAD(P)H oxidase activity is increased 2-fold and is in part responsible for the increase in O$_2^-$ . Recent studies in ApoE-deficient mice also implicate uncoupling of eNOS as a mechanism of O$_2^-$ production in atherosclerosis. 21

The mechanism by which oxidative stress is increased by hyperlipidemia could involve the renin–angiotensin system. In the rabbit model, both endothelial dysfunction and lesion area are improved by treatment with an angiotensin II receptor antagonist. 20 In rats made hypertensive by angiotensin II infusion, NAD(P)H oxidase subunit expression and O$_2^-$ production are increased. 22 Both the resulting impaired endothelial-dependent vasodilation and the hypertension can be corrected by infusion of modified forms of SOD that penetrate the vessel wall. 22 Moreover, angiotensin II dramatically accelerates lesion formation in ApoE$^{-/-}$ mice, 23 to the point that survival is compromised after 8 weeks. Another potentially proatherogenic effect of angiotensin II, as well as of cytokines produced by inflammatory cells, is upregulation of adhesion molecule expression, thus promoting further macrophage recruitment. Because LDL upregulates angiotensin II receptor type 1 (AT$_1$) receptor expression, 24 the effects of angiotensin II can be exacerbated by hypercholesterolemia. Conversely, angiotensin II can upregulate the expression of receptors for oxidized LDL 25 and could in fact contribute to oxidation of LDL. Finally, angiotensin II causes hypertrophy of vascular smooth muscle in a ROS-dependent fashion, which can participate in arterial thickening. Other mediators can share these properties of angiotensin II. Thus, suppression of thromboxane formation and antagonism of the thromboxane receptor (the TP) both retard atherosclerosis. 26, 27

Indeed, TP antagonism is more effective in retarding atherosclerosis than combined inhibition of cyclooxygenases 1 and 2. 28 The explanation for this discrepancy could be the incidental activation of the TP by lipid peroxidation products, including the iPFs, 29 as well as by the conventional cyclooxygenase-derived ligands, prostaglandin H$_2$ (PGH$_2$) and thromboxane A$_2$. Indeed, suppression of iPF generation with vitamin E augments the impact of combined cyclooxygenase inhibition on atherosclerosis. 30

ROS can also modulate collagen degradation, which is important in lesion instability, by modifying the activity of matrix metalloproteinases (MMPs). MMP-2 (gelatinase A, which degrades collagen IV) and MMP-9 (gelatinase B, which acts on collagen I fibers) are secreted by macrophages and vascular myocytes, and the expression of MMP-9 is increased in the rupture-prone shoulders of atherosclerotic plaques. 31 Rajagopalan et al 31 demonstrated that pro-MMP-9 and pro-MMP-2 from vascular smooth muscle cells (VSMCs) are activated in vitro by ROS. Moreover, neoadjuvant chemotherapy treatment prevents MMP-9 expression and activation by foam cells. 32 MMP activation by ROS could thus contribute to plaque rupture. Indeed, Mallat and colleagues 33 found that the content of lipid peroxidation products of arachidonic acid (AA) were higher in carotid atherosclerotic plaques obtained from patients with symptomatic versus asymptomatic cerebrovascular disease. 33

Another potential effect of elevated ROS in atherosclerosis is a compensatory increase in a mutant form of a functionally active extracellular SOD (ecSOD). 34 Excessive O$_2^-$ is converted into extracellular H$_2$O$_2$ as a consequence of ecSOD upregulation. This, in turn, in the presence of myeloperoxidase and nitrite, could modify LDL. 35 These observations raise the possibility that it is H$_2$O$_2$ rather than O$_2^-$ , that is the deleterious ROS in atherosclerosis. If this is the case, antioxidant therapies directed primarily toward O$_2^-$ should be relatively ineffective in preventing atherosclerosis.

Hypertension

Abundant evidence supports a role for O$_2^-$ in various animal models of hypertension. Vascular O$_2^-$ is increased in the DOCA-salt, 36, 37 angiotensin II–infusion, 38, 39 2-kidney 1-clip, 40 1-kidney 1-clip, 41 chronic inhibition of eNOS, 52 and SHR90-92 models of hypertension, and appears to be functionally important because administration of antioxidants, heparin–binding SOD, or genetic deletion of the NAD(P)H oxidase partially normalizes the blood pressure of these rats. 36, 41, 43– 47 In contrast, norepinephrine-induced hypertension does not increase O$_2^-$ production or oxidase expression, 58 suggesting that O$_2^-$ could be variably involved in the human syndrome of hypertension.

In many forms of hypertension, the increased ROS are derived from NAD(P)H oxidases, 36, 39, 49 which could serve as a triggering mechanism for uncoupling endothelial ROS by oxidants. 49 Wang et al 50 showed that overexpression of human SOD in mice does not alter the hypertrophic response to angiotensin II, suggesting that it is H$_2$O$_2$, rather than O$_2^-$, that regulates VSMC growth in vivo, similar to in vitro results. 51 These results suggest that O$_2^-$ and H$_2$O$_2$ contribute to hypertension by different mechanisms: O$_2^-$ by inactivating NO and H$_2$O$_2$ by altering vascular remodeling.

Diabetes Mellitus

There is also evidence that oxidative stress is increased in the vascular dysfunction that accompanies diabetes mellitus. In animal models of diabetes, antioxidant defense capacity is diminished in certain tissues. 52 In vivo studies linking ROS and diabetes have been performed in rats treated with streptozotocin (a model of type I diabetes), 53– 56 genetically diabetic rats (a model of type II diabetes), 57 as well as in human patients with type II diabetes. 58 Increased vascular O$_2^-$ production, decreased tissue glutathione, impaired endothelial-dependent relaxation, and increased NAD(P)H oxidase activity leading to uncoupling of eNOS have all been demonstrated. 54, 55, 57, 58 These results clearly indicate an association between ROS and diabetes, although the full complement of vascular consequences remains to be determined.

Restenosis

On the basis of animal studies, a role for ROS in the intimal hyperplasia that results in restenosis after arterial balloon angioplasty has been proposed. Evidence has been presented that ROS contributes to the initial VSMC apoptosis, as well as remodeling, proliferation, and migration of medial VSMCs or adventitial myofibroblasts. A remarkable increase in O$_2^-$ production occurs immediately after balloon injury, and
levels remain 20-fold higher than those in control uninjured arteries for up to 10 minutes.\(^6\) Glutathione levels fall by roughly 60% within 30 minutes after injury, coincidental with medial cell apoptosis, suggesting that this early step in the response to injury is associated with severe oxidant stress. Importantly, administration of antioxidants (probucol, vitamin C and E, butylated dihydroxytoluene, or \(L\)-cysteine) reduces neointimal proliferation and oxidants (probucol, vitamin C and E, butylated dihydroxytoluene, or \(L\)-cysteine) reduces neointimal proliferation and glutathione peroxidase activity is decreased compared with uninjured arteries 10 to 14 days after balloon injury of pig coronary arteries or rat aorta when neointimal cells are actively proliferating.\(^{50,63}\) Moreover, treatment with a variety of antioxidants (probucol, vitamin C and E, butylated dihydroxytoluene, or \(L\)-cysteine) reduces neointimal proliferation and promotes vessel remodeling in several animal models.\(^{52,63}\) Although the identity of the oxidase activity stimulated by injury is unclear, involvement of inflammatory cells in neointimal formation is minimal. Szöcs et al\(^{63}\) showed that the novel NAD(P)H oxidases, nox1 and nox4, are increased after injury, and Souza et al\(^{59}\) provided evidence that oxidase activity is increased during injury. Because balloon injury necessarily reduces NO levels, the potential deleterious effects of increased \(O_2^-\) could be exacerbated.

**ROS and Vascular Disease: Human Studies**

Traditional approaches to the assessment of oxidative stress in humans relied on ex vivo indices such as the lag time to oxidation of LDL\(^6\) and in vivo indices of questionable veracity such as thiobarbituric acid reactive substances\(^{66}\) and plasma malondialdehyde.\(^67\) However, more contemporary biomarkers of increased ROS generation now suggest that elevated oxidant stress is a characteristic of a spectrum of cardiovascular diseases.

Like in hypercholesterolemic rodents, patients with hypercholesterolemia have evidence of increased ROS generation (Figure). Urinary iP excretion is increased,\(^{68,69}\) as are circulating antibodies to oxLDL.\(^70\) Levels of iP in circulating LDL are also increased in such patients. Recent evidence suggests that reduction in hypercholesterolemia with a statin can also reduce ROS, perhaps because they interfere with geranylgeranylation of the small molecular weight G protein Rac, a component of vascular NAD(P)H oxidases. De Caterina and colleagues\(^71\) have reported that simvastatin reduced urinary immunoreactive iP\(_{F_2\alpha-III}\) by approximately one third in an uncontrolled study of 43 hypercholesterolemic patients. These results, if confirmed, would suggest that statins and perhaps other hypolipidemic therapies might obviate any benefit from antioxidants and severely confound their evaluation in clinical trials. Interestingly, Sinzinger and colleagues\(^72\) have reported an association of elevations of the same iP with statin-induced myopathy.

Much attention has focused on the role of inflammation in atherogenesis, and urinary iP is increased dramatically in response to inflammatory stimuli such as the administration of lipopolysaccharide in humans.\(^73\) Cyclooxygenases are also upregulated in leucocytes ex vivo by lipopolysaccharide, and augmented prostaglandin formation coincides with systemic “flu-like” symptoms. However, although prior administration of cyclooxygenase inhibitors suppresses the prostaglandin response, the increase in iP is unaltered. Thus, in contrast to the suggestion with statins, cyclooxygenase inhibitors and antioxidants would be expected to express distinct efficacies when inflammation and oxidant stress coincide.

Many of the risk factors and some of the consequences of atherosclerosis have been associated with evidence of oxidant stress in vivo. Thus, urinary and circulating iP is increased, dose-dependently, in cigarette smokers and they fall on quitting.\(^74,75\) Oxidative modification of proteins, specifically of fibrinogen,\(^76\) is also increased in smokers, in whom fibrinogen levels are elevated.\(^77\) Urinary 8-oxodeoxyguanosine is also elevated in the sperm DNA of smokers.\(^78\) Thus, cigarette smoke, which itself contains oxidants, results in cellular activation leading to augmentation of diverse biomarkers of ROS in humans, as has also been shown in rodents.\(^79\)

The vascular reactivity to both acetylcholine and flow has been used to assess endothelial cell function in vivo. Endothelial cell function is impaired in patients with atherosclerosis and could antecede the development of overt evidence of the disease. Cigarette smoke augments leucocyte–endothelial interactions, thought to be relevant to the early stages of atherogenesis ex vivo and in vitro,\(^80,81\) and this effect is neutralized by vitamin C.\(^81\) Congruently, endothelial cell function is also impaired by passive exposure to cigarette
smoke⁶² and in apparently healthy chronic smokers, in whom the defect is largely corrected with vitamin C.⁸¹ Interestingly, vitamin C, but not vitamin E, depresses elevated urinary iPs in smokers.⁷⁸ Exposure to secondhand smoke also impairs acutely coronary endothelial function,⁸⁴ and acute smoking is associated with a rapid depletion of endogenous nitrate and nitrite, reflecting NO generation and endogenous antioxidants.⁸⁵

Oxidant stress has also been documented in patients exhibiting other risk factors for cardiovascular disease. Thus, urinary iPs are increased in patients with hyperhomocysteinemia,⁶⁶ diabetes mellitus,⁶⁷,⁸⁸ alcohol,⁸⁹ lupus anticoagulant,⁹⁰ and renovascular hypertension.⁹¹ Elevated levels of immunoreactive iPF₂-III have been reported in pericardial fluid of patients with heart failure⁹², and some iPs, but not all, are increased in the urine of patients with severe heart failure.⁹³ Recently, elevated immunoreactive iPF₂-III was described in conjunction with evidence of platelet activation in women with android obesity. Both parameters fell on weight loss,⁹⁴ raising the possibility of a role for peroxidation products in platelet activation.⁷⁹

Urinary iPs confirm, in patients, the oxidative burst that accompanies reperfusion after a period of ischemia. Thus, urinary iPs increase coincident with reperfusion in patients subject to coronary angioplasty or therapeutic thrombolysis.⁹⁵ This accords also with evidence obtained using electron spin resonance, spin-trapping radicals ex vivo.⁹⁶ The ischemic episodes in patients with unstable angina also are characterized by increased lipid peroxidation.⁵⁷

Clinical Trials of Antioxidants
The association of diverse risk factors for cardiovascular disease with elevated indices of oxidant stress in vivo would seem to indicate the likely importance of this mechanism in clinical cardiology. We have recounted the emergence of evidence, obtained with credible methodology for increased oxidant stress, in a range of cardiovascular diseases. Furthermore, proof of principle of the efficacy of antioxidants, or of interfering with systems that generate ROS, has been obtained in animal models of atherogenesis, atherosclerosis regression, and reperfusion injury. Yet most,⁹⁸–¹⁰¹ but not all,¹⁰²–¹⁰⁵ clinical trials of antioxidants have reached disappointing conclusions. How can this be so?

Inappropriate End Points
First, it is possible that oxidant stress, while elevated, is a marginal player in the end points (usually myocardial infarction, persistent angina, stroke, and vascular death) measured in clinical trials. It is possible that ROS generation is more relevant to initiation of the process, rather than its culmination.¹⁰⁶ Most trials are initiated when atherosclerosis is established. Adjuvant therapies can also diminish the opportunity for antioxidants to express their efficacy. As discussed previously, the reduction in iP generation in patients with hypercholesterolemia with statins suggests that they, and perhaps other drugs, could confound the assessment of antioxidants in hyperlipidemic individuals. Interestingly, there is also some preliminary evidence to suggest that concomitant vitamins could diminish the benefits from statins, although this has been disputed.¹⁰⁷

Inappropriate Patients
It is also possible that the patients included in these trials were inappropriate for testing the hypothesis. Thus, no clinical trials have been performed in which patient inclusion was based on biochemical evidence of elevated ROS generation. In other words, patients who do not have increased oxidative stress would not be expected to benefit from antioxidant therapy. The efficacy of exogenous antioxidants in vitro systems is highly conditioned by the degree of depletion of the diverse repertoire of endogenous antioxidant defenses. Meagher and colleagues¹⁰⁸ conducted a double-blind, placebo-controlled evaluation of a range of doses of vitamin E (200 to 2000 IU/day) in healthy individuals with intact endogenous antioxidant defense and normal levels of urinary iPF₂-III and iPF₂-IV and of 4-hydroxynonenal, all measured by mass spectrometry. Although these doses were administered to achieve steady state and embraced the dose range most commonly used in clinical trials (400 to 800 IU/day), they had no impact on the indices of lipid peroxidation. Patrignani and colleagues¹⁰⁹ obtained congruent results. They found that urinary immunoreactive iPF₂-III was not altered by administration of vitamin E at 300, 600, or 1200 IU per day. Furthermore, they related inversely the degree of suppression of the iP by vitamin E to the magnitude of the elevation of iP generation in different conditions. Thus, inclusion of patients who did not exhibit evidence of oxidant stress in vivo could have diluted the population susceptible to benefit and undermined the sample size calculations for the clinical trials.

Ineffective Antioxidants
Remarkably little progress has been reported on the development of novel antioxidants. Most trials have been conducted with vitamins E and C. However, both can exert pro-oxidant effects in vitro,¹¹⁰,¹¹¹ and both are incompletely effective at suppressing elevated levels of biomarkers in humans.⁷⁵,⁸⁹ Furthermore, the rate constant for the reaction of vitamin E with O₂⁻ is 5 orders of magnitude slower than the rate of reaction of O₂⁻ with endogenous antioxidant enzymes and molecules such as SOD and nitric oxide.¹¹² Because oral intake of vitamin E only modestly increases its plasma and tissue levels,¹⁰² this slow rate of reaction with ROS means that vitamin E, at the concentrations reached in tissue, is unlikely to affect biological outcomes. Rational evaluation of combination¹¹⁴ and novel¹¹³ antioxidants therapies seems desirable. Although proof of principle in animal models has been obtained with these and other antioxidants, it is important to remember that much higher doses of these vitamins have been used in model systems than are administered in clinical trials. Finally, it is possible that administered antioxidants could fail to intercept radical generation from discrete sources, especially those that occur in the cytoplasm, nucleus, and interstitial space. For example, myeloperoxidase is emerging as a major source of ROS generation in inflammatory syndromes.¹¹⁵ However, myeloperoxidase-catalyzed lipid peroxidation is resistant to inhibition by vitamin E.¹¹⁶

The Future
The “oxidative modification hypothesis”¹¹⁷ remains to be tested definitively. Negative trials of the cardioprotective
effect of aspirin in inappropriate populations and underpowered trials of statins long delayed appreciation of the efficacy of these 2 mainstays of cardiovascular therapy. Novel biomarkers have emerged but, for the most part, they remain complex and expensive to perform. The development of simplified assays, applicable to high-throughput analysis, is urgently needed. Assessment of the oxidative status of the patient and the true efficacy of antioxidant therapies can then be made so that the effective therapy is directed to the appropriate patient population. Refinement of clinical trial design to incorporate such indices would seem necessary to ensure recruitment of appropriate patients and to identify and subject novel, efficient antioxidant dosing regimens to controlled analysis. In the interim, pessimism and rejection of the hypothesis would seem premature.

The best lack all conviction
While the worst are full of passionate intensity
Surely some revelation is at hand.

William Butler Yeats, The Second Coming

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