Abstract—There is compelling evidence that oxidative stress plays a key role in the pathophysiology of several major cardiovascular diseases. In atherosclerosis, hypertension, stroke, diabetes, and heart failure, expression of superoxide is increased in blood vessels, and endothelial vasomotor function is impaired, presumably caused in large part by inactivation of nitric oxide by superoxide. Endothelial dysfunction is predictive of cardiovascular risk, and probably plays a key role in the pathophysiology of atherosclerosis and its complications. In preliminary studies in hypercholesterolemic mice and in older humans, we have found high levels of superoxide in the aortic valve, as well as aorta. We speculate that superoxide, in addition to playing a key role in atherogenesis, may play a key role in signaling that leads to calcific aortic valvular stenosis. Antioxidant enzymes, especially the three isoforms of superoxide dismutase (SOD), modulate basal levels of superoxide and protect against vasomotor dysfunction. A common gene variant of extracellular SOD (ecSOD) is associated with increased risk of ischemic heart disease. We have made recombinant adenoviruses to examine cardiovascular effects of ecSOD and its heparin-binding domain. This approach might be used to study the almost 500 other proteins with a heparin-binding domain. Finally, several key unanswered questions in relation to oxidative stress and atherosclerosis are raised, and proposed as fruitful areas of research. (Arterioscler Thromb Vasc Biol. 2006;26:689-695.)

Key Words: endothelial function □ atherosclerosis □ oxidative stress □ antioxidant mechanisms

One of the hottest topics in vascular biology is the role of reactive oxygen species in signaling4 and in pathophysiology.7 Oxidative stress is associated with several cardiovascular diseases, including atherosclerosis, hypertension, heart failure, stroke, and diabetes.3–5 Oxidation of low density lipoprotein (LDL) appears to play a key role in the pathogenesis of atherosclerosis.6 Oxidative stress also plays a key role in endothelial dysfunction associated with these diseases, as superoxide (O₂⁻) inactivates nitric oxide (NO) and thereby produces endothelial dysfunction.7,8 Because endothelial dysfunction is associated with, and may contribute to, an increased risk of cardiovascular events,9,10 oxidative stress may be causally related to, as well as associated with, cardiovascular events.

There is a paradox, however, in relation to oxidative stress and cardiovascular disease. Although there is compelling evidence in both humans and experimental animals for oxidative stress in a wide variety of vascular diseases and in risk factors for cardiovascular disease, stimuli that attenuate oxidant stress surprisingly are not consistently beneficial in humans and experimental animals.3,11 For example, in most studies, antioxidant vitamins fail to reduce cardiovascular events in humans. Furthermore, although one might expect that superoxide dismutases (SODs) might protect against atherosclerosis, mice that are deficient in extracellular SOD (ecSOD) do not have accelerated atherosclerosis,12 and over-expression of CuZnSOD does not protect against atherosclerosis.13

The goal of this review is to provide a perspective about: (1) the role of oxidative stress in some major cardiovascular diseases; (2) endogenous mechanisms that protect against oxidative stress; (3) examples of failure of endogenous antioxidant mechanisms that predispose to cardiovascular diseases; and (4) some very recent studies of oxidative stress in cardiovascular disease. The topic is enormously broad. I focus on a few studies from our laboratory and therefore apologize to the many authors who have made great contributions to this area of research but who do not always receive due credit in this review.

Structural and Functional Changes
I begin the story with a description of some structural and functional changes during atherosclerosis and regression of atherosclerotic lesions. Atherosclerotic lesions are character-
In the words of my teacher in medical school, Seymour Glagov, “Logic is not Truth.” One cannot rely on logic, Glagov said; one must perform experiments to discover the truth.

The study by Armstrong et al., which is a “citation classic,” inspired a generation of investigators to examine consequences of treatment of hypercholesterolemia in humans. A series of studies demonstrated in humans that treatment of hypercholesterolemia reduces cardiovascular events and leads to structural improvement in atherosclerotic vessels. These studies, and many intervention trials to treat hypercholesterolemia, have revolutionized our treatment of patients with hypercholesterolemia.

Evidence for structural improvement in vessels during regression of atherosclerosis was consistent, but modest in magnitude. In contrast to the modest improvement in structure, we and others found evidence for enormous, relatively rapid improvement in vasoconstriction function of arteries during regression of atherosclerosis (Figure 1). In monkeys, we found that regression of atherosclerosis produces great improvement in endothelial function and reduction of vasocostrictr hyperresponsiveness, with only limited resolution of structural changes. These findings in monkeys were confirmed in humans, as endothelial function improves relatively rapidly during treatment of hypercholesterolemia. We described this as improvement of the “sick vessel syndrome.”

An important question is whether responses to acetylcholine and other endothelium-dependent vasodilators should be viewed only as a tool to study vasoconstriction regulation, or whether responses to acetylcholine also are an index of other functions of endothelium. For example, is the anticoagulant role of endothelium impaired during atherogenesis and improved during regression, in parallel with changes in vasoconstrictor regulation?

As discussed previously, when a thrombus forms at sites of arterial injury, one might predict that the thrombotic process would propagate down the artery, unless a potent anticoagulant mechanism terminated propagation of the thrombus. In otherwise normal arteries, however, thrombosis is confined to the site of injury, in part because thrombin binds to thrombomodulin on intact endothelium downstream from the thrombus and activates protein C in blood. Activated protein C (APC) is a remarkably potent anticoagulant, which inhibits propagation of a thrombus. We found in atherosclerotic monkeys, however, that impairment of endothelial vasomotor function is accompanied by a defect in the thrombomodulin/protein C anticoagulant system. Furthermore, regression of atherosclerosis in monkeys is accompanied by improvement in both endothelial vasomotor function and the anticoagulant protein C system.

The studies of effects of atherosclerosis and regression on the thrombomodulin/protein C anticoagulant system are important because they may provide insight into one of the mechanisms by which atherosclerosis predisposes to arterial thrombosis, and insight into mechanisms by which treatment of hypercholesterolemia reduces cardiovascular events. The studies also imply that endothelial vasomotor function and endothelial antithrombotic function may change in parallel during atherosclerosis and regression. Mechanisms by which atherosclerosis impairs the endothelial thrombomodulin pathway, and the possible role of oxidative stress, are not yet clear.

**Superoxide and Endothelial Function**

What causes endothelial dysfunction in atherosclerosis? Expression of endothelial nitric oxide synthase (eNOS) apparently is normal or increased in atherosclerotic vessels, at least until atherosclerotic lesions are very advanced. The finding that eNOS expression is normal or increased suggests that generation of NO by endothelium is not reduced in atherosclerotic vessels, and does not account for impairment of endothelial dysfunction, if substrate and cofactors for eNOS are sufficient.

Classic studies demonstrated that superoxide can inactivate NO, and superoxide thereby produces endothelial dysfunction by reduction of NO bioavailability. It appears that levels of superoxide are increased in atherosclerotic vessels, and inactivation of NO by superoxide plays a key role in endothelial dysfunction of atherosclerotic arteries.

Many of us assumed that the primary source of superoxide in atherosclerotic arteries is inflammatory cells in the vessel wall. Thus, the finding that smooth muscle cells in the media of atherosclerotic arteries are an important source of superoxide was surprising and important. It is likely that NAD(P)H oxidase, possibly activated in part by angiotensin-II, is an important mechanism for generation of superoxide in smooth muscle cells in atherosclerotic vessels.
enzymes, however, also may be important sources of superoxide, including xanthine oxidase and “uncoupling” of the NO synthases, as NOS produces superoxide in addition to NO.

What accounts for striking and rapid improvement of endothelial function during regression of atherosclerosis? Improvement of endothelial function is accompanied by reduction of superoxide in arterial media, and decreased expression of NAD(P)H oxidase. In addition, the number of inflammatory cells in atherosclerotic arteries decreases during regression of atherosclerosis. During regression of atherosclerosis, it is of interest that reduction of inflammatory cells (perhaps by apoptosis) occurs primarily in intima, not adventitia, and inflammatory cells in adventitia persist.

Human monocytes contain angiotensin II, which is a potent stimulus for NAD(P)H oxidase. Macrophages in atherosclerotic lesions of monkeys also contain angiotensin II, and the number of macrophages that contain angiotensin II decreases during regression of atherosclerosis. It is likely that reduction of angiotensin II-containing macrophages, combined with decreased expression of NAD(P)H oxidase, diminish the generation of superoxide in atherosclerotic lesions during regression of atherosclerosis.

Effects of superoxide on NO-mediated vasomotor responses have been studied extensively during the past 2 decades, but a relatively new concept is that superoxide and other reactive oxygen species may modulate NO-independent relaxation in vascular muscle. Atherosclerosis impairs relaxation produced by activation of K+ channels, and superoxide can inhibit opening of K+ channels on vascular muscle cells directly, or by combining with NO and generating peroxynitrite. Because activation of K+ channels on vascular muscle produces hyperpolarization and relaxation, inhibitory effects of superoxide on K+ channels may predispose to vasoconstriction. It should be noted that other oxidative species may activate, as well as inhibit, several different K+ channels.

Oxidative Stress in Other Cardiovascular Diseases

Several groups have made key observations about the role of superoxide in hypertension. Superoxide is increased in the kidney in experimental hypertension, and appears to play a key role in elevation of arterial pressure. Delivery of SOD by liposomes to rats with angiotensin-induced hypertension improves endothelial function in aorta and reduces arterial pressure. We confirmed that superoxide is increased in arteries in spontaneously hypertensive rats (SHR) and, because gene transfer of ecSOD improved endothelial function and reduced blood pressure, suggested that the interaction of superoxide and NO occurs primarily in the extracellular space.

Diabetes also is associated with increased oxidative stress. Superoxide levels are increased in arteries from diabetic animals. Urinary isoprostanes increase in diabetic patients and decrease during treatment with vitamin E. Hyperglycemia impairs endothelium-dependent vasodilatation, and responses are restored by the antioxidant vitamin C. Several potential mechanisms may contribute to endothelial dysfunc-

tion in diabetes, including mitochondrial superoxide, activation of NAD(P)H oxidase, and depletion of tetrahydrobiopoterin with generation of superoxide from “uncoupling” of a nitric oxide synthase, as well as advanced glycation end products (AGEs).

Superoxide also is increased in patients and experimental animals with heart failure. Gene transfer of ecSOD improves endothelial function in experimental animals with heart failure. The findings of vascular oxidative stress in heart failure and improvement of endothelial function by ecSOD are important because endothelial dysfunction predicts survival in patients with heart failure.

Thus, in atherosclerosis, hypertension, diabetes, and heart failure: (1) expression of superoxide is increased in blood vessels; (2) endothelial modulation of vasomotor tone is impaired; and (3) antioxidants generally improve endothelial function in experimental animals.

Antioxidant Mechanisms

There are 3 isoforms of superoxide dismutase which catalyze the dismutation reaction from O2− (superoxide) to H2O2. Copper-zinc SOD (CuZnSOD) is localized primarily in cytosol, manganese SOD (MnSOD) in mitochondria, and extracellular SOD (ecSOD) in the extracellular space. CuZn-SOD and ecSOD are the predominant isoforms in blood vessels.

Although expression of MnSOD is much less than CuZn-SOD and ecSOD in blood vessels, MnSOD nevertheless plays a critical role in protection against mitochondrial damage during oxidative stress. MnSOD is upregulated by oxidative stress and transiently upregulated by atherosclerosis. In blood vessels from normal mice, we were unable to demonstrate an important role for MnSOD in protection against oxidative stress. MnSOD, however, protects against mitochondrial damage and development of atherosclerosis. Recently, we have made preliminary observations that Mn-SOD protects against endothelial dysfunction in apolipoprotein (apo)E-deficient mice. Thus, although expression and activity of MnSOD in blood vessels are relative low, localization of MnSOD in mitochondria plays a critical role in protection against oxidative stress.

CuZnSOD is the predominant isofrom of SOD in blood vessels. CuZnSOD appears to play a critical role in regulation of basal levels of O2− in blood vessels, and therefore of NO bioavailability and endothelial function. It is surprising, therefore, that oxidation of lipids and development of atherosclerosis are not attenuated in mice that overexpress CuZnSOD. The finding that overexpression of both CuZnSOD and catalase reduced isoprostane levels and slowed the development of atherosclerosis suggests that H2O2 may play an important role in atherogenesis.

ecSOD is the only isofrom of SOD that is released from cells and expressed in the extracellular space. ecSOD is synthesized in vascular muscle, but not endothelium, and is localized especially between smooth muscle and endothelium. A major function of ecSOD may be to protect NO, because it diffuses from endothelium to vascular muscle.

It is surprising that development and progression of atherosclerosis are not accelerated in ecSOD-deficient mice.
possibly because the study was underpowered.58 ecSOD decreases $O_2^-$ in blood vessels, however, and the enzymatic reaction also increases $H_2O_2$ (although ecSOD also may have peroxidase activity).59 Perhaps $H_2O_2$ plays a more important role than $O_2^-$ in the pathophysiology of structural changes in blood vessels during development of atherosclerosis.13 There is little question that superoxide, which inactivates NO, is of central importance in endothelial dysfunction in several vascular diseases. But, the SODs (which dismute $O_2^-$ to $H_2O_2$ and thereby improve endothelial function) may not attenuate structural changes, if $H_2O_2$ plays a key role.

Since its discovery by Marklund,60 ecSOD seems like the “forgotten” SOD, except by investigators who study blood vessels. Perhaps there is less focus on ecSOD because expression of ecSOD is very low in the liver, brain, and heart, where CuZnSOD and MnSOD (together with GPX and other antioxidant enzymes) are the major antioxidant enzymes. Levels of CuZnSOD are 50- to 1000-fold higher than levels of ecSOD in liver, brain, and heart.61,62 But, in contrast to low levels of expression of ecSOD in liver, brain, and heart, expression of ecSOD is comparable to CuZnSOD in arteries.63 Thus, based on high levels of expression in arteries, it seems likely that ecSOD plays an important protective role against oxidant stress in blood vessels.

The heparin-binding domain (HBD) of ecSOD accounts for binding of ecSOD to heparan sulfate proteoglycans on the external surface of cells (Figure 2).57,60,63 A recent important study reported that a common gene variant of ecSOD (ecSODR213G), which is carried by 3% to 6% of humans, is associated with increased risk of ischemic heart disease.58 The R213G gene variant occurs in the HBD of ecSOD. As described previously,64 enzymatic function of ecSOD is normal in the R213G variant. Because the enzyme fails to bind normally to the outer surface of endothelium and other cells (because of impaired binding by the HBD of ecSOD), serum levels of ecSOD protein and activity are increased in humans with the R213G gene variant.

The HBD of ecSOD is essential for normal function of ecSOD.43 In rats, gene transfer of ecSOD protects against endothelial dysfunction in hypertension,43 after endotoxin,65 and in heart failure,59 but gene transfer of ecSOD after deletion of the HBD fails to protect the endothelium.43,49,65 Recently, we made a recombinant adenovirus that expresses the R213G gene variant of ecSOD. ecSODR213G binds poorly to endothelium, and fails to protect against endothelial dysfunction in hypertension66 and, in preliminary studies in our laboratory, after endotoxin and in heart failure.67 Thus, the HBD of ecSOD is essential for normal function of ecSOD,43,49,65 and the R213G human gene variant in the HBD greatly impairs function of ecSOD.66,67

We speculate that the experimental approach that we have used to study vascular effects of gene transfer of ecSOD and ecSODR213G might be valuable in other studies. First, after gene transfer of ecSOD to liver, ecSOD protein is released into the circulation, and there is strong expression of ecSOD in renal glomeruli.43 Glomerular basement membrane has a strongly negative charge, and the positive charge of the HBD of ecSOD presumably accounts for binding to glomeruli. We speculate that ecSOD may be protective against oxidant stress in glomeruli, and may be useful in studies of (and perhaps therapy for) glomerular disease associated with oxidative stress.

Second, Henry Keen, Yi Chu, and Curt Sigmund searched for heparin-binding motifs in protein sequence databases with a computer script using the pattern-matching capabilities of the Perl programming language.68 They found 483 such proteins (excluding different isoforms of the same protein), although not all of the proteins have been demonstrated to bind heparin (Figure 3). We speculate that the approach that we have used to make a recombinant virus and study effects of gene transfer of ecSOD with and without the HBD43 and with a gene variant in the HBD66 may be useful for study of other proteins with an HBD. Thus, effects of deletion or alteration of the HBD of many proteins (Figure 3) might be examined using recombinant adenoviruses.

In relation to the homology search, 10 proteins have the atypical HBD found in ecSOD, and 473 have the canonical HBD. It is intriguing that, of the 10 characterized eukaryotic proteins (not prokaryotic or predicted proteins) with the 6
Amino acid HBD that is identical to ecSOD (RKKRRR basic amino acid sequence), 9 are nuclear proteins and 1 (ecSOD) is expressed extracellularly! It is surprising, and intriguing, that “extracellular” SOD has an HBD that is identical to several nuclear transcription factors.

**Aortic Valvular Stenosis**

A major cause of aortic valvular stenosis, in addition to congenital bicuspid valve and rheumatic heart disease, is “degenerative” calcific aortic stenosis. Nonrheumatic, tricuspid aortic valvular stenosis has many of the morphological characteristics of atherosclerosis, including lipid deposition, inflammation, and calcification. Furthermore, risk factors are similar for aortic valvular stenosis and atherosclerosis, including hypercholesterolemia, male gender, and smoking. Thus, it seems reasonable to suggest that the pathophysiology of aortic valvular stenosis and atherosclerosis may be similar.

Because oxidative stress plays a key role in the pathophysiology of atherosclerosis, we have postulated recently that superoxide also may play a role in the pathophysiology of “degenerative” calcific aortic valvular stenosis. We have found increased levels of superoxide in the aortic valve of apoE mice and hypercholesterolemia “Reversa” mice, and strong echocardiographic and hemodynamic evidence for severe aortic valvular stenosis in Reversa mice. Furthermore, levels of superoxide in the aortic valve appear to be much higher in humans older than 55 than in men younger than 25 (unpublished observations).

Based on these preliminary findings, we speculate that superoxide may play a key role in the pathophysiology of calcific aortic valvular stenosis. Many genes that regulate calcification of bone and blood vessels are redox-sensitive. We speculate that superoxide in the aortic valve may activate these genes and thereby contribute to calcification and valvular stenosis.

**Future Directions**

The role of oxidative stress in cardiovascular disease is an important area of research, which is likely to continue to be fruitful. Several directions of research may be especially exciting.

First, a key question is why oxidative mechanisms appear to be extremely important in pathophysiology of atherosclerosis and its risk factors, and yet antioxidant vitamins do not consistently reduce risk of cardiovascular disease. It seems likely that a better understanding of mechanisms by which oxidant injury predisposes to atherosclerosis may lead to design of approaches that will demonstrate efficacy of antioxidants in protection against cardiovascular disease in patients.

Second, with development of drugs that are extremely effective in reducing levels of low-density lipoprotein in humans, there have been great advances in understanding mechanisms and consequences of regression of atherosclerosis, including the role of oxidative stress. It is now clear that inhibitors of cholesteryl ester transfer protein (CETP) are remarkably effective in raising levels of high-density lipoproteins in humans. It is not yet clear, however, whether CETP inhibitors also reduce cardiovascular events in humans. If CETP inhibitors reduce the risk of cardiovascular events, it is likely that the inhibitors will be a useful approach to obtain better insight into mechanisms (including, perhaps, effects on oxidative stress) by which high-density lipoprotein modulates vascular function.

Third, rupture of the “vulnerable plaque,” with consequent thrombosis, is a major cause of arterial occlusion in atherosclerotic arteries. Superoxide activates matrix metalloproteinases, and oxidative stress is increased in plaques of patients with acute coronary syndrome. The role of oxidative stress in the vulnerable plaque, and in the pathophysiology of acute coronary syndrome, is an important area of research.

Fourth, most research in cardiovascular effects of oxidative stress has focused on NO-mediated endothelial vasomotor mechanisms. Some under-studied areas of research include the role of oxidative stress on endothelium-dependent, antithrombotic mechanisms, NO-independent vasomotor regulation (eg, effects on K+ channels), and calcification of the aortic valve.

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