

This MiniReview is part of a thematic series on **Oxidant Signaling in Cardiovascular Cells**, which includes the following articles:

NAD(P)H Oxidase: Role in Cardiovascular Biology and Disease

Oxidant Signaling in Vascular Cell Growth, Death, and Survival

Potential Antiatherogenic Mechanisms of Ascorbate (Vitamin C) and  $\alpha$ -Tocopherol (Vitamin E)

Endothelial Dysfunction in Cardiovascular Diseases: The Role of Oxidant Stress

Vascular Nitric Oxide and Eicosanoid Signaling

Vascular Oxygen Species Generation

David G. Harrison, Guest Editor

## Endothelial Dysfunction in Cardiovascular Diseases The Role of Oxidant Stress

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**Abstract**—Accumulating evidence suggests that oxidant stress alters many functions of the endothelium, including modulation of vasomotor tone. Inactivation of nitric oxide (NO<sup>•</sup>) by superoxide and other reactive oxygen species (ROS) seems to occur in conditions such as hypertension, hypercholesterolemia, diabetes, and cigarette smoking. Loss of NO<sup>•</sup> associated with these traditional risk factors may in part explain why they predispose to atherosclerosis. Among many enzymatic systems that are capable of producing ROS, xanthine oxidase, NADH/NADPH oxidase, and uncoupled endothelial nitric oxide synthase have been extensively studied in vascular cells. As the role of these various enzyme sources of ROS become clear, it will perhaps be possible to use more specific therapies to prevent their production and ultimately correct endothelial dysfunction. (*Circ Res.* 2000;87:840-844.)

**Key Words:** superoxide ■ nitric oxide ■ endothelium ■ NADH/NADPH oxidase ■ xanthine oxidase

Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives produced in all aerobic cells. Excessive production of ROS, outstripping endogenous antioxidant defense mechanisms, has been implicated in processes in which they oxidize biological macromolecules, such as DNA, protein, carbohydrates, and lipids. This condition has commonly been referred to as oxidant stress. An increasing body of evidence suggests that oxidant stress is involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure. In this review, mechanisms by which oxidant stress affects vascular function and ultimately contributes to vascular disease will be discussed.

### Chemical Characteristics of Reactive Oxygen Species

Many ROS possess unpaired electrons and thus are free radicals. These include molecules such as superoxide anion

(O<sub>2</sub><sup>•-</sup>), hydroxyl radical (HO<sup>•</sup>), nitric oxide (NO<sup>•</sup>), and lipid radicals. Other reactive oxygen species, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), peroxynitrite (ONOO<sup>-</sup>), and hypochlorous acid (HOCl), are not free radicals per se but have oxidizing effects that contribute to oxidant stress. The cellular production of one ROS may lead to the production of several others via radical chain reactions. For example, reactions between radicals and polyunsaturated fatty acids within cell membrane may result in a fatty acid peroxy radical (R-COO<sup>•</sup>) that can attack adjacent fatty acid side chains and initiate production of other lipid radicals. Lipid radicals produced in this chain reaction accumulate in the cell membrane and may have a myriad of effects on cellular function, including leakage of the plasmalemma and dysfunction of membrane-bound receptors. Of note, end products of lipid peroxidation, including unsaturated aldehydes and other metabolites, have cytotoxic and mutagenic properties.<sup>1</sup>

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## Endothelial Dysfunction

The term endothelial dysfunction has been used to refer to several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling.<sup>2</sup> However, in much of the literature this term has been used to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide (NO) bioactivity in the vessel wall. Several human studies have shown that traditional risk factors for atherosclerosis predispose to endothelial dysfunction. This form of endothelial dysfunction is not merely a laboratory curiosity. Impaired endothelium-dependent vasodilation in the coronary circulation of humans has profound prognostic implications in that it predicts adverse cardiovascular events and long-term outcome.<sup>3</sup>

A decline in NO bioavailability may be caused by decreased expression of the endothelial cell NO synthase (eNOS),<sup>4</sup> a lack of substrate or cofactors for eNOS,<sup>5</sup> alterations of cellular signaling such that eNOS is not appropriately activated,<sup>6</sup> and, finally, accelerated NO degradation by ROS.<sup>7</sup>

Even before it was known to be NO, early studies showed that the endothelium-derived relaxing factor (EDRF) could be inactivated by  $O_2^{\cdot-}$  and stabilized by superoxide dismutase (SOD).<sup>8</sup> Now that EDRF is known to be nitric oxide, this chemistry is much better understood. The interaction between NO and  $O_2^{\cdot-}$  occurs at an extremely rapid rate of  $6.7 \times 10^9$  mol/L<sup>-1</sup> · s<sup>-1</sup>.<sup>9</sup> This is 3 times faster than the reaction rate for  $O_2^{\cdot-}$  with SOD. Given this rapid reaction rate, there is likely always some  $O_2^{\cdot-}$  reacting with NO within cells and in the extracellular space. Under physiological conditions, endogenous antioxidant defenses minimize this interaction and maintain what seems to be a tenuous balance between  $O_2^{\cdot-}$  and NO.

This tenuous balance seems to be altered in a variety of common disease states. One of the first examples of this came from studies of hypercholesterolemic rabbits. These animals have severely impaired endothelium-dependent vascular relaxation, suggesting a lack of NO. Paradoxically, the production of total nitrogen oxides (NO and oxidation products of NO) was increased by as much as 3-fold in these vessels. Furthermore, nitrogen oxide production increased appropriately on stimulation with either acetylcholine or the calcium ionophore A23187, suggesting that signaling pathways leading to eNOS activation were intact in these vessels.<sup>10</sup> These findings led to the speculation that hypercholesterolemia could result in oxidation of NO to vaso-inactive nitrogen oxides (such as nitrite and nitrate). Subsequently, it was shown that treatment of cholesterol-fed rabbits with polyethylene-glycolated-SOD could markedly enhance endothelium-dependent vascular relaxation but have no effect in normocholesterolemic animals.<sup>11</sup> This observation strongly supported the concept that in hypercholesterolemia, nitric oxide bioavailability is reduced by  $O_2^{\cdot-}$ .

Subsequently, altered endothelium-dependent vascular relaxation has been associated with enhanced degradation of NO by ROS in animal models of many different diseases. These include hypertension, diabetes, cigarette smoking, and

heart failure.<sup>12-15</sup> These studies have been extended to humans. Antioxidant vitamins have been shown to enhance endothelium-dependent vasodilation in both the coronary and forearm circulations in subjects with many of the same diseases examined in animal models.<sup>16-18</sup>

Superoxide is probably not the only radical that can react with NO. Lipid radicals (LO<sup>·</sup> and LOO<sup>·</sup>) can react with NO to form, respectively, LONO and LOONO.<sup>19</sup> It is of interest that oxidized LDL, but not native LDL, added to isolated vessels inhibits endothelium-dependent vascular relaxation.<sup>20</sup> The oxidation of LDL leads to production of linoleic hydroperoxy and alkoxy radicals that could participate in such reactions with NO. Recently, it has been shown that hydroxyl radical may react with NO.<sup>21</sup>

## Sources of ROS in Vascular Cells

In mammalian cells, potential enzymatic sources of ROS include the mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, cytochrome p450s, xanthine oxidase, NADH/NADPH oxidases, NO synthase, peroxidases, and other hemoproteins. Although many of these sources could potentially produce ROS that inactivate NO, 3 have been studied rather extensively in cardiovascular system. These include xanthine oxidase, NADH/NADPH oxidase, and NO synthase. These will be discussed separately below.

## Xanthine Oxidase

The xanthine oxidoreductase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. Xanthine oxidoreductase can exist in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. The former reduces NAD<sup>+</sup>, whereas the latter prefers molecular oxygen, leading to the production of both  $O_2^{\cdot-}$  and H<sub>2</sub>O<sub>2</sub>. In endothelial cells, the activity and expression of xanthine oxidase is enhanced by interferon- $\gamma$ .<sup>22</sup> The first suggestion that  $O_2^{\cdot-}$  derived from xanthine oxidase might alter NO bioavailability came from studies of spontaneously hypertensive rats (SHRs). In these animals, a recombinant form of SOD modified to bind to heparin-binding sites dramatically lowered blood pressure but had no effect on blood pressure in nonhypertensive rats. In these same animals, the xanthine oxidase inhibitor oxypurinol also lowered blood pressure, strongly suggesting that xanthine oxidase played a role in this process.<sup>23</sup> There is also evidence that free radical production is increased in the microcirculation of SHRs and that this can be prevented by a xanthine oxidase inhibitor.<sup>24</sup> Previous studies have shown that early stages of experimental atherosclerosis caused by diet-induced hypercholesterolemia are associated with increased  $O_2^{\cdot-}$ , presumably from xanthine oxidase, because  $O_2^{\cdot-}$  production in this setting can be normalized by oxypurinol.<sup>25</sup> In humans with hypercholesterolemia, administration of oxypurinol, an inhibitor for xanthine oxidase-mediated  $O_2^{\cdot-}$  production, improved impaired vasodilation in hypercholesterolemic patients.<sup>26</sup>

Recent work has shown that xanthine oxidase may exist in a molybdenum-deficient form. In this state, the enzyme is unable to use xanthine as a substrate and is not inhibited by

oxypurinol but can use NADH as an electron donor to form  $O_2^{\cdot-}$ .<sup>27</sup> Using classical assays of homogenates of tissues, one could easily conclude that such a form of xanthine oxidase is an NADH oxidase (see below). There are some technical difficulties for enabling additional studies of the enzyme. A sufficiently sensitive assay of xanthine oxidase enzyme activity that would permit identification of the enzyme in small amounts of tissue has not been successfully developed. A widely available specific antibody against the enzyme that could facilitate studies of enzyme expression is also not available. These problems have prevented an in-depth understanding of the role of xanthine oxidoreductase in endothelial dysfunction.

### NADH/NADPH Oxidase

In several studies, investigators have attempted to define the source of ROS using homogenates of either vascular cells or tissues. In such experiments, the relevant enzymes are characterized on the basis of their substrate preference and, in some cases, specific inhibitors. When homogenates of endothelial and vascular smooth muscle cells have been studied in this fashion, the predominant substrate capable of driving  $O_2^{\cdot-}$  production has been NADH and, to a lesser extent, NADPH, no matter what detection system has been used. In fact, in these studies, there is little evidence that other enzyme systems, such as xanthine oxidase, cyclooxygenase, or cytochrome p450, serve as important sources of  $O_2^{\cdot-}$ . A caveat with these studies is that the assay systems may not have been optimum for demonstrating  $O_2^{\cdot-}$  production from some of these other sources. Nevertheless, on the basis of such studies, it has been proposed that the predominant superoxide-producing enzyme is an NADH/NADPH oxidase. The structure and function of these enzymes has been the subject of recent reviews,<sup>28</sup> including one in this series of MiniReviews. Importantly, the activity of the vascular NADH/NADPH oxidase is regulated by cytokines, hormones, and mechanical forces that are known to be involved in the pathogenesis of vascular disease. Stimulation of vascular smooth muscle cells with angiotensin II, thrombin, platelet-derived growth factor, tumor growth factor- $\alpha$ , and lactosylceramide all increase activity of the vascular ROS formation and NADH/NADPH oxidase activity.<sup>29–33</sup> Exposure of human umbilical endothelial cells to 5 or 20 dyne/cm<sup>2</sup> unidirectional laminar shear stress resulted in a transient elevation in NADH-dependent  $O_2^{\cdot-}$  formation, whereas oscillatory shear caused a sustained increase in oxidase activity.<sup>34</sup>

Several studies have demonstrated a critical role of NADH/NADPH oxidase in angiotensin II-induced hypertension. In cultured rat vascular smooth muscle cells, angiotensin II is able to stimulate  $O_2^{\cdot-}$  generation by increasing the activity of NADH/NADPH oxidase.<sup>31</sup> Similarly, in rats made hypertensive by chronic angiotensin II infusion, vascular  $O_2^{\cdot-}$  production is dramatically increased, as is NADH/NADPH oxidase activity.<sup>35</sup> Blood pressure and vascular reactivity are restored by exogenous liposome-encapsulated SOD in those rats.<sup>36</sup> Additional studies have shown that the mRNA expression of p22phox is increased in angiotensin II-induced hypertension.<sup>37</sup> Accumulating evidence suggests that the NADH/NADPH oxidase may be responsible for excessive  $O_2^{\cdot-}$

generation in other cardiovascular diseases. Both basal and NADH-stimulated  $O_2^{\cdot-}$  production is significantly elevated in rats with heart failure secondary to chronic myocardial infarction.<sup>38</sup> Treatment with SOD improved endothelium-dependent vasorelaxation markedly in those rats.<sup>38</sup> Zalba et al<sup>39</sup> recently reported that NADH/NADPH oxidase-derived  $O_2^{\cdot-}$  production is increased in SHR. In segments of human saphenous veins obtained from patients undergoing routine coronary artery bypass surgery, Guzik et al<sup>40</sup> reported that both diabetes and hypercholesterolemia are associated with increased NADH-dependent  $O_2^{\cdot-}$  production.

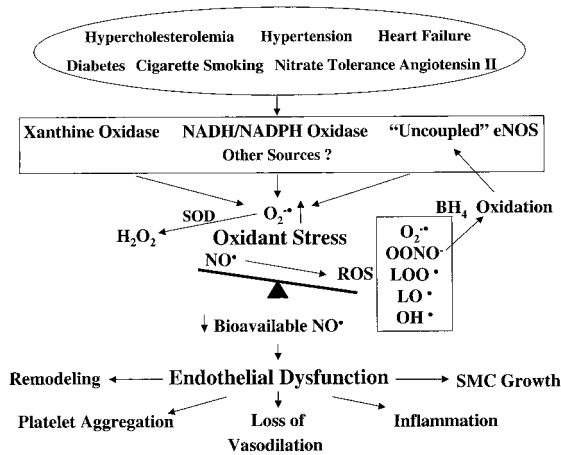
There remain several questions about the NADH/NADPH oxidases of vascular tissues. The subunits of these enzymes have not been identified precisely, and how they interact is not understood. The precise manner in which their activity is modulated is not well understood. Nevertheless, the prevailing evidence suggests that activation of this source of  $O_2^{\cdot-}$  can lead to endothelial dysfunction by reducing NO<sup>•</sup> bioavailability. This phenomenon likely plays an important role in the genesis of vascular disease in several pathophysiological conditions.

### Endothelial Nitric Oxide Synthase

A third source of vascular ROS production that has received substantial attention is eNOS. eNOS is a cytochrome p450 reductase-like enzyme that catalyzes flavin-mediated electron transport from the electron donor NADPH to a prosthetic heme group. The enzyme requires tetrahydrobiopterin, bound near this heme group, to transfer electrons to a guanidino nitrogen of L-arginine to form nitric oxide. In the absence of either L-arginine or tetrahydrobiopterin (BH<sub>4</sub>), eNOS can produce  $O_2^{\cdot-}$  and H<sub>2</sub>O<sub>2</sub>. This phenomenon has been referred to as NOS uncoupling. There have been several demonstrations of this phenomenon in studies of the purified enzyme.<sup>5,41,42</sup>

During the last 2 to 3 years, there has also been evidence presented that eNOS can become uncoupled in vivo in a variety of pathophysiological conditions. In the aorta of stroke-prone spontaneously hypertensive rat,  $O_2^{\cdot-}$  production is increased, and this can be normalized by treatment with L-NAME or removal of the endothelium.<sup>43</sup> Preliminary studies in our group have suggested that  $O_2^{\cdot-}$  produced in aortas of mice with deoxycorticosterone acetate-salt hypertension may come from eNOS, because it is attenuated by L-NAME treatment and endothelium removal and does not occur in eNOS-deficient mice with deoxycorticosterone acetate-salt hypertension. Recently, nitrate tolerance has been associated with an increase in vascular  $O_2^{\cdot-}$  production via uncoupling of eNOS.<sup>44</sup> Impaired endothelium-dependent vasorelaxation has been observed in rats made insulin resistant by high-fructose feeding and has been normalized by supplement with BH<sub>4</sub>.<sup>45</sup> Intra-arterial infusion of BH<sub>4</sub> has been shown to improve endothelium-dependent vasodilation in chronic smokers, suggesting that depletion of BH<sub>4</sub> may have impact on turning eNOS into a  $O_2^{\cdot-}$ -generating enzyme in human.<sup>46</sup>

The mechanisms whereby eNOS can become uncoupled in vivo remain unclear. Recent studies from our laboratory have suggested that peroxynitrite, the product of the reaction between NO<sup>•</sup> and  $O_2^{\cdot-}$ , can oxidize BH<sub>4</sub> and that this may lead



Mechanisms for oxidant stress-induced endothelial dysfunction in cardiovascular diseases.

to uncoupling of eNOS in vivo.<sup>47</sup> BH<sub>4</sub> biosynthesis is carried out via an enzymatic pathway involving GTP cyclohydrolase I (GTP-CH), 6-pyruvoyl-tetrahydropterin synthase, and sepiapterin reductase. Mutations in GTP-CH, the first-step enzyme catalyzes the biosynthesis of BH<sub>4</sub>, have been shown to cause deficiency in BH<sub>4</sub>.<sup>48</sup> In GTP-CH-deficient (hph-1) mice, NO/cGMP production is reduced in brain, and this can be corrected by peripheral administration of BH<sub>4</sub>.<sup>48</sup>

Uncoupling of eNOS in the endothelium may lead to oxidative stress and endothelial dysfunction via at least 3 mechanisms. First, the enzymatic production of NO<sup>•</sup> is diminished, allowing the radicals that it normally might react with to attack other cellular targets. Second, the enzyme begins to produce O<sub>2</sub><sup>•-</sup>, contributing to oxidative stress. Finally, it is likely that eNOS can become partially uncoupled, such that both O<sub>2</sub><sup>•-</sup> and NO<sup>•</sup> are produced simultaneously. Under this circumstance, eNOS may become a peroxynitrite generator, leading to a dramatic increase in oxidative stress.

### Summary

During the last 2 decades, a large body of evidence has suggested that endothelial dysfunction may be caused by accelerated inactivation of NO<sup>•</sup> by reactive oxygen species. This phenomenon has been implicated in many pathophysiological conditions, including hypercholesterolemia, atherosclerosis, cigarette smoking, hypertension, diabetes, and heart failure. As summarized in the Figure schematically, 3 major enzyme systems, namely xanthine oxidase, NADH/NADPH oxidase, and eNOS, are likely enzymatic sources contributing to increased production of reactive oxygen species in these various pathophysiological states. It is likely that other enzyme systems also contribute to radical production in the vascular wall. Recent preliminary studies have indicated that cytochrome p450, a probable source of the so-called endothelium-derived hyperpolarizing factor, may also be an important source of superoxide and contribute to inactivation of NO<sup>•</sup> (Dr Rudi Büsse, personal communication, August 2000). As the role of these various enzyme sources of ROS become clear, it may be possible to use more specific therapies to

prevent their production and ultimately prevent endothelial dysfunction.

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