

Oxidative Stress and Cardiovascular Injury Part I: Basic Mechanisms and In Vivo Monitoring of ROS

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Oxidative stress has been associated with diverse pathophysiological events, including cancer, renal disease, and neurodegeneration. More recently, it has become apparent that reactive oxygen species (ROS) also play a role in the development of vasculopathies, including those that define atherosclerosis, hypertension, and restenosis after angioplasty. The "response to injury" hypothesis developed by Russell Ross in the late 1970s suggested that atherosclerosis, at least, resulted from an initial injury to endothelial cells, leading to impaired endothelial function and subsequent macrophage infiltration and smooth muscle dysfunction. Many investigators then focused on oxidation of LDL and its interaction with the endothelium as the initial injury leading to the formation of fatty streaks and ultimately atherogenesis. It is now clear not only that diverse ROS are produced in the vessel wall, but that they individually and in combination contribute to many of the abnormalities associated with vascular disease.

Reactive Oxygen Species

There are many ROS that play central roles in vascular physiology (Figure 1) and pathophysiology, the most important of which are nitric oxide (NO[•]), superoxide (O₂^{•-}), hydrogen peroxide (H₂O₂) and peroxynitrite (ONOO⁻). NO[•] is normally produced by endothelial nitric oxide synthase (eNOS) in the vasculature, but in inflammatory states, inducible NOS can be expressed in macrophages and smooth muscle cells and contributes to NO[•] production. NO[•] is a crucial mediator of endothelium-dependent vasodilation and may also play a role in platelet aggregation and in maintaining the balance between smooth muscle cell growth and differentiation. Superoxide results from one electron reduction of oxygen by a variety of oxidases (Figure 2). When O₂^{•-} is produced in concert with NO[•], they rapidly react to form the highly reactive molecule ONOO⁻. ONOO⁻ is an important mediator of lipid peroxidation and protein nitration, including oxidation of LDL, which has dramatic proatherogenic effects. In the absence of immediately accessible NO[•], O₂^{•-} is rapidly dismutated to the more stable ROS, H₂O₂, by superoxide dismutase, which is then converted to H₂O by either catalase or glutathione peroxidase. The effects of O₂^{•-}

and H₂O₂ on vascular function depend critically on the amounts produced. When formed in low amounts intracellularly, they can act as intracellular second messengers, modulating the function of biochemical pathways mediating such responses as growth of vascular smooth muscle cells (VSMCs) and fibroblasts. Higher amounts of ROS can cause DNA damage, significant toxicity, or even apoptosis, as demonstrated in endothelial cells¹ and smooth muscle cells.²

Cellular and Enzymatic Sources of ROS in the Vessel Wall

Each of these ROS derives from specific enzymatic or chemical reactions. NO[•] is produced in endothelial cells by activation of eNOS during the normal functioning of the vessel wall. Vasodilator hormones raise intracellular Ca²⁺, leading to an increase in eNOS activity and NO[•] release. Physical forces, such as fluid shear stress, activate eNOS via protein kinase A- or Akt-dependent phosphorylation. Pathophysiological expression of inducible NOS in both macrophages and VSMCs elevates cytokine levels, resulting in localized inflammation. This, in turn, results in production of NO[•] in the absence of further stimuli. Moreover, under some circumstances, eNOS becomes uncoupled and O₂^{•-} is made rather than NO[•].³ The NOS enzymes are thus potentially important sources of both NO[•] and O₂^{•-}, depending on the surrounding environment.

Virtually all types of vascular cells produce O₂^{•-} and H₂O₂.⁴ In addition to mitochondrial sources of ROS, O₂^{•-} and/or H₂O₂ can be made by many enzymes (Figure 2). Two of the most important sources in the normal vessel are thought to be cytochrome P450 and the membrane-associated NAD(P)H oxidase(s).^{5,6} A cytochrome P450 isozyme homologous to CYP 2C9 has been identified in coronary arteries and has been shown to produce O₂^{•-} in response to bradykinin.⁷ NAD(P)H oxidases that are similar in structure to the neutrophil respiratory burst NADPH oxidase, but produce less O₂^{•-} for a longer time, have been identified in vascular cells. The endothelial, VSMC, and fibroblast enzymes are not identical but have unique subunit structures and mechanisms of regulation.⁴ One important aspect of ROS production by at least the VSMC NAD(P)H oxidase is that it occurs largely

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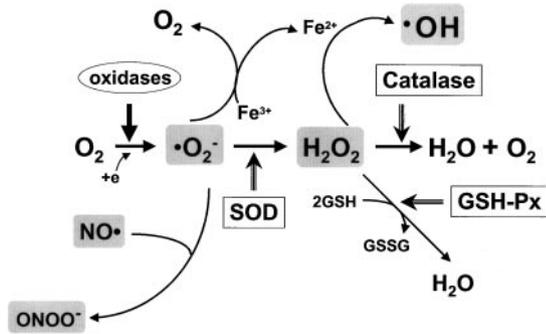


Figure 1. Vascular ROS. Highlighted in gray are some of the most important ROS in vascular cells. Oxidases convert oxygen to $O_2^{\cdot-}$, which is then dismutated to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be converted to H_2O by catalase or glutathione peroxidase (GSH-Px) or to hydroxyl radical ($\cdot OH$) after reaction with Fe^{2+} . In addition, $O_2^{\cdot-}$ reacts rapidly with nitric oxide (NO) to form peroxynitrite ($ONOO^-$).

intracellularly, making it ideally suited to modify signaling pathways and gene expression.

The activity of the NAD(P)H oxidases can be modulated by vasoactive hormones and the small molecular weight G-protein rac-1 (for review, see Griendling et al⁸). Angiotensin II, tumor necrosis factor- α , thrombin, and platelet-derived growth factor all increase oxidase activity and raise intracellular levels of $O_2^{\cdot-}$ and H_2O_2 in VSMCs. Angiotensin II and lactosylceramide activate the endothelial cell enzyme, whereas fibroblasts increase $O_2^{\cdot-}$ production in response to angiotensin II, tumor necrosis factor- α , interleukin-1, and platelet-activating factor. Physical forces, including cell

stretch, laminar shear stress, and the disturbed oscillatory flow that occurs at branch points, are also potent activators of $O_2^{\cdot-}$ production in endothelial cells. There are two major mechanisms by which hormones and physical forces activate the NAD(P)H oxidase: (1) acutely, whereby expressed enzyme is activated by phosphorylation, GTPase activity, and production of relevant lipid second messengers⁹; and (2) chronically, when expression of rate-limiting subunits of the enzyme is induced, thereby providing higher levels of enzyme susceptible to activation.¹⁰

Macrophages are perhaps the major vascular source of $O_2^{\cdot-}$ in disease states. They oxidize LDL via activation of diverse enzymes. Neutrophils and monocytes may also secrete myeloperoxidase, which appears to initiate lipid peroxidation.¹¹ Two potential diffusible candidates to initiate myeloperoxidase-dependent lipid peroxidation are tyrosyl radical and nitrogen dioxide (NO_2). Deletion of myeloperoxidase reduces markedly the formation of F_2 -isoprostanes (F_2iPs), quantitative indices of lipid peroxidation in vivo (vide infra) in an experimental model of peritonitis.¹²

Biochemical Consequences of ROS Production in the Vessel Wall

As noted above, ROS are involved in some of the most fundamental functions of the vessel wall. NO is a critically important mediator of endothelium-dependent vasodilation, whereas $O_2^{\cdot-}$ and H_2O_2 mediate VSMC growth, differentiation, and apoptosis. The lipid peroxidation and protein nitration induced by $ONOO^-$ are some of the earliest atherogenic events. Because macrophages release ROS extracellularly,

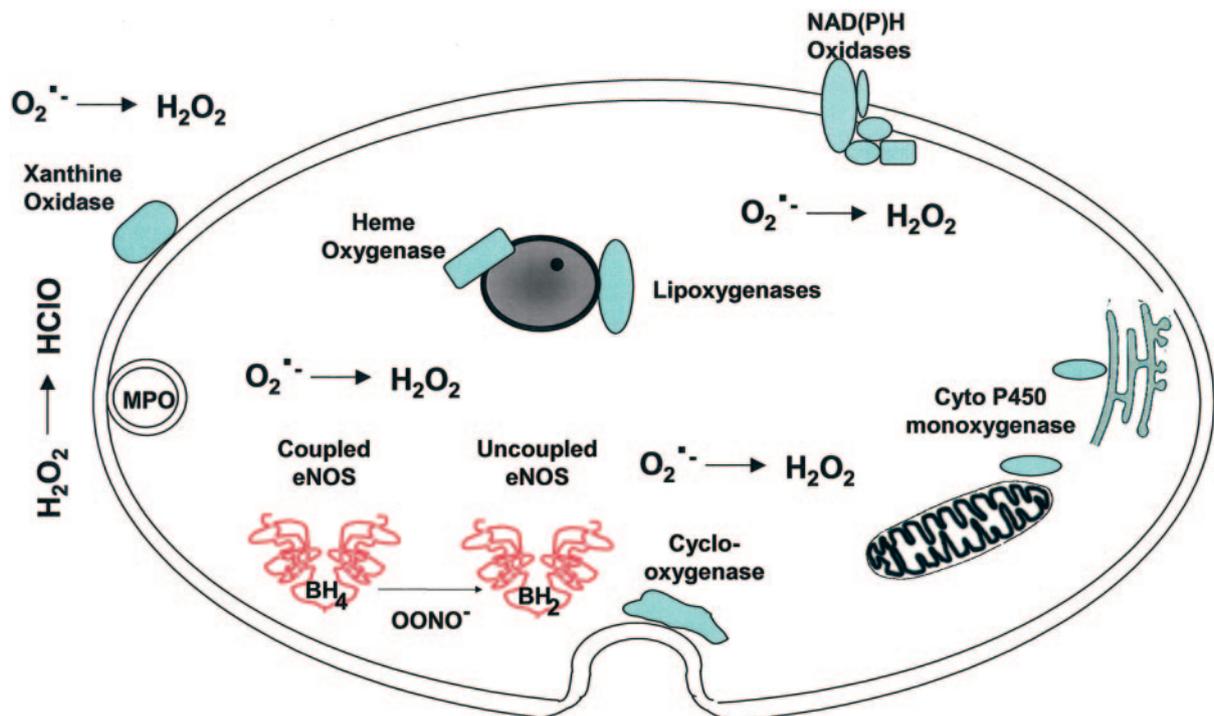


Figure 2. Potential sources of ROS. Many enzymes, including those in the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidases, cytochrome P450 monooxygenase, uncoupled NOS, heme oxygenases, peroxidases, and NAD(P)H oxidases, produce ROS. According to their location in the cell, these ROS are generated intracellularly, extracellularly, or in specific intracellular compartments.

they activate matrix metalloproteinases (MMPs) MMP-2 and MMP-9.^{13,14} Once activated, MMPs can degrade the collagen-based extracellular matrix, contributing to weakening of the fibrous cap and plaque rupture.¹³ In VSMCs, ROS exert their effects via activation of specific intracellular signaling pathways and can profoundly influence both normal physiology and the course of vascular disease. Furthermore, it is becoming evident that stable products of ROS may also influence cellular function by adduction of signaling molecules or by serving as incidental ligands for both membrane and nuclear receptors in vascular cells.

Just as NO[•] mediates vasodilation by activating the VSMC guanylate cyclase, O₂^{•-} and H₂O₂ can alter the activity of selected intracellular proteins. Unlike NO[•], no specific target for these ROS has been identified, nor has the identity of the actual reactive species been elucidated, although *in vitro* studies show that both O₂^{•-} and H₂O₂ are able to inhibit protein phosphatases. O₂^{•-} and H₂O₂ or their products can modulate the activity of signaling pathways. For example, attenuation of agonist-induced ROS production by antisense inhibition of NAD(P)H oxidase expression in VSMCs leads to reduction of angiotensin II-induced hypertrophy, platelet-derived product-stimulated tissue factor expression, and serum-induced growth.^{15–17} These effects appear to be mediated in part through activation of the c-Src, p38 mitogen-activated protein kinase, and the cell survival kinase (Akt) in the case of angiotensin II, and extracellular signal-regulated kinases in the case of platelet-derived growth factor. These signaling pathways, in turn, control gene expression. In some cases, regulation of the gene is redox sensitive because of the susceptibility of these upstream signaling pathways to ROS. However, the affinity of certain transcription factors for their cognate DNA binding sites can also be directly modified by ROS, particularly nuclear factor- κ B and activator protein-1 (AP-1) transcription factors.¹⁸

ROS regulate several general classes of genes, including adhesion molecules and chemotactic factors, antioxidant enzymes, and vasoactive substances. Some of these are clearly an adaptive response, such as the induction of superoxide dismutase and catalase by H₂O₂.¹⁹ Upregulation of adhesion molecules (vascular cell adhesion molecule-1, intracellular adhesion molecule-1) and chemotactic molecules (monocyte chemoattractant protein-1) by oxidant-sensitive mechanisms is of particular relevance to vascular pathology.¹⁸ These molecules promote adhesion and migration of monocytes into the vessel wall. Conversely, transcriptional induction of adhesion molecules by cytokines is inhibited by NO donors in a cyclic guanosine monophosphate-independent manner.²⁰ These mechanisms combine to suppress adhesion molecule expression in the normal vessel wall and induce its expression in vasculopathies.

Monitoring ROS Formation In Vivo

ROS are evanescent species. Consequently, their measurement within integrated systems, such as animal models and humans, has proven to be a complex challenge. Traditionally, *ex vivo* indices, such as the oxidizability of LDL or spin trapping approaches, have been deployed, but increasingly, attention has focused on the development of *in vivo* biomar-

kers of oxidant stress. Essentially, the approach has been indirect and configured on the identification of chemically stable, free radical-catalyzed products of lipid peroxidation (such as isoprostanes), modified proteins (such as nitrated fibrinogen), and indices of free radical-catalyzed modification of DNA (such as 8-oxo-deoxyguanosine).^{21–23}

Much of the earlier literature has been confounded by limitations reflective of *ex vivo* methodology or intrinsic to the specific approach. These include the nonspecific route to formation of the anlylate, the imprecision with which the anlylate is quantified, and the possibility that ROS generation is related nonlinearly to alterations in the anlylate. Finally, ROS generation can result in modification of lipids, protein, and DNA.^{24–26} Approaches to quantification of ROS generation *in vivo* have tended to focus on a single anlylate within one of these broad categories, and an integrated approach, using modern spectroscopic methods, has yet to be applied. Earlier studies have focused most commonly on products of lipid peroxidation. These have included the measurement of thiobarbituric acid-reacting substances, including malonyldialdehyde. However, these compounds can be formed nonspecifically (malonyldialdehyde is a byproduct of cyclooxygenase turnover), and *ex vivo* platelet activation may seriously confound measurements.²⁷ Furthermore, comparative analysis with high-performance liquid chromatography and mass spectrometry have shown that the most commonly applied fluoroscopic methods are quantitatively inaccurate.²⁸

An example of the more recently discovered anlylates formed *in vivo* are the isoprostanes (iPs), chemically stable, free radical-catalyzed products of arachidonic acid.²⁹ These compounds are free radical-catalyzed isomers of traditional enzymatic products of arachidonic acid metabolism. They are formed initially *in situ* in the phospholipid domain of cell membranes subject to ROS attack and are then cleaved by phospholipases, released extracellularly, circulated, and excreted in urine.^{25,30,31} A range of mass spectroscopic assays have emerged on the basis of authentic standards for individual F₂ iPs.^{32–38} Current immunoassays directed against iPF_{2 α} -III (also known as 8-*iso* PGF_{2 α}) are more commonly used. However these are semiquantitative estimates, and iPF_{2 α} -III itself is less than an ideal target anlylate as it may also be formed by cyclooxygenases-1 and -2.^{33,39} For this reason, attention has switched to iPF_{2 α} -VI³⁴ and the even more abundant 8,12-*iso* iPF_{2 α} -VI³⁵ as indices of lipid peroxidation. Immunoassays for these compounds are under development.

One of the consequences of increased ROS production is oxidation of LDL, which modifies its bioactivity extensively *in vitro*, conferring properties associated with disease pathogenesis. Witztum's group^{40,41} has developed a range of antibodies directed against oxidation-dependent epitopes in LDL (anti-oxLDL) and demonstrated their utility in the quantification of lipid peroxidation in animal models and in humans. The increase in F₂iP generation that accompanies atherogenesis in hypercholesterolemic mice correlates closely with titers of anti-oxLDL, and both are depressed in a gene dose-dependent fashion by deletion of the 12/15-lipoxygenase.⁴² Indeed, the epitopes against which these antibodies are directed appear themselves to have functional significance. An anti-oxLDL that is directed against an oxidized moiety in

phosphorylcholine, accessible after oxidation of phosphatidylcholine, blocks oxLDL uptake by macrophages in vitro.⁴³ Furthermore, the acute-phase C-reactive protein, which has been linked to cardiovascular outcome, also binds to a similar, if not identical, moiety.⁴⁴

Mass spectrometry has also been used to identify oxidized amino acids in inflammatory lesions⁴⁵ and in plasma and urine.^{46,47} The pattern of oxidative modification seems to relate to the pathways that initiate oxidation. Furthermore, the application of proteomic approaches may identify analytes that could permit the development of noninvasive “fingerprint” urinary analyses to guide therapy.⁴⁵ Quantitative methods for assessing oxidative modifications of DNA are at an earlier stage of development. Ex vivo and intra-assay artifacts have complicated interpretation of levels of modified bases, such as 8-oxo-deoxyguanosine and 8-oxo-guanine.⁴⁸ However, these methods continue to be refined,⁴⁹ and new biomarkers of DNA modification, such as 1,N⁶-etheno-2'-deoxyadenosine and 1,N²-etheno-2'-deoxyguanosine have begun to emerge.⁵⁰

Conclusion

In vitro studies unequivocally demonstrate that all vascular cells produce ROS and that ROS mediate diverse physiological functions in these cells. The short half-life of these species makes them ideal signaling molecules, but it also confounds their measurement in complex biological systems. Nonetheless, advances have been made in the development of reliable assays, and in Part II of this review, we will discuss their in vivo application and the data supporting a role for oxidative stress in the development of cardiovascular disease.

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