Detection of Superoxide in Vascular Tissue

Thomas Münzel, Igor B. Afanas'ev, Andrei L. Kleschyov, David G. Harrison

Abstract—During the past decade, it has become apparent that reactive oxygen species play a critical role in the genesis of many vascular diseases. The superoxide anion is among the most important of these, not only because of its rapid reaction with NO but also because it serves as a progenitor for many other reactive oxygen species. Although there are many approaches to detecting and quantifying superoxide in chemical systems, its detection in intact tissues is more difficult. The validity of the most popular and frequently used assay for this purpose, lucigenin-enhanced chemiluminescence, has been recently questioned. It has been suggested that lucigenin itself, especially at high concentrations (>50 μmol/L), may act as a source for superoxide via redox cycling. Lower lucigenin concentrations (5 μmol/L) do not participate in redox cycling to an important extent in intact tissues and, therefore, provide an accurate assessment of the rate of superoxide production in such samples. Other useful assays for superoxide include those using the fluorescent dye dihydroethidine, 2-methyl-6-phenyl-3,7-dihydroimidazo(1,2-α)pyrazin-3-one (CLA), and 2-(p-hydroxybenzyl)-6-(p-hydroxyphenyl) 8-benzylimidazo[1,2-α]pyrazin-3-one (coelenterazine). The chemiluminescent compound 5-amino-2,3-dihydroxy-1,4-phthalalineidone (luminol) may also be used to detect various reactive oxygen species and may be made specific for various oxidants, such as hydrogen peroxide, superoxide, and peroxynitrite, by altering the experimental conditions. Although each of these methods may be associated with potential artifacts, the use of ≥2 different techniques that yield similar results provides a reliable approach for the study of reactive oxygen species in intact vascular tissues. (Arterioscler Thromb Vasc Biol. 2002;22:1761-1768.)

Key Words: superoxide ■ intact tissue ■ lucigenin ■ NO synthase III uncoupling

During the past decade, a growing body of evidence suggests that reactive oxygen species play a major role in vascular disease and that pathological conditions such as hypercholesterolemia, atherosclerosis, diabetes, cigarette smoking, heart failure, and nitrate tolerance increase the production of these molecules. Reactive oxygen species play a critical role in vascular smooth muscle cell growth, vascular remodeling, inflammation, and modulation of vascular tone. Among reactive oxygen species, the superoxide anion (O$_2^-$) is of critical importance. Many others, including H$_2$O$_2$, peroxynitrite, hypochlorous acid, the hydroxyl radical, and lipid radicals, are derived from O$_2^-$.

Importantl, O$_2^-$ rapidly reacts with NO at a rate that is diffusion-limited and leads to a marked alteration of vascular tone. Thus, in all of the diseases mentioned above, there is evidence to suggest that this interaction between O$_2^-$ and NO is important in altering vasodilatation and promoting vasoconstriction. Scavenging of O$_2^-$ has proven useful in restoring endothelium-dependent vasodilatation in virtually all of these conditions. Of particular importance is a recent study from Heitzer et al., who used the degree of improvement in acetylcholine-induced forearm vasodilatation caused by vitamin C as a marker of oxidative stress. In a 5-year follow-up, this marker of oxidative stress was a powerful predictor of subsequent major adverse coronary events. That study emphasized the importance of oxidative stress, as characterized by oxidative inactivation of NO, in determining prognoses in patients with coronary artery disease.

Despite the evidence that reactive oxygen species are important in vascular disease, there continues to be concern about the specificity, sensitivity, and validity of the various methods used to quantify them in vascular tissues. The currently available methods include chemiluminescence techniques, fluorescence-based assays, enzymatic assays, and electron paramagnetic resonance spin trapping (EPR). We will discuss each of these, focusing on their utility in studies of intact vascular segments.

Chemiluminescence-Based Assays

Because of its sensitivity, chemiluminescence is frequently used to detect O$_2^-$ in neutrophils and vascular tissue. On exposure to O$_2^-$, chemiluminescent probes release a photon, which in turn can be detected by a scintillation counter or a luminometer. Because most of these compounds are cell permeable, the O$_2^-$ measured reflects extracellular as well as intracellular O$_2^-$ production. Among these chemiluminescent compounds, bis-N-methylacridinium nitrate (lucigenin) remains the most widely used. Other com-

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pounds that may be used include 5-amino-2,3-dihydroxy-1,4-phthalalynedione (luminol) and cypridina luciferin analogues, such as 2-methyl-6-phenyl-3,7-dihydroimidazo (1,2-\alpha)pyrazin-3-one (CLA), 2-methyl-6-(p-methoxyphenyl)-3,7-dihydroimidazo (1,2-\alpha)pyrazin-3-one (MCLA), and 2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl) 8-benzyl-3,7-dihydroimidazol[1,2-\alpha]pyrazin-3-one (coelenterazine).

Lucigenin-Enhanced Chemiluminescence: Theoretical Considerations

Lucigenin has been widely used as an indicator of \(O_2^-\) production\(^2\) from isolated enzymes and inflammatory cells, such as macrophages and neutrophils.\(^3\) In 1991, it was introduced as a tool to measure \(O_2^-\) in vascular tissue by Wolin’s group (Omar et al\(^4\)). Subsequently, lucigenin has been used to detect \(O_2^-\) production in numerous situations in which endothelium-dependent vasodilation is abnormal, including hypercholesterolemia,\(^5,7\) diabetes mellitus,\(^8,9\) nitrate tolerance,\(^10,11\) and different forms of hypertension, such as that produced by angiotensin II infusion,\(^12\) that found in spontaneously hypertensive rats,\(^13\) that produced by deoxy-corticosterone acetate salt treatment,\(^14\) 2-kidney 1-clip hypertension,\(^15\) and that produced by aortic banding.\(^16\)

A thorough understanding of lucigenin-enhanced chemiluminescence requires insight into its potential reactions with \(O_2^-\) and other molecules. The reactions (1 through 4) involved in lucigenin-amplified chemiluminescence (CL) are as follows: reaction 1, \(O_2^- + \text{LC}^2+ \rightarrow \text{LC}^+ + O_2\); reaction 2, \(\text{LC}^+ + O_2^- \rightarrow \text{LCO}_2^-\); and reaction 3, \(\text{LCO}_2^- \rightarrow 2\text{N-methylacridone} + h\nu\), where \(\text{LC}^+\) is the lucigenin cation radical, and \(\text{LCO}_2^-\) is lucigenin dioxetane.

In accord with this mechanism, \(O_2^-\) reduces lucigenin to its cation radical, which reacts with a second \(O_2^-\) to form the energy-rich dioxetane molecule emitting a photon.

In 1997, the credibility of this method was questioned\(^17\) on the grounds that \(O_2^-\) production might be artificially overestimated because of a phenomenon known as redox cycling (reaction 4): \(\text{LC}^+ + O_2 \leftrightarrow O_2^- + \text{LC}^2+\).

The major reason for this proposal was the fact that in some cases, especially when high lucigenin concentrations (up to 250 \(\mu\)mol/L) were used, \(O_2^-\) concentration measured by lucigenin-enhanced chemiluminescence was higher than that determined by other methods. Furthermore, in these cases, an increase in oxygen consumption was observed that was supposedly due to reaction 4.

Reaction 4 occurs at all concentrations of lucigenin, and the extent to which reaction 4 proceeds also depends on the concentration of \(O_2^-\). In biological systems with low \(O_2^-\) levels, the relative contribution of \(O_2^-\) via redox cycling to the measured \(O_2^-\) will be greater than the contribution if \(O_2^-\) content in the biological system is higher. Higher concentrations of lucigenin favor redox cycling, but it is quite clear that this phenomenon does not occur to an appreciable extent when lower concentrations (<20 \(\mu\)mol/L) are used. Li et al\(^18\) showed that higher concentrations of lucigenin could react with a variety of flavin-containing enzymes to yield \(O_2^-\) but that concentrations of lucigenin <20 \(\mu\)mol/L could not (Figure 1A). Similarly, we found that lower concentrations of lucigenin (5 \(\mu\)mol/L) failed to stimulate \(O_2^-\) production from vascular segments, as detected by electron paramagnetic resonance (EPR, Figure 1B).\(^19\) Likewise, Tarpey et al\(^20\) showed that concentrations of lucigenin ≤10 \(\mu\)mol/L had no effect on the production of hydrogen peroxide by cultured endothelial cells. Thus, these findings from various investigators clearly indicate that redox cycling is minimal or nonexistent when low concentrations of lucigenin are used.

One convincing argument against lucigenin redox cycling has been provided by its chief critics: In their first article examining this method, Liochev and Fridovich\(^17\) showed that \(O_2^-\) produces chemiluminescence in its reaction with lucigenin in aqueous solution. This finding is in agreement with reactions 1 to 3 above and has been confirmed by several other groups. Surprisingly, in subsequent discussions, this direct reaction between \(O_2^-\) and lucigenin has simply been ignored, and a great deal of emphasis has been placed on the potential artifactual production of \(O_2^-\) by redox cycling that has been observed in test tube studies. We believe that these very contrived in vitro experiments do not reflect the manner in which lucigenin-enhanced chemiluminescence detects \(O_2^-\).
in intact tissues. Major arguments against the lucigenin redox cycling have been thoroughly reviewed.\textsuperscript{21–23}

**One-Electron Reduction Potential of Lucigenin**

The 1-electron reduction potential of lucigenin (E\(_{1/2}\)[LC\(^{2+}/LC\(^{+}\)]) is 0.19 V.\textsuperscript{22,25} In view of the fact that the 1-electron potential of oxygen (E\(_{1/2}\)[O\(_2/O_2^-\)]) is –0.16 V, the equilibrium constant for reaction 1 (K\(_1\)) is 10\(^6\). Thus, the equilibrium of reaction 1 is so shifted to the right that the back reaction (reaction 4 above) is not favored. Even if one takes into account the relatively high concentration of oxygen in a biological system, the amount of O\(_2^-\) that could be generated by reaction 4 would only minimally contribute (<10%) to the total amount of O\(_2^-\) produced by cells or tissues. Spasovic et al.\textsuperscript{24} have recently measured the 2-electron reduction potential of lucigenin as ≈0.14±0.02 V by using rapid cyclic voltammetry. However, 2-electron potentials cannot be used to calculate equilibrium constants for 1-electron transfer reactions and are irrelevant for measurements of O\(_2^-\), the 1-electron reduction product of oxygen.

**Reduction of Lucigenin by Xanthine Oxidase and Other Flavin-Containing Enzymes**

It has been suggested that direct reduction of lucigenin by xanthine oxidase and other flavin-containing enzymes is a main reason for O\(_2^-\) overproduction in this assay.\textsuperscript{17} If reaction 4 is unlikely, however, then the enzymatic reduction of lucigenin should result in a decrease rather than an increase in O\(_2^-\) production because of the competition between lucigenin and oxygen for an electron. This suggestion has been confirmed for reactions involving xanthine–xanthine oxidase and NADH–xanthine oxidase systems\textsuperscript{21–23} (Figure 2).

**Comparison of Results**

The simplest but perhaps most convincing validation of the lucigenin assay is the comparison of the results obtained by various methods of O\(_2^-\) detection. There are excellent correlations between data obtained by lucigenin-enhanced chemiluminescence and cytochrome c reduction methods in various cell-free and cellular systems (Table).\textsuperscript{23} Likewise, excellent correlations between lucigenin-enhanced chemiluminescence and other chemiluminescence techniques have been observed. Recent studies examining the activity of NADPH oxidase in membranes of vascular smooth muscle cells\textsuperscript{26} and endothelial cells\textsuperscript{27} using the spin trap DEPMPO and EPR have largely confirmed prior findings made with lucigenin.

One of the concerns about lucigenin-enhanced chemiluminescence is that it seems to detect O\(_2^-\) when other methods fail to do so. This is at least in part explained by comparisons of the rate constants for reactions of O\(_2^-\) with the various electron acceptors used in other assays. The reaction of O\(_2^-\) with lucigenin is ≈10\(^8\) mol/L per second,\textsuperscript{21,23} whereas it is 2.6×10\(^7\) mol/L per second for cytochrome c\(_2^\) and 10 to 15 mol/L per second for DMPO.\textsuperscript{2} Thus, O\(_2^-\) reacts with lucigenin ≈1000 times faster than it reacts with cytochrome c and 10\(^3\) times faster than it reacts with DMPO. Therefore, many of these other methods are simply too insensitive, and the ability of lucigenin to detect O\(_2^-\) when it is produced at low levels, e.g., in vascular cells, is simply due to its high sensitivity. In addition, most of the other chemiluminescent probes have significant redox-sensitive backgrounds originating from their oxidation by substances such as transition metals and the impurities present in buffers.

Finally, lucigenin might predominantly exist in tissue as the reduced form Luc\(^-\) due to the presence of numerous reductants there. This will double the lucigenin chemiluminescence produced by reaction 2 and increase oxygen consumption because of the absence of reaction 1.

**Lucigenin-Enhanced Chemiluminescence: Practical Considerations**

To estimate O\(_2^-\) production with the use of lucigenin-enhanced chemiluminescence, vascular segments have been dissected from the experimental animal and cleaned of adhering fat by using a dissecting microscope or similar magnification. For mouse aortas, 2 ring segments of ≈2.5 mm were used in each assay. The vessels were allowed to equilibrate in a Krebs’ buffer containing HEPES buffer for 30 minutes at 37°C and were then transferred to a scintillation vial containing the same buffer with lucigenin (5 \(\mu\)mol/L). Counts were obtained after the vessels were in this buffer for 15 minutes and at each minute for 5 minutes thereafter and averaged. After the assay, the tissues were removed from the buffer and dried at 90°C for 24 hours, and the counts were expressed per milligram of dry weight or per luminal surface area. The assay, like most other methods, does not provide precise quantification of O\(_2^-\) production but allows compar-

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**Table: Correlation Between Lucigenin-Amplified Chemiluminescence and Rates of Cytochrome c Reduction**

<table>
<thead>
<tr>
<th>System</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthine-XO</td>
<td>0.979</td>
<td>41.5</td>
</tr>
<tr>
<td>NADH-XO</td>
<td>0.978</td>
<td>0.47</td>
</tr>
<tr>
<td>Neutrophils*</td>
<td>0.978</td>
<td>92</td>
</tr>
<tr>
<td>Monocytes*</td>
<td>0.992</td>
<td>105</td>
</tr>
<tr>
<td>WBC*</td>
<td>0.930</td>
<td>10.8</td>
</tr>
<tr>
<td>WBC\†</td>
<td>0.994</td>
<td>5500</td>
</tr>
</tbody>
</table>

\*White blood cells were stimulated with the phorbol ester PMA
\†White blood cells were stimulated with opsonized zymosan
ison of relative rates of $O_2^{-}$ production between various vessels.

There are several important caveats to be considered when this assay is used. First, the type of counter to be used is critically important. Luminometers typically used for luciferase or other similar assays may be not sensitive enough to detect the low counts yielded by $O_2^{-}$ reactions with 5 μmol/L lucigenin. We have found that scintillation counters switched to the out-of-coincidence mode are optimal for this purpose. It is ideal if one has a devoted counter for these studies. Counters used to detect very high levels of radioactivity develop high backgrounds because of photobleaching of the photomultiplier tube, and they become unreliable for detecting low levels of scintillation caused by the reaction of $O_2^{-}$ with lucigenin. It is also essential that the reaction vial be “dark-adapted” (kept in the scintillation counter or wrapped in aluminum foil) for 20 to 30 minutes before adding the vascular segments to reduce background counts. Before adding the tissue to the scintillation vial, the background counts must be very stable; ie, the background counts should not vary >200 cpm over a period of 5 to 10 minutes.

By varying the experimental conditions, one can gain insight into the cellular and enzymatic sources of $O_2^{-}$ by using lucigenin-enhanced chemiluminescence. For example, endothelial denudation can be performed before the equilibration step described above. In a number of prior studies, such a maneuver has been used to show the contribution of the endothelium to overall vascular $O_2^{-}$ production. There has been some suggestion that lucigenin may preferentially detect endothelial $O_2^{-}$; however, these results may reflect the fact that the endothelium is a predominant source of $O_2^{-}$ in many disease states. Another useful approach is the addition of a variety of inhibitors to the vascular segments to examine the enzymatic source of $O_2^{-}$. For example, the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA) or its methyl ester Nω-nitro-L-arginine methyl ester can be used to inhibit $O_2^{-}$ production by uncoupled forms of NO synthase.28 (Figure 3). Interestingly, recent studies with human tissue confirmed the fact that the endothelium is a predominant source of $O_2^{-}$.

To investigate mitochondria as a source of $O_2^{-}$, it is necessary to use membrane-permeable molecules such as tiron or manganese tetrakis (4-benzoic acid) porphyrin (TBAP) or to pretreat animals or vessels with diphenylene iodonium (DPI) or to pretreat animals or vessels with diphenylene iodonium. Apocynin has been used to inhibit $O_2^{-}$ production from NADPH oxidase, although the specificity of this compound has never been fully substantiated. Moreover, this compound blocks the assembly of NADPH oxidase and may not be effective if this enzyme system has already been activated. Diphenylene iodonium is often used as an inhibitor of flavin-containing oxidoreductases. It is very nonspecific, and when a reduction in $O_2^{-}$ is observed, one can only say that a flavin-containing enzyme is involved.

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Lucigenin has also been used in homogenates of tissues and membrane fractions to examine activities of various enzymes, such as NADPH oxidase. It is strongly recommended that lucigenin concentrations do not exceed 10 μmol/L when such assays are used. Importantly, recent studies of NADH/NADPH oxidase in endothelial and smooth muscle cells with the use of EPR have yielded results that are identical to those previously obtained with lucigenin-enhanced chemiluminescence.26,27 Several investigators have attempted to examine the activity of NADH/NADPH oxidase by adding either NADH or NADPH to intact vascular segments.32,33 Such studies must be interpreted with caution because these substrates (NADH and NADPH) do not likely...
gain access to the intracellular space but may directly reduce oxygen to form $\text{O}_2^-$ if present in high concentrations.

If one uses the precautions described above, lucigenin can be used as a powerful approach in the assessment of vascular $\text{O}_2^-$ production. It is reproducible, inexpensive, and reliable. Despite recent criticisms, an enormous amount of insight has been gained about the role of $\text{O}_2^-$ in the genesis of vascular disease with the use of this experimental tool.

**Lipophilic Luminophore Compounds**

**Coelenterazine, CLA, and MCLA**

The luminophore coelenterazine has been reported to be a useful tool in the detection of $\text{O}_2^-$ in cell-free systems, in cultured cells, and in vascular tissue. Coelenterazine has no effect on xanthine oxidase–dependent oxygen consumption, endothelial hydrogen peroxide release, or endothelium-dependent relaxation. In contrast to lucigenin, however, it also reacts with peroxynitrite. Thus, to determine whether $\text{O}_2^-$ or peroxynitrite contributes to coelenterazine-induced chemiluminescence, scavengers such as ebselen or uric acid must be used. Although the concomitant detection of $\text{O}_2^-$ and peroxynitrite seems at first glance to be a disadvantage, it allows quantification of all $\text{O}_2^-$ produced by vessels, including that which has already reacted with NO. Coelenterazine (1 μmol/L) has recently been used in assays identical to the assay described above using lucigenin to detect increased $\text{O}_2^-$ in the vessels of animals with experimental diabetes. Importantly, the chemiluminescence signals were almost completely inhibited by manganese TBAP, the SOD mimetic.

The usefulness of CLA, another amplifier of chemiluminescence with high specificity for $\text{O}_2^-$, as an alternative to lucigenin to measure $\text{O}_2^-$ has recently been described. CLA has been previously reported to assay specifically the formation of $\text{O}_2^-$ formed enzymatically in a xanthine–xanthine oxidase system. A major drawback for the use of CLA seems to be a background of high chemiluminescence when HEPES buffer is used. This high background signal can be quenched by adding desferrioxamine and diethyldithiocarbamate to chelate transition metals present in the buffer. CLA has been used to demonstrate increased $\text{O}_2^-$ production in experimental animals with hyperlipidemia and diabetes.

Another chemiluminescent probe similar to CLA is the agent MCLA. MCLA has predominantly been used to detect $\text{O}_2^-$ in cultured cells, and reports on the quantification of $\text{O}_2^-$ production in vascular tissue are scarce. Recently, Sumi et al. showed that in vessels from cholesterol-fed rabbits, $\text{O}_2^-$ production was significantly increased compared with that in vessels from normocholesterolemic rabbits. The MCLA concentration was 1 μmol/L, and the experiments were performed without transition metal chelators.

**Luminol**

Luminol-derived chemiluminescence may be evoked by a variety of reactive oxygen species, including $\text{O}_2^-$, hydroxyl radical, hydrogen peroxide, and peroxynitrite. The selective use of various scavengers, such as ebselen, uric acid, catalase, and SOD, is necessary to determine which of these species is responsible for the signal produced by luminol. For example, a recent study has used the scavengers uric acid and ebselen to show that peroxynitrite formation is markedly elevated in the aortas of apoE-deficient mice (Figure 4). During the past several years, a number of groups have used fluorescent probes to detect reactive oxygen species in cultured cells and in sections of vascular tissues. In cultured cells, confocal microscopy has been used to directly image the fluorescence produced by these agents. These immuno-fluorescence approaches are semiquantitative but can provide important information about the topographical location of reactive oxygen species in the vessel wall. Cells can also be analyzed with the use of a fluorescent plate reader or by fluorescence-activated cell sorter counting to provide quantitative information. Even when these more quantitative approaches are used, these fluorescent probes are subject to quenching and may not increase linearly as the levels of reactive oxygen species increase.

**Dihydroethidium**

Currently, the most popular probe to measure $\text{O}_2^-$ with fluorescent techniques is dihydroethidium (DHE). For these studies, vessels are harvested from experimental animals, and 30-μm frozen sections are obtained. The sections are then allowed to thaw and are incubated with DHE. DHE is cell permeable and reacts with $\text{O}_2^-$ to form ethidium, which in turn intercalates with DNA, providing nuclear fluorescence at an excitation wavelength of 520 nm and an emission wavelength of 610 nm. DHE is reasonably specific for $\text{O}_2^-$ but it may also react with OH• and H₂O₂.

As shown previously with experimental animal studies and summarized in Figure 5, fluorescence caused by DHE has been found to be increased in vessels of animals with angiotensin II–induced hypertension, in diabetic rats, and in nitroglycerin tolerance. These studies have also shown that increased $\text{O}_2^-$ levels are not only in the endothelial cell layer but also in the media and adventitia. Very recently, Sorescu et al. have used DHE to show that $\text{O}_2^-$ production is rather dramatically increased in human atherosclerotic lesions, especially in the shoulder region of plaques. In addition, recent studies using DHE have shown increased $\text{O}_2^-$ levels in
levels, suggesting that the use of DCFH to measure reactive oxygen species may be problematic. Recently, concerns have been raised about the possibility that DCF may fluoresce in the presence of nitroxyl and SOD; however, these experimental conditions are unlikely relevant to most in vivo experiments. We are not aware that this compound has been used for the detection of $O_2^-$ in vascular segments.

**Enzymatic Assays**

**Nitro Blue Tetrazolium**

Nitroblue tetrazolium (NBT) undergoes reduction by $O_2^-$ to form diformazan, a dark insoluble precipitate. Similar to DHE, NBT detects intracellular $O_2^-$; however, it is less sensitive and specific for $O_2^-$ than is DHE. NBT is susceptible to reduction by several tissue reductases and, in fact, is used as the substrate in the blue formazan reaction used to detect NO synthase. Thus, the specificity for $O_2^-$ should be confirmed by inhibition of NBT staining by SOD.

**Aconitase**

Aconitase is a citric acid cycle enzyme belonging to the family of dehydrogenases containing iron sulfur (4Fe-4S) centers that catalyze the conversion of citrate to isocitrate. The mitochondrial and the cytosolic forms of aconitase are inactivated by $O_2^-$, so that its activity has been proposed to reflect intracellular levels of $O_2^-$ production, with low levels of enzyme activity reflecting high levels of $O_2^-$.

**Cytochrome c Reduction**

Reduction of cytochrome $c$ is a widely used and well-accepted technique for measurement of $O_2^-$ production by isolated enzymes, cell homogenates, and neutrophils. In general, $O_2^-$ is measured as the SOD-inhibitable reduction of cytochrome $c$, determined in a spectrophotometer by the increase in absorbance at 550 nm. Because of its inability to penetrate cells, it can be used only to measure extracellular $O_2^-$. Consideration should also be given that reduced cytochrome $c$ can be reoxidized by cytochrome oxidases, peroxidases, and oxidants (including hydrogen peroxide and peroxynitrite), thereby underestimating the rate of $O_2^-$ production. There are a few reports of use of this assay in intact vessels, but it has not been widely used, probably because of the problems mentioned above.

**EPR Spin Trapping**

By far, the most elegant method for detection of radical species is EPR. EPR detects molecules with unpaired elec-
rons, and because most such species are very short-lived, one cannot directly detect them by use of this method only. Two approaches are widely used in biological experiments. The most well known is the use of spin traps, which on reaction with the radical, incorporate the radical into their structure. The most popular spin traps are the nitrons, such as DMPO and DEPMPO, which form $–OH$ and $–O{{\text{H}}_2}$ adducts on reaction with the hydroxyl and $O_{2}^{−}$ radicals, respectively. There are a few reports in which these spin traps have been used with intact vessels; however, precaution must be taken with the use of these compounds. In the presence of reductants present in tissues, such as ascorbate, the spin active adducts are rapidly reduced to EPR silent species. Thus, the absence of a signal in intact cells or tissues may not reflect the absence of radicals but simply the instability of the spin adduct. For this reason, the nitron spin traps are more widely used for studies of isolated enzymes and membranes. Recently, DEPMPO has been used to demonstrate the presence of the NADPH oxidase in membranes of cultured endothelial cells and vascular smooth muscle cells, yielding results that are qualitatively similar to prior studies using lucigenin chemiluminescence.

An emerging approach to the use of EPR in vascular biology has been the use of cyclic hydroxylamines. These molecules are not spin traps, in that they do not “trap” radicals, but they are oxidized to form very stable radicals with half-lives of several hours, which can readily be detected by EPR. One of these spin probes is 1-hydroxy-3-carboxy-piperidine (CPH), which is oxidized by $O_{2}^{−}$ and peroxynitrite to form the CP radical. Another recently developed compound is an acyl-protected cyclic hydroxylamine, which is resistant to auto-oxidation until it enters cells, where it undergoes esterase cleavage to form a typical cyclic hydroxylamine.

These molecules have successfully been used with intact cells and have also been used in vivo to detect reactive oxygen species released in the circulation. Other cyclic hydroxylamines are being developed that may be very useful in the study of intact vessels. Thus, the cyclic hydroxylamines and similar compounds may prove very useful in the detection of $O_{2}^{−}$ and other reactive oxygen species in vascular tissues in the near future.

Conclusions
During the past several years, an enormous body of literature has shown that reactive oxygen species contribute to vascular disease. This research has used a variety of methodologies to detect reactive oxygen species and, in total, has provided reasonably consistent results, independent of the methodologies used. Despite theoretical criticisms, lucigenin-enhanced chemiluminescence has provided useful and reliable estimates of $O_{2}^{−}$ production in intact vascular segments and has tremendously enhanced our understanding of vascular pathophysiology. Other useful approaches include the use of several chemiluminescent probes and fluorescent agents that react with reactive oxygen species. EPR, especially with the use of nitron spin traps, is a very promising approach for the detection of $O_{2}^{−}$ in vascular segments but currently has limitations that prohibit its use under most experimental conditions. It is implausible to assume that all of the reactive oxygen species one hopes to detect are reacting with the probe used; therefore, it is unwise to attempt to precisely quantify the amount of $O_{2}^{−}$ released in such experiments. Because all of these methods may be subject to potential artifacts, it is wise to use $≥2$ estimates of reactive oxygen species levels for any experimental situation.

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