Methods of Detection of Vascular Reactive Species
Nitric Oxide, Superoxide, Hydrogen Peroxide, and Peroxynitrite

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Abstract—The evanescent nature of reactive oxygen and nitrogen species, the multiple cellular mechanisms evolved to maintain these substances at low (submicromolar) concentrations within the vascular system, and the often multifaceted nature of their reactivities have made measurement of these compounds within the vasculature problematic. This review attempts to provide a critical description of some of the most common approaches to quantification of nitric oxide, superoxide, hydrogen peroxide, and peroxynitrite, with attention to key issues that may influence the utility of a particular assay when adapted for use in vascular cells and tissues. (Circ Res. 2001;89:224-236.)

Key Words: free radical ■ nitric oxide ■ superoxide ■ hydrogen peroxide ■ peroxynitrite

Reactive oxygen and nitrogen species play a central role in the maintenance of vascular homeostasis and injury. Nitric oxide (NO)-dependent cell signaling, including endothelial-dependent relaxation, is modulated by both superoxide (O$_2^-$) and superoxide dismutase (SOD), the family of enzymes catalyzing the formation of hydrogen peroxide (H$_2$O$_2$) and molecular oxygen from O$_2$. Alterations in both the rates of formation and extents of scavenging of O$_2^-$ have been implicated in vascular dysfunction seen in atherosclerosis, hypertension, diabetes, and chronic nitrate tolerance as well as in postischemic myocardium. Increased rates of vascular production of O$_2^-$ and H$_2$O$_2$ contribute to initiation of proinflammatory events, with transcriptional regulation of the gene expression of vascular cellular adhesion molecule-1 and monocyte chemotactic protein-1 sensitive to changes in cellular oxidant production as well as modulation of cell-signaling events. Peroxynitrite (ONOO$^-$), the diffusion-limited reaction product of O$_2^-$ and NO, although limiting the bioavailability of NO, has been proposed to mediate many of the cytotoxic effects associated with NO because of the multiplicity of its reactions with cellular thiols, lipids, proteins, and DNA. In addition to its putative role in pathologic processes, ONOO$^-$ may also serve a physiological function as a cell-signaling molecule.

Sensitive techniques for the analysis of these reactive species are required for developing better insight into the complex interactions that characterize the roles these substances play in vascular homeostasis. However, measurement of the vascular production of reactive species is difficult for several reasons. For example, low intracellular steady-state concentrations of O$_2^-$ occur as a result of the balance between endogenous rates of partial reduction of oxygen to O$_2^-$ and scavenging of O$_2^-$ by highly efficient cytoplasmic and mitochondrial SODs, resulting in intracellular O$_2^-$ con-
centrations estimated to rarely exceed 1 nmol/L.\textsuperscript{18} Extracellular release of small proportions of intracellularly formed $O_2^-$ may occur via anion channels.\textsuperscript{19,20} In addition, $O_2^-$ formed from plasma membrane–bound oxidases remains at relatively low levels in vascular tissues because of serum and extracellular fluid components, including low molecular weight (LMW) oxidant scavengers and the heparin-binding extracellular (EC)-SOD.\textsuperscript{3,21–24} Thus, the relatively short half-life (seconds) of reactive species and the efficient and redundant systems that have evolved to scavenge them require that any detection technique must be sensitive enough to effectively compete with these intracellular and extracellular antioxidant components for reaction with the substance in question. Additionally, methods for analysis of reactive species must have intracellular access to faithfully reflect the intracellular situation.

Although not intended to serve as an exhaustive review, this article critically explores commonly used approaches to detect reactive species, with particular attention paid to misconceptions that may limit the usefulness of an assay when adapted for vascular cells and tissues.

**Nitric Oxide Detection**

**Electrochemical Detection**

Nitric oxide is an electrochemically reactive species that can be oxidized at the surface of a metallic, carbon, or carbon-modified anode. Oxidation of NO proceeds by a 1-electron transfer from NO to the anode, generating a nitrosonium cation ($NO^+$), which is ultimately converted to nitrite ($NO_2^-$):

$$
-e^- + OH^- \rightarrow NO^+ \rightarrow HONO \rightarrow NO_2^-
$$

Current generated from the oxidation of NO is directly proportional to NO concentration, with detection limits of $\approx 10$ nmol/L. NO. A selective gas-permeable hydrophobic membrane is used to allow diffusion of NO to the electrode surface while limiting access of $NO_2^-$ and other ionic species. Commercially available electrochemical NO electrodes use Clark-type platinum electrodes (1 to 2 mm diameter) or microelectrodes (30 to 200 $\mu$m) comprised of coated metal electrodes or carbon fibers. Electrochemical detection has been used in measurements of NO from cultured cells\textsuperscript{25} as well as NO released into the coronary effluent of isolated working rat hearts.\textsuperscript{26} A porphyrinic microsensor has been developed to measure vascular NO in humans; however, it is not presently commercially available.\textsuperscript{27} Additionally, carbon fiber/porphyrinic sensors also readily detect catecholamines, such as dopamine and norepinephrine\textsuperscript{28}; thus, in circumstances in which elevated catecholamines are anticipated, such as single-cell recordings, verification of NO concentrations must be confirmed by another technique.\textsuperscript{29}

**Hemoglobin Oxidation**

The reaction of oxyhemoglobin ($HbO_2$) with NO to form methemoglobin (metHb) and nitrate ($NO_3^-$) serves as the basis of a common method to determine rates of NO production. The rapid reaction of NO and $HbO_2$ ($3.7 \times 10^7$ [mol/L]$^{-1} \cdot s^{-1}$)\textsuperscript{30}

$$
HbFe^{2+}O_2 + NO \rightarrow HbFe^{3+} + NO_3^-
$$

ensures that under most conditions, NO will be consumed stoichiometrically. When there is concern that $O_2^-$ may compete for NO, the addition of $> 100$ U/mL SOD will increase the effectiveness of this assay as well as limit $O_2^-$-dependent redox-cycling of Hb.\textsuperscript{31} An alternative approach is to simultaneously assay NO and $O_2^-$ using both metHb formation and cytochrome $c$ reduction.\textsuperscript{32}

Various spectrophotometric methods have been used to determine rates of NO-dependent formation of metHb. Among the most sensitive is dual-wavelength spectroscopy. By using the wavelength pair 401/410 and a band width of 1 nm, there is a detection limit of $\approx 1$ nmol/L NO. The change in concentration of metHb is represented by the following equation:

$$
\Delta C_{metHb} = \Delta (\Delta A_{401–410})/\Delta e_{401–410}(metHb-oxyHb)
$$

If there is interference, for example because of tissue hemoproteins absorbing strongly in the Soret region of hemoglobin, wavelength pairs using other absorbance maxima and isosbestic points can be used (eg, 577/590), although sensitivity of the assay is then reduced.

There are several critical elements to assure accuracy of this method in determining rates of NO formation. First, variations in hemoglobin concentration, eg, superfusion experiments, can profoundly affect results. The ratio of the absolute absorbance at the isosbestic point over time will provide a correction factor:

$$
\Delta C_{metHb} = \Delta A_{401} + (1 - A_{410}/A_{401}) \times A_{401}/\Delta e_{401–410}
$$

Second, appropriate controls should be used to ensure that the formation of metHb is attributable to NO and not other oxidants potentially present in samples. In the case of cellular or tissue extracts, NO synthase (NOS) inhibitors should inhibit metHb production. Likewise, absence of an exogenous NO donor should yield diminished rates of metHb formation. An additional concern when NO donors are used is the concomitant generation of $NO_3^-$, which can independently oxidize $HbO_2$. When high levels of $NO_3^-$ are anticipated, the contribution of $NO_2^-$ to metHb formation must be determined and corrected for.

Third, the pH of incubations should also remain constant over the course of the measurement, because the absorption spectrum of metHb is pH-dependent and the experimental conditions under which NO is released may be altered by fluctuations in pH. Nitrite-mediated oxidation of $HbO_2$ is also pH dependent.

**Chemiluminescent Detection of NO**

Nitric oxide will react with ozone ($O_3$) to form nitrogen dioxide ($NO_2$), a portion of which is in an excited state. As this $NO_2$ species returns to ground state, light is emitted.

$$
O_3 + NO \rightarrow O_2 + NO_2^* \rightarrow NO_2 + h\nu
$$
The rapid rate of reaction of NO with \( \mathrm{O}_3 \) (\( \approx 10^7 \) [mol/L]\(^{-1}\) \cdot s\(^{-1}\)) and the sensitivity of the chemiluminescent detection of light emitted by \( \mathrm{NO}_2 \) (\( \approx 100 \) pmol/L) thus allows real-time monitoring of NO formation. Although gaseous NO can be detected in a straightforward manner, much of biologically formed NO will be rapidly oxidized to \( \mathrm{NO}_2^- \) and \( \mathrm{NO}_3^- \). To detect these NO metabolites in biological fluids, they must first undergo reduction. This can be achieved with the use of nitrate reductase to reduce \( \mathrm{NO}_3^- \) to \( \mathrm{NO}_2^- \), which is then further reduced to NO by reaction with iodide under acidic conditions. An alternative method to detect NO metabolites is to reduce \( \mathrm{NO}_2^- \) to NO with acidic vanadium (III) at room temperature. After the return of the chemiluminescent tracing to baseline, the temperature is raised to 90°C, which results in vanadium-dependent reduction of \( \mathrm{NO}_3^- \) to NO. As with other methods of \( \mathrm{NO}_2^-/\mathrm{NO}_3^- \) measurement, appropriate controls must be performed to ensure that determinations are the result of NO metabolism and are not attributable to the presence of preformed substances present in culture media or buffer solutions, gastrointestinal bacterial metabolism, or dietary ingestion of \( \mathrm{NO}_2^-/\mathrm{NO}_3^- \).

**Griess Reaction**

Nitrite, a stable oxidation product of NO, is measured in a spectrophotometric assay using the Griess reagents, sulfanilamide, HCl, and N-(1-naphthyl)-ethylenediamine (NED). To measure total accumulated NO oxidation products, any \( \mathrm{NO}_3^- \) must first be reduced to \( \mathrm{NO}_2^- \), most commonly with NADH-dependent nitrate reductase. Because NADH interferes with subsequent spectrophotometric detection of \( \mathrm{NO}_2^- \), excess NADH is removed by LDH-catalyzed reaction with pyruvate.

The reactions are as follows:

\[
\begin{align*}
\text{NO}_2^- + \text{NAD}^+ & \rightarrow \text{NO} + \text{NADH} \\
\text{NADH} & \rightarrow \text{LDH} \\
\text{LDH} & \rightarrow \text{NAD}^+ \\
\text{NO}_3^- + \text{H}^+ & \rightarrow \text{HONO}
\end{align*}
\]

\[
\begin{align*}
\text{HONO} + \text{H}_2\text{N} & \rightarrow \text{HON}=\text{N} - \text{SO}_2\text{NH}_2 + \text{NH}_2^-=\text{SO}_2\text{NH}_2 + \text{OH}^-
\end{align*}
\]

\[
\begin{align*}
\text{H}_2\text{N} & \text{CH}_2\text{CH}_2\text{NH}_2 + \text{H}_2\text{N} \text{CH}_2\text{CH}_2\text{NH}_2 & \rightarrow \text{H}_2\text{N} \text{CH}_2\text{CH}_2\text{NH} + \text{N}=\text{N} - \text{SO}_2\text{NH}_2
\end{align*}
\]

(6)

The reaction can be monitored at \( \text{A}_550 \) in a standard spectrophotometer, or it can be adapted for a microplate reader using a 550-nm filter. Standard curves are generated using \( \text{NaNO}_3^- \) or \( \text{NaNO}_2^- \) in the range of 0.1 to 40 \( \mu \text{mol/L} \), with sensitivity of \( \approx 1 \) \( \mu \text{mol/L} \). Deproteination of samples can improve detection by elimination of turbidity attributable to precipitate formation in protein-rich specimens. Furthermore, sensitivity can be improved by initial addition of acid and sulfanilamide followed by addition of NED, thus reducing competition of NED for sulfanilic acid and carrying out the reaction at low temperature (4°C) to increase the stability of the diazo product. Appropriate controls must be performed to rule out the contribution of background \( \text{NO}_2^-/\text{NO}_3^- \).

**Arginine-Citrulline Conversion**

Nitric oxide synthase catalyzes oxidation of the guanidino nitrogen of arginine, resulting in formation of NO and stoichiometric amounts of citrulline. Thus, the net potential of a tissue or cell extract to generate NO can be estimated by rates of formation of citrulline from arginine in the presence of saturating concentrations of NOS cofactors FAD, FMN, NADPH, tetrahydrobiopterin, calcium, and calmodulin (in the case of neuronal and endothelial NOS). To initiate the reaction, radiolabeled arginine and cofactors are added to cell or tissue preparations; reactions are terminated by the addition of EDTA pH 5.5 to bind calcium, resulting in enzyme inactivation. Samples are then eluted on a cation exchange column; under these conditions, arginine binds to the exchange resin, whereas the zwitterionic citrulline is collected and measured in a liquid scintillation counter.

There are several critical controls to ensure that this assay reflects NOS-catalyzed formation of citrulline. Radiolabeled arginine, particularly \( ^1\text{H}-\text{arginine}, \) can decompose over time; thus, it is essential to demonstrate that applied arginine is retained on the column. To verify that citrulline is the major radiolabeled compound in the flow-through, thin layer chromatography on silica gel G can be performed using the solvent system \( \text{CHCl}_3/\text{CH}_3\text{OH/} \text{NH}_4\text{OH/} \text{H}_2\text{O} \) (0.5:4.5:2.0:1.0). The \( R_f \) values for arginine, ornithine, and citrulline with this system are 0.53, 0.92, and 0.77. Citrulline recycling can occur via argininosuccinate synthase and argininosuccinase to reform arginine and limit apparent rates of citrulline formation. Preparations from liver, a tissue with complete urea cycle activity, can result in production of citrulline via ornithine. To confirm that citrulline production is the result of NOS activity, a competitive NOS inhibitor can be added or NADPH can be omitted from the reaction mixture.

**Superoxide Detection**

**Cytochrome c Reduction**

Reduction of ferricytochrome \( c \) has been used to measure rates of formation of \( \text{O}_2^- \) by numerous enzymes, tissue extracts, and whole cells. This reaction occurs with a rate constant of \( \approx 1.5 \times 10^7 \) [mol/L]\(^{-1}\) \cdot s\(^{-1}\) at pH 8.5 and room temperature.

\[
\text{Fe}^{3+} \text{cyt} + \text{O}_2^- \rightarrow \text{Fe}^{2+} \text{cyt} + \text{O}_2
\]

The reaction is followed spectrophotometrically at 550 nm; the extinction coefficient for ferricytochrome \( c \) is 0.89 \times 10^4 [mol/L]\(^{-1}\) \cdot cm\(^{-1}\), whereas the extinction coefficient for ferrocyanochrome \( c \) is 2.99 \times 10^5 [mol/L]\(^{-1}\) \cdot cm\(^{-1}\). Therefore, \( \Delta E_{\text{e}} \) \text{max} = 2.1 \times 10^4 [mol/L]\(^{-1}\) \cdot cm\(^{-1}\).

There are several precautions when using this reaction to detect \( \text{O}_2^- \).
First, cytochrome c reduction is nonspecific for O$_2$·$.^1$ Tissue extracts contain compounds such as ascorbate and glutathione that can reduce cytochrome c as well as cellular reductases that enzymatically catalyze cytochrome c reduction. Additionally, enzymes such as xanthine oxidase are capable of reducing quinones or redox-active dyes that may also be present and whose reduced forms are capable of directly reducing cytochrome c. Specificity of this reaction for O$_2$·$^-$ is achieved in part by the extent of inhibition of cytochrome c reduction by added SOD.$^43$

Second, reduced cytochrome c can be reoxidized by cytochrome oxidases, peroxidases, and oxidants, including H$_2$O$_2$, and ONOO$^-$. This reoxidation, by diminishing apparent rates of cytochrome c reduction, will underestimate the rate of O$_2$·$^-$ formation. Enzyme inhibitors (10 μmol/L CN for cytochrome oxidase) or scavengers of reactive species (100 U/mL catalase for H$_2$O$_2$ and 10 mmol/L urate for ONOO$^-$) can be added to avoid this pitfall.

Third, to enhance specificity of the assay for O$_2$·$,^-$ cytochrome c can be acetylated.$^{45}$ Acetylation of ferricytochrome c lysine residues decreases direct electron transfer by mitochondrial and microsomal reductases to cytochrome c and from cytochrome c to cytochrome oxidase while preserving the capacity of O$_2$·$^-$ to reduce cytochrome c.$^{46}$ Succinoylation of cytochrome c is more effective in decreasing reduction of cytochrome c by NADPH-cytochrome P-450 reductase or cytochrome b$_5$ and oxidation by cytochrome c oxidase. However, the rate constant for reduction of succinoylated cytochrome c by O$_2$·$^-$ is ≈10% of native cytochrome c, thus requiring higher concentrations of succinoylated cytochrome c.$^{47}$

### Nitroblue Tetrazolium

Nitro-substituted aromatics, such as nitroblue tetrazolium (NBT), can be reduced by O$_2$·$^-$ via one-electron transfer reactions. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction. Reduction of NBT chloride, a dication, to di-formazan proceeds in 2 steps, yielding the partially reduced reaction.

The first step of this reaction occurs through a one-electron process to form the NBT radical (NBT$^+$):

$$\text{NBT}^{2+} + \text{O}_2^- \rightarrow \text{NBT}^+ + \text{O}_2$$

The NBT radical undergoes a dismutation reaction with another NBT$^+$, or it can accept a second electron yielding the monoformazan (NBT$^-$).

$$\text{H}^+ + \text{NBT}^+ + \text{NBT}^+ \rightarrow \text{NBT}^{2+} + \text{NBTH}^+$$

or

$$\text{H}^+ + \text{NBT}^+ + \text{O}_2^- \rightarrow \text{NBTH}^+ + \text{O}_2$$

However, the reduction of the dication NBT$^{2+}$ is not exclusive to O$_2$·$.^-$ Numerous other substances including cellular reductases can donate an electron to NBT, forming the NBT radical:

$$(11) \quad \text{NBT}^{2+} + e^- \rightarrow \text{NBT}^+.$$

A complication in the use of this detector molecule then arises when under aerobic conditions NBT radical also reacts with environmental O$_2$ to generate O$_2$·$.^-$

$$(12) \quad \text{NBT}^+ + \text{O}_2 \rightarrow \text{NBT}^{2+} + \text{O}_2^-.$$
marker for O$_2^-$ production. First, cytochrome c is also capable of oxidizing HE. This may be of importance when mitochondria are the primary source of O$_2^-$ production or when cytochrome c is released into the cytosol under conditions leading to apoptosis. Because oxidant stress has been suggested to initiate apoptotic processes and apoptosis itself has been proposed to increase mitochondrial O$_2^-$ production, it would be difficult to ascribe HE conversion to ethidium solely to O$_2^-$; thus, localization of cellular sites of O$_2^-$ generation would be suspect under these circumstances. Second, quantitation of O$_2^-$ production using HE will be inaccurate because of its capacity to enhance rates of O$_2^-$ dismutation to H$_2$O$_2$. Thus, under these conditions, initial rates of O$_2^-$ formation will be underestimated.

Electrochemical Detection

There have been several methods reported for the electrochemical detection of O$_2^-$. These include direct oxidation of O$_2^-$ on a carbon microfiber microelectrode with an Ag counter-electrode, used to detect O$_2^-$ release from single neutrophils. A carbon microelectrode covered by a polypyrrole/horseradish peroxidase (HRP) membrane with an additional adsorbed layer of SOD has been described; O$_2^-$ is dismutated to H$_2$O$_2$, which is then reduced by HRP, resulting in oxidation at the electrode surface. In a somewhat similar fashion, a Clark-type platinum microelectrode has been coated with a polypyrrole film in which SOD is immobilized; the H$_2$O$_2$ generated from the dismutation of O$_2^-$ becomes oxidized at the electrode surface. However, systems that depend on formation of H$_2$O$_2$ would be unable by themselves to discriminate between processes that primarily form O$_2^-$ and those that are directly linked to H$_2$O$_2$ generation. Presently, there are no commercially available electrochemical detection O$_2^-$ systems. The ability to use microelectrodes and to image real-time fluxes of O$_2^-$ would represent a noteworthy advance in our capacity to understand the significance of oxidant biology.

Electron Spin Resonance and Spin Trapping

The only analytical approach that permits the direct detection of free radicals is electron spin resonance (ESR), also termed electron paramagnetic resonance spectroscopy. This sister technique to nuclear magnetic resonance spectroscopy reports on the magnetic properties of unpaired electrons and their molecular environment. These unpaired electrons can exist in two orientations, either parallel or antiparallel with respect to an applied magnetic field. The energy differences of these states correspond to the microwave region of the electromagnetic spectrum. Although unpaired electrons of species such as NO, -OH, or O$_2^-$ are too low in concentration and short-lived to be directly detected by ESR in biological systems, this dilemma can be circumvented by ESR measurement of more stable secondary radical species formed by adding exogenous spin-traps—molecules that react with primary radical species to give longer-lasting radical adducts with characteristic ESR signatures that can accumulate to levels permitting detection. These spin traps, frequently nitroxide and nitrone derivatives, can also be used to label biomolecules and probe basal and oxidation-induced events in protein and lipid microenvironments. Interestingly, this spin-trap approach can also be used to measure tissue oxygen consumption and to noninvasively spatially map oxygen concentrations in living tissues. With a sensitivity limit of $\approx 10^{-9}$ mol/L, ESR spectroscopy is also capable of detecting the more stable free radical–derived species produced in the vascular compartment during oxidative and inflammatory injury, including ascorbyl radical, tocopheroxyl radical, and heme-nitrosyl complexes. The use of spin traps such as 5-phenyl-tert-butylnitrotrone (PBN) and 5,5-dimethylpyrroline-N-oxide (DMPO) has had an illustrious history in detecting organic radical products of lipid peroxidation (PBN), -OH, and O$_2^-$ (DMPO). However, uncertainties about influences on NO metabolism (PBN) and the ability of ferric ion to oxidize DMPO resulting in the classic 4-line spectrum associated with -OH metabolism have led to development of more selective species. More recently, a more O$_2^-$-specific and stable spin trap, 5-diethoxypyrophosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO), has proven increasingly useful in measuring extents of cell and tissue O$_2^-$ production. Reaction of NO with endogenous heme and non-heme iron proteins leads to formation of iron-nitrosyl complexes with characteristic spectra. Addition of exogenous iron-dithiocarbamates and nitronyl nitroxides has also been used to detect NO formation. Both reactive oxygen species and NO have been detected in vivo with ESR. Although instability, tissue metabolism, the sometimes broad reactivity of spin traps, and the cost of ESR spectrometers can be problematic, when combined with parallel strategies for detecting specific reactive species, ESR has proven to be a useful and revealing free radical detection strategy.

Chemiluminescence Reactions

Chemiluminescent methods of O$_2^-$ detection in vascular tissues have been used frequently because of the potential for access to intracellular sites of O$_2^-$ generation, the alleged specificity of reaction of the probe with O$_2^-$, minimal cellular toxicity, and purported increased sensitivity compared with chemical measurements. Several compounds have been used for chemiluminescent detection of O$_2^-$, including Luc$^2^+$, luminol (LumH$_2$), and coelenterazine [2-(4-hydroxybenzyl)-6-(4-hydroxyphenyl)-8-benzyl-3,7-dihyridimido[1,2-$\alpha$]pyrazin-3-one] and its analogs CLA (2-methyl-6-phenyl-3,7-dihyridimido[1,2-$\alpha$]-pyrazin-3-one) and MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihyridimido[1,2-$\alpha$]pyrazin-3-one). Luciferin and LumH$_2$ and the light they can produce have long been studied in the hope that understanding these chemiluminescences would lead to insight into the mechanism of bioluminescence. Thus, Gleu and Petsch knew in 1935 that oxidants such as hypochlorite or ferricyanide would elicit LumH$_2$ luminescence, provided that O$_2$ was also present, but these oxidants did not cause Luc$^2^+$ to emit light. In contrast, reductants, such as dithionite or stannite, caused Luc$^2^+$ to luminesce aerobically but failed to induce LumH$_2$ luminescence. Hydrogen peroxide, which at elevated pH can act both as a reductant and as an oxidant, was also able to elicit LumH$_2$ luminescence. The mechanism proposed at that time was incorrect, but the observations must be accommodated by any mechanism now espoused.
Totter et al. noted that the aerobic xanthine oxidase (XO)/hypoxanthine reaction could cause Luc⁺ luminescence, and they proposed that O₂ radicals were involved in this process. Greenlee et al. then proceeded to study the effect of pO₂ on the Luc⁺ and LumH₂ luminescences in the XO system, and they noted that raising pO₂ from 0% to 100% increased the LumH₂ luminescence over that entire range but that Luc⁺ light production was optimal at 2% O₂ and declined at higher [O₂]. They also noted that Luc⁺ was reduced by XO/xanthine under anaerobic conditions and that a reduction intermediate of Luc⁺ was autoxidizable. Moreover, the catechol, tiron, which had already been seen to inhibit the O₂⁻ -dependent reduction of cytochrome c in this system, also inhibited Luc⁺ luminescence. Thus it was apparent that the conditions leading to luminescence of Luc⁺ and of LumH₂ were fundamentally different in that the former required reduction and the latter oxidation. Superoxide dismutase was applied to probe for the involvement of O₂⁻ in the luminescence of LumH₂ elicited by several oxidants, including ferricyanide, persulfate, hypochlorite, and the XO/xanthine reactions. In all these cases, SOD was strongly inhibitory, implicating O₂⁻. The mechanism proposed involved the univalent oxidation of LumH₂ to a radical (LumH·), which could autoxidize with production of O₂⁻. The subsequent reaction of LumH· with O₂⁻ would yield an unstable endoperoxide whose decomposition produced N₂ plus aminophthalate in an excited state, which then emitted light during its return to the ground state. These results indicate that LumH₂ luminescence is not a reliable indicator of O₂⁻, even when O₂⁻ is a participant in the reactions leading to light emission, because LumH₂ could mediate O₂⁻ formation by a variety of oxidants. Moreover, there are O₂⁻-independent routes to LumH₂ luminescence such that caused by HRP plus H₂O₂.

The case with Luc⁺ is somewhat analogous but different in that Luc⁺ has to be univalently reduced to Luc⁻, which can autoxidize with production of O₂⁻. Then, in a radical-radical addition reaction, Luc⁻ and O₂⁻ combine, yielding an unstable dioxetane, whose decomposition produces two molecules of N-methyl acridone, one of which is in an excited state and emits light on return to the ground state. Lucigenin is very similar to paraquat in that both are dications that can be reduced to an autoxidizable monocation radical. Thus, Luc⁺, like LumH₂, can mediate O₂⁻ production, with the difference that Luc⁺ does so catalytically by a cycle of reduction followed by autoxidation, whereas LumH₂ does so by two successive univalent oxidations, the first by hydroxyl radical, OCl⁻, ferricyanide, or another oxidant; and the second by O₂.

Because of the frequent misuse of LumH₂ and Luc⁺ luminescence as supposedly specific detectors of O₂⁻, this chemistry has been previously reviewed. However, such is the triumph of hope over reality that the inappropriate use of these compounds continues. We then reported that Luc⁺ could increase the production of O₂⁻ in the XO/xanthine as well as the glucose oxidase plus glucose reactions, much as does paraquat, and in Escherichia coli, Luc⁺ induced the soxRS regulon, again much as does paraquat. Others have also shown that Luc⁺ mediates O₂⁻ production by use of spin-trapping and other methods. The magnitude of overestimation of O₂⁻ concentration appears related to the dose of Luc⁺ used and has encouraged some to advocate use of a safe concentration of Luc⁺. However, as Luc⁺ undergoes redox-cycling to varying extents under different experimental conditions, the uncertainty concerning the precise mechanisms of enhanced Luc⁺-dependent O₂⁻ formation, particularly in a cell- or tissue-based experimental system, precludes knowledge of such a safe Luc⁺ concentration. There are those whose faith in these luminescence methods remain unshaken; however, as is the case with NBT reduction, chemiluminescent techniques using Luc⁺ compounds may aid in discriminating between the contributions of O₂⁻ and ONOO⁻ to chemiluminescence under these circumstances.

**Aconitase Inhibition**

Aconitase, present in both the cytosol and mitochondria, catalyzes the interconversion of citrate to isocitrate. Aconitase is inactivated by O₂⁻ because of oxidation followed by reversible loss of Fe from its cubane [4Fe-4S] cluster, with a rate constant of 10⁶ to 10⁷ [mol/L]⁻¹·s⁻¹. Because of basal endogenous production of O₂⁻, a small proportion of aconitase at any moment is inactive but capable of reactivation; this ratio of inactive/active aconitase increases as rates of O₂⁻ production are augmented. Aconitase activity is determined by the relative rates of inactivation by O₂⁻ or other oxidants and the rate of reactivation by reduction and restoration of iron to the cluster. Alterations in aconitase activity have been used as a sensitive index of changes in steady-state levels of O₂⁻. Using this method, steady-state O₂⁻ concentrations have been estimated at ~8 to 30 pmol/L in A549 lung epithelial cells under normal conditions (86% active aconitase) and 50 to 200 pmol/L in cells when 50% of aconitase is in the inactive form.

Aconitase activity is measured in cell or tissue homogenates by monitoring spectrophotometrically the conversion of isocitrate to cis-aconitate at A₅₄₉. Background interference may be avoided with a coupled assay where citrate serves as the aconitase substrate and the isocitrate formed is converted...
to $\alpha$-ketoglutarate by NADP$^-$-dependent isocitrate dehydrogenase, permitting monitoring of NADPH formation at A340nm. Reactivation of aconitase activity is accomplished by addition of the reducing agent dithiothreitol, ferrous ammonium sulfate, and Na$_2$S to cellular extracts and remeasuring aconitase activity.102

Other oxidants can modulate aconitase activity, in addition to O$_2^-$. Peroxynitrite will oxidize the [4Fe-4S] cluster of the enzyme with subsequent loss of activity, which is restored by the addition of thiol reagents and reduced iron.98,99 The rate constant of this bimolecular reaction has been estimated at $10^4$ [mol/L$^{-1}$] $\cdot$ s$^{-1}$. It has also been reported that NO itself can inhibit aconitase activity, but it is unclear if, in vivo, NO is directly responsible for significant enzyme inactivation or it is the result of ensuing ONOO$^-$ formation. The effect of NOS inhibition on extents of aconitase inactivation may help to determine the role of O$_2^-$ and NO-derived species in enzyme inactivation. Oxygen and H$_2$O$_2$ have also been noted to inhibit aconitase activity, albeit with rate constants 4 to 5 orders of magnitude lower than those noted for O$_2^-$ or ONOO$^-$.97 Thus, a direct contribution of oxygen and H$_2$O$_2$ to oxidant-mediated aconitase inactivation is minimal.

**Hydrogen Peroxide Production**

Because of the rapid dismutation of O$_2^-$ to H$_2$O$_2$ (spontaneous, 10$^3$ [mol/L$^{-1}$] $\cdot$ s$^{-1}$; SOD-catalyzed, 10$^4$ [mol/L$^{-1}$] $\cdot$ s$^{-1}$), endogenous rates of cellular H$_2$O$_2$ production can be used as an indirect measure of O$_2^-$ formation, recognizing that such measurements will also reflect direct divalent reduction of molecular oxygen to H$_2$O$_2$. Intracellular steady-state H$_2$O$_2$ can be estimated by aminotriazole-mediated catalase inactivation.103 A significant portion of cellularly derived H$_2$O$_2$ can also traverse the cell membrane and thus be measured in the extracellular space by HRP-catalyzed oxidation of homovanillic acid or $p$-hydroxyphenylacetic acid (see below). Use of a scavenger such as uric acid will control for ONOO$^-$-dependent oxidation of phenolic fluorophores.

**Protection by SOD or LMW Scavengers of Reactive Species**

A role for enhanced production of O$_2^-$ can be inferred when the addition of SOD or LMW SOD mimetics alters the effect seen in the absence of the antioxidant.5,104,105 Robust conclusions, however, can only be drawn when scavengers are used appropriately. For example, the lack of an effect of native CuZn SOD does not imply that O$_2^-$ is not involved in a given process if O$_2^-$ is formed at a site to which CuZn SOD is unable to gain access either because of size or charge considerations. Additionally, many SOD mimetics can also catalyze peroxidase and peroxynitrite isomerase reactions and thus may not be entirely specific for O$_2^-$:106–108 Another approach is to use methods such as transgenic animals or transient transfection to study the effects of overexpression of SOD,109–111 a strategy complicated by frequent compensatory responses of cell antioxidant defense mechanisms.

**Enhancement of Effect by Inhibition of SOD**

In a similar manner, enhanced O$_2^-$ formation has been implicated when diminished SOD activity results in an alteration in response variables. Decreases in SOD activity can be attributable to chromosomal abnormalities,112 the addition of inhibitors such as metal chelators,4 or the use of transgenic animals113–115 and again may be complicated by nonspecific effects of inhibitors on other metalloproteins or compensatory tissue responses.

**Hydrogen Peroxide Detection**

**Horse Radish Peroxidase–Linked Assays**

Several assays for the detection of H$_2$O$_2$ depend on the oxidation of a detector compound. In the presence of H$_2$O$_2$, hydrogen donors are oxidized by HRP:

$\begin{align*}
(13) \text{HRP} + \text{H}_2\text{O}_2 & \rightarrow \text{HRP} - \text{H}_2\text{O}_2 \, [\text{Compound I}] \\
(14) \text{HRP} - \text{H}_2\text{O}_2 + \text{AH}_2 & \rightarrow \rightarrow \text{HRP} + \text{2H}_2\text{O} + \text{A}
\end{align*}$

The amount of H$_2$O$_2$ present is estimated by following the decrease in fluorescence of initially fluorescent probes, such as scopoletin (7-hydroxy-6-methoxy-coumarin),116 or by monitoring the increase in fluorescent products from previously nonfluorescent hydrogen donors, such as diacetyldichlorofluorescin,117 $p$-hydroxyphenylacetate,118 homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid),119 or N-acetyl-3,7-dihydroxyphenoxazine.120 Spectrophotometric assays using the same principle include oxidation of tetramethylbenzidine121 or phenol red.122 Several caveats are necessary to reasonably use and interpret reporter molecule test systems when detecting H$_2$O$_2$ production in biological systems.

First, numerous biological substances, including thiol compounds and vitamin C, can serve as substrates for HRP and thus compete with the detector molecule for oxidation, leading to underestimation of H$_2$O$_2$ formation.

Second, competition with HRP by endogenous catalase for H$_2$O$_2$ can also lead to underestimation of H$_2$O$_2$. For example, in the scopoletin-HRP–coupled assay, rates of H$_2$O$_2$ formation in isolated mitochondria or submitochondrial particles ranged from 2% to 76% of those detected by either oxygen consumption or by H$_2$O$_2$-induced cytochrome c peroxidase Compound I formation.116

Third, quenching of fluorescent signals by cell and tissue components can lead to overestimation of H$_2$O$_2$ in the case of scopoletin or underestimation, as with homovanillic acid. When measuring cellular or subcellular H$_2$O$_2$ accumulation, accuracy of the assay can be improved by separation of the incubation media from cellular components before addition of the detection system, thus limiting confounding interactions of the detection system with cellular elements.121

Use of HRP-coupled reactions with spectrophotometric detection can also have limitations. Direct reduction of the oxidized detector molecule by electron transport components can limit the utility of tetramethylbenzidine in H$_2$O$_2$ determination, eg, in mitochondria.121 Although spectrophotometric detection of H$_2$O$_2$ with phenol red has been used successfully in purified enzyme–substrate mixtures and to detect H$_2$O$_2$ release from activated leukocytes,122 the phenol red–HRP assay is pH-dependent and less sensitive than fluorescent methods, reducing its utility when attempting to detect H$_2$O$_2$ from vascular tissue.
Catalase-Based Assays

The intermediate complex that is formed in catalase-dependent metabolism of H_{2}O_{2} is stable; thus, steady-state monitoring of a H_{2}O_{2}-catalase complex (Compound I) can be followed with dual-wavelength spectrophotometry at 660 to 640 nm. The fraction of catalase heme present as Compound I depends not only on the rate of H_{2}O_{2} formation but is influenced by the total catalase heme concentration and on the concentration of a hydrogen donor.\textsuperscript{123}

Additional assays for H_{2}O_{2} detection are based on the ability of catalase to act as a peroxidase. In the presence of H_{2}O_{2}, catalase will oxidize ^{14}C-methanol to ^{14}C-formaldehyde. To enhance the efficiency of the peroxidase reaction and to diminish competition from endogenous hydrogen donors, high concentrations of the hydrogen donor should be used relative to expected H_{2}O_{2} levels. The potential for side reactions to occur with either substrate, e.g., alcohol dehydrogenase, or product, e.g., aldehyde dehydrogenase, must also be considered.\textsuperscript{123}

Intracellular production of H_{2}O_{2} can be estimated by using the catalase inhibitor, aminotriazole, which reacts with the Compound I intermediate; thus, catalase is inhibited by aminotriazole only in the presence of H_{2}O_{2}.\textsuperscript{124} By determining the half time of inactivation of catalase, steady-state H_{2}O_{2} concentration can be approximated according to the following equation:\textsuperscript{103,125}

\begin{equation}
[H_{2}O_{2}] = \frac{k_{cat}}{k_{1}}
\end{equation}

where

\[
0.5/t_{1/2} = -k_{cat} \text{ and } k_{1} = 1.7 \times 10^{7} \text{[mol/L]}^{-1} \text{s}^{-1}.
\]

Under conditions where glutathione peroxidase-reductase significantly contributes to metabolism of H_{2}O_{2}, calculations of H_{2}O_{2} concentrations by this method will be underestimated.\textsuperscript{125}

Ferrithiocyanate

Hydrogen peroxide oxidizes ferrous iron to the ferric state. Addition of thiocyanate to the ferric ions results in formation of a red complex with a peak in the 450 to 480 nm range.\textsuperscript{126} This method has been used to estimate H_{2}O_{2} formation in hepatic microsomes,\textsuperscript{127} fibroblasts,\textsuperscript{128} and endothelial cells\textsuperscript{96} when fluorescent quenching and interference by tissue chromophores prevented use of alternative approaches.

Dichlorofluorescein Fluorescence

The oxidation of 2′-7′-dichlorofluorescin (DCFH) to the fluorescent compound 2′-7′-dichlorofluorescein (DCF) was initially thought to be a relatively specific indicator of H_{2}O_{2} formation\textsuperscript{129} and has been used extensively in the detection of oxidants produced during the respiratory burst in inflammatory cells, either in cell lysates or in intact cells using flow cytometry.\textsuperscript{130,131} The diacetate form of DCFH (DCFH-DA) is taken up by cells, where intracellular esterases cleave the molecule to DCFH, which has been suggested to remain trapped intracellularly. In the presence of H_{2}O_{2}, DCFH is oxidized to DCF; fluorescence is measured with excitation at 498 nm and emission at 522. However, there are limitations to the interpretation of DCF fluorescence as a specific marker for quantitative intracellular H_{2}O_{2} formation.

First, the H_{2}O_{2}-dependent oxidation of DCFH to DCF occurs slowly, if at all, in the absence of ferrous iron.\textsuperscript{132} DCF formation is greatly enhanced in the presence of heme-containing substances, such as hematin, peroxidases, or cytochrome c.\textsuperscript{132–135} Importantly, peroxidases are capable of inducing DCFH oxidation in the absence of H_{2}O_{2}.\textsuperscript{134,136,137} Thus, alterations in cellular peroxidase activity are likely to be equally if not more important than H_{2}O_{2} in determining rates of DCF formation and cellular fluorescence.

Second, intracellularly formed DCFH does not necessarily remain within the confines of the intracellular space but rather can reaccumulate in extracellular media, where it would be available for reaction with extracellular oxidants.\textsuperscript{138}

Third, the peroxidase-dependent formation of DCF from DCFH, both in the presence and absence of H_{2}O_{2}, is complex and generates both the DCF semiquinone free radical DCF•\textsuperscript{135} and the DCF phenoxyl radical DCF•\textsuperscript{139} as well as O_{2}•−. The dismutation of O_{2}•− to H_{2}O_{2} would then yield artifactual DCFH oxidation and amplification of DCF fluorescence.

In addition to peroxidase-dependent oxidation of DCFH, other substances are capable of directly inducing DCF formation in the absence of H_{2}O_{2}, including ONOO• and HOCl.\textsuperscript{140,141} In the presence of heme-containing compounds, lipid peroxides are also capable of generating DCF fluorescence.\textsuperscript{133}

Because of the multiple pathways that can lead to DCF fluorescence and the inherent uncertainty relating to endogenous versus artifactual oxidant generation, this assay may best be applied as a qualitative marker of cellular oxidant stress rather than a precise indicator of rates of H_{2}O_{2} formation.

Peroxynitrite Detection

Dihydorhodamine Oxidation

Dihydorhodamine 123 (DHR) is a cell-permeant, mitochondrial-avid analog of 2,7 dichlorodihydrofluorescein that can undergo oxidation to the fluorophore rhodamine 123. The oxidized rhodamine tends to be retained within the cell after tautomerization of its equivalent amino groups.\textsuperscript{138} Peroxynitrite readily oxidizes DHR 123; however, several cell-derived oxidants are also capable of oxidizing DHR 123. In addition to ONOO−, HOCl will also directly oxidize DHR 123.\textsuperscript{140} The direct reaction of DHR 123 with H_{2}O_{2} does not
occur but can be catalyzed by heme-containing peroxidases, such as HRP, or other heme compounds, including cytochrome c. Selective application of SOD, catalase, various NOS inhibitors, and ONOO− scavengers are required to provide for more precise identification of the substances responsible for DHR oxidation; thus, cellular oxidation of DHR 123 to rhodamine is at best a qualitative probe for ONOO−.

**Chemiluminescent Reactions**

Peroxynitrite reacts with LumH2 to yield chemiluminescence, a reaction enhanced by CO2 present in bicarbonate-containing systems attributable to formation of nitrosoperoxocarbonate (ONOOCO2). Whereas O2− does not directly react with LumH2, SOD inhibits ONOO−-dependent LumH2 chemiluminescence. The mechanisms for SOD inhibition of ONOO−-dependent LumH2 chemiluminescence include inhibition of ONOO− formation, SOD-dependent catalysis of ONOO− decomposition, and inhibition of O2−-mediated oxidation of preformed LumH2 radical. Excess NO can cause inhibition of ONOO−-dependent LumH2 chemiluminescence via reaction with LumH2 radicals. Although not well described, ONOO− also rapidly reacts with another chemiluminescent probe used for detection of O2−, coelenterazine. One potential strategy to thus discriminate between chemiluminescence that is principally derived from O2− or ONOO− would be to also determine Luc− chemiluminescence under identical conditions, because ONOO− does not result in Luc−-dependent chemiluminescence.

**Nitrotyrosine Formation**

Formation of free and protein 3-nitrotyrosine (NO2Tyr) derivatives has been used as a probe for reactive nitrogen species (RNS)-mediated vascular injury in recent years. Tissue NO2Tyr may simply serve as a dosimeter to indicate extents of RNS reaction or may be present in proteins at levels that will impair protein, cell, and organ function. Nitrating of protein tyrosine residues can readily alter function, because incorporation of a bulky NO2 group into tyrosine lowers the pKa of the phenolic group and imposes structural residues or at critical catalytic and structural residues of a few highly susceptible proteins, then low yields of NO2Tyr do not exclude NO2Tyr as a mediator of altered tissue structure and function. It is interesting to note that the yields of NO2Tyr in inflammatory events are similar in magnitude to the extents of tyrosine phosphorylation during cell signaling. It may also be plausible that tyrosine nitration can, in some cases, mimic tyrosine phosphorylation or adenylation as a novel mechanism for modulating protein function during inflammation. The identification of a NO2Tyr denitrase activity, conceptually similar to the protein phosphatases of tyrosine kinase signaling events, infers the reversibility of tyrosine nitration and supports nitration as a potential signaling event.

The principal mechanisms underlying tyrosine nitration in vascular diseases are subjects of ongoing investigation. Peroxynitrite will directly nitrate tyrosine in low yields, with transition metals catalyzing additional increases in yield. Peroxynitrite also reacts rapidly with adventitious CO2 to yield ONOOCO2−, with the radical products produced during decomposition of this intermediate viewed to be responsible for most ONOO−-mediated nitration reactions. Peroxynitrite is commonly and often erroneously implicated as the principal mediator of tyrosine nitration. Other facile tyrosine nitration pathways can also be operative during inflammatory oxidative reactions; myeloperoxidase (MPO) and other metalloproteins capable of peroxidase activity can catalyze tyrosine nitration in the vascular compartment. For example, neutrophil-derived MPO is intimately linked with inflammatory events, concentrates in the subendothelial matrix after neutrophil degranulation, and frequently displays spatial and temporal colocalization with tyrosine nitration. Because substrates supporting peroxidase-dependent tyrosine nitration (NO, NO2−, and hydroperoxides) become abundantly available for MPO-dependent nitration reactions, it is likely that multiple mechanisms will account for tissue NO2Tyr formation in diverse vascular inflammatory events. The aforementioned ONOO− and peroxidase-mediated nitration reactions all yield and occur via a common final and proximal nitrating species, NO2−. Interestingly, acidification of NO2− will also result in a chemistry that can result in tyrosine nitration. It remains to be demonstrated, however, whether acidification of intracellular microenvironments occurs to an extent that leads to nitrative chemistry.

It is important to note that many acidic and peroxide-containing tissue preparations and analytical reaction conditions are used in the mass spectroscopic and immunohistochemical quantitation of tissue NO2Tyr formation. It has recently been convincingly demonstrated that these pitfalls
can result in the artificial generation of NO$_2$-Tyr and overestimation of tissue NO$_2$-Tyr content.\cite{162,163} Despite the present uncertainty as to proximal mediators of tyrosine nitration and the extent to which this occurs (eg, NO$_2$Tyr/1000 tyr content of a protein or tissue preparation), the widespread occurrence and diverse mechanisms leading to tyrosine nitration underscore the significance of this process in inflammation.

In the future, it will be revealing to quantify in concert the multiple NO-dependent modifications that occur in vascular inflammatory responses.\cite{144} Multiple NO-dependent modifications that occur in vascular inflammatory responses provides a marker for pharmacological modulation at early candidate mechanisms underlying oxidative tissue injury and provides a marker for pharmacological modulation at early points in vascular inflammatory responses.

**Summary and Recommendations**

From the above discussion, it is apparent that no single technique will accurately reflect quantitative rates of formation of a particular reactive species under all circumstances, ie, when measuring free radical and oxidizing species in vascular cells and tissues, one size will not fit all. Because of the often-overlapping capacity of reactive oxygen and nitrogen species to chemically react with detector molecules, robust conclusions concerning rates of formation of reactive species will be markedly enhanced when a multifaceted approach is used. With the provided mechanisms of reaction for reactive species and the detector molecules used in a particular assay system and the recognition and accounting for potentially significant side reactions, experimentalists should be able to more reliably use detection methods yielding data that accurately reflect cell and tissue conditions.

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**References**


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