20

Detection of Reactive Oxygen Species and Nitric Oxide in Vascular Cells and Tissues

Comparison of Sensitivity and Specificity

Hua Cai, Sergey Dikalov, Kathy K. Griendling, and David G. Harrison

Summary

Reactive oxygen and nitrogen species are thought to contribute to pathogenesis of many cardiovascular diseases including hypertension, atherosclerosis, restenosis, heart failure, and diabetic vascular complications. Some of these reactive oxygen species also play an important role in vascular signaling. In this chapter, we describe various techniques that we have successfully employed to reliably measure superoxide and hydrogen peroxide. Because reactive oxygen species are capable of rapidly inactivating nitric oxide and because endothelial function characterized by nitric oxide bioavailability is an important indicator of vascular health, we have also included novel techniques capable of directly measuring nitric oxide radical from vascular cells and tissues.

Key Words: Reactive oxygen species; Nitric oxide; Vascular smooth muscle cells; Methods.

1. Introduction

Increased production of reactive oxygen species outstripping endogenous anti-oxidant defense systems has been referred to as oxidant stress, which in turn contributes to pathogenesis of many cardiovascular diseases including hypertension, atherosclerosis, restenosis, heart failure, and diabetic vascular complications ***(1–4)***. In mammalian cells, potential enzymatic sources of reactive oxygen species include the mitochondrial electron transport chain, the arachidonic acid metabolizing enzymes lipoxygenase and cycloxygenase,

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the cytochrome P450s, xanthine oxidase, NAD(P)H oxidases, uncoupled nitric oxide synthase (NOS), peroxidases, and other hemoproteins. Among biologically relevant and abundant reactive oxygen species, superoxide ($O_2^{\bullet-}$) and its dismutation product hydrogen peroxide (H_2O_2) appears most important in vascular signaling ***(1–4)***. On the contrary, recent studies suggest that $O_2^{\bullet-}$ and H_2O_2 may have differential signaling roles in the vasculature under various conditions ***(5–7)***. Both can be simultaneously produced by xanthine oxidase and some recently identified reduced nicotinamide dinucleotide (phosphate) (NAD(P)H) oxidases ***(6)***. Thus, production of H_2O_2 may correlate with formation of $O_2^{\bullet-}$. It does not, however, always reflect rate of $O_2^{\bullet-}$ generation, because other systems such as mitochondria or uncoupled eNOS produce $O_2^{\bullet-}$ solely. The efficacy of $O_2^{\bullet-}$ dismutation into H_2O_2 is affected by the abundance of intracellular superoxide dismutase (SOD) and small reducing molecules such as glutathione. Herein, we describe various techniques that we have successfully employed to reliably measure $O_2^{\bullet-}$ or H_2O_2 differentially. Because reactive oxygen species are capable of rapidly inactivating nitric oxide ($NO^{\bullet}$) and endothelial function characterized by $NO^{\bullet}$ bioavailability is an important indicator of vascular health ***(1,2)***, this review also discusses novel techniques capable of directly measuring $NO^{\bullet}$ from vascular cells and tissues.

Comprehensive reviews on methodologies detecting reactive oxygen species are available ***(8,9)***. This chapter instead primarily focuses on simplified and user-friendly experimental protocols and technical notes that are practically applicable to vascular biological studies. Taking into consideration the complexity of the biology of reactive oxygen species and limitations of individual techniques, it is generally recommended that reactive oxygen species should be measured with at least two different assays.

1.1. Superoxide Analysis

Though superoxide ($O_2^{\bullet-}$) is very short lived, largely cell impermeable and less likely to serve as a dominant signaling intermediate compared to other reactive oxygen species including H_2O_2 and $NO^{\bullet}$ it remains the precursor of many biologically relevant and important reactive oxygen species. More importantly, it rapidly reacts with $NO^{\bullet}$ to inactivate $NO^{\bullet}$ in a diffusion-limited fashion, which in turn leads to $NO^{\bullet}$ deficiency and endothelial dysfunction, hallmarks of many vascular diseases, including hypertension, atherosclerosis, and diabetic vascular complications ***(1–4)***. Many assays have been used to detect $O_2^{\bullet-}$ in test tubes, with cultured cells or intact blood vessels. Only the assays with which we are personally experienced are discussed in this chapter.

1.1.1. Dihydroethidium Staining

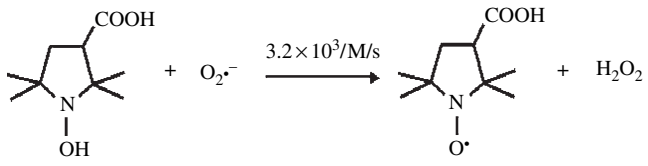
Dihydroethidium (DHE) or hydroethidine is a cell-permeable compound that, upon entering the cells, interacts with $O_2^{\bullet -}$ to form oxyethidium ***(10)***, which in turn interacts with nucleic acids to emit a bright red color detectable qualitatively by fluorescent microscope ***(8)***. Recent studies by Zhao et al. ***(10)*** demonstrated that $O_2^{\bullet -}$ oxidation of DHE yields oxyethidium, rather than the previously assumed ethidium bromide. The authors characterized this novel compound using HPLC. Fink et al. ***(11)*** adapted this high-performance liquid chromatography (HPLC)-based DHE assay for specific and quantitative detection of $O_2^{\bullet -}$ from biological samples, including cultured endothelial cells and isolated murine blood vessels.

1.1.2. Electron Spin Resonance with Superoxide-Specific Spin Traps

Free radicals such as $O_2^{\bullet -}$ and $NO^{\bullet}$ have unpaired electrons, and thus are paramagnetic and detectable by electron spin resonance (ESR). When conjugated to specific "spin traps"—compounds capable of selectively reacting with reactive oxygen species to prolong the half-lives of these molecules—free radicals-spin trap adducts generate characteristic signature spectrum that can be quantitatively analyzed using ESR ***(12)***. **Figure 1** illustrates $O_2^{\bullet -}$ conjugates after reacting with the traditional nitrone spin traps DMPO and DEPMPO. The nitrone family of $O_2^{\bullet -}$ spin traps has been thoroughly reviewed by Zweier and colleagues ***(13)***.

Recent studies however have demonstrated that cyclic hydroxylamines such as 1-hydroxy-3-carboxy-2,2,5,-tetramethyl-pyrrolidine hydrochloride (CPH) can react with $O_2^{\bullet -}$ to form a stable nitroxide radical with a much longer half-life (*see* **Fig. 2**). This is a distinct advantage over nitrone spin traps such as DEPMPO and DMPO, which form unstable $O_2^{\bullet -}$ adducts in biological samples ***(14)***. The reaction of cyclic hydroxylamines with $O_2^{\bullet -}$ is also a 100-fold faster than those with nitrone spin traps ***(15)***.

More importantly, cyclic hydroxylamines have been recently shown to specifically detect $O_2^{\bullet -}$ with high sensitivity in biological samples. They were



CPH CP

Fig. 1. Reaction of 1-hydroxy-3-carboxy-2,2,5,-tetramethyl-pyrrolidine hydrochloride (CPH) with $O_2^{\bullet -}$.

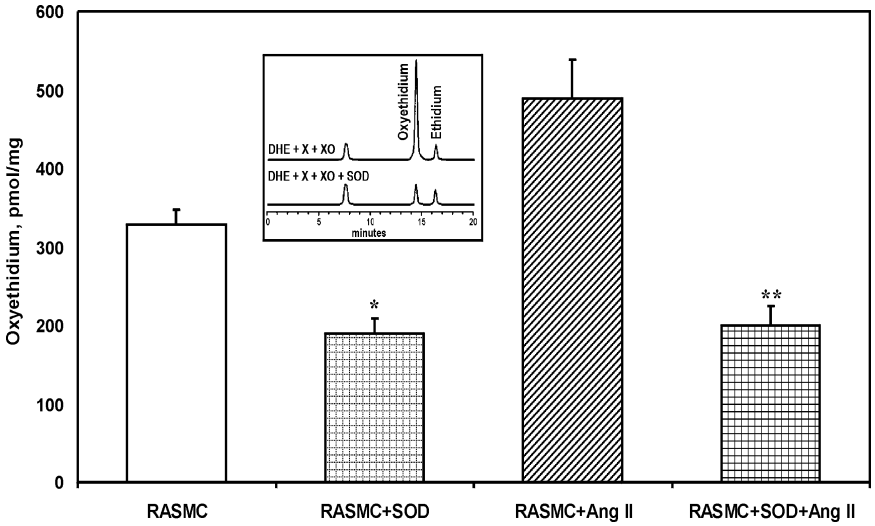


Fig. 2. Measurements of intracellular $O_2^{\bullet-}$ by dihydroethidium–HPLC. Inset shows typical HPLC diagram illustrating the formation $O_2^{\bullet-}$-specific adduct, oxyethidium. Treatment of rat aortic smooth muscle cells (RASMC) with polyethylene glycol (PEG)–superoxide dismutase (SOD) (18-h incubation with 25 U/ml) strongly inhibited oxyethidium formation in angiotensin II-stimulated (100 nmol/l, 4 h) cells. Data are presented as mean ± SEM. $^{*}p < 0.01$ versus RASMC, $^{**}p < 0.01$ versus angiotensin II.

used to detect $O_2^{\bullet-}$ formation from angiotensin II-stimulated or oscillatory shear stress-stimulated endothelial cells ***(16,17)*** or coronary artery homogenates of heart failure patients ***(18)***. ESR has a detection limit of approximately 1 nmol/L (10^{-9} M) for $O_2^{\bullet-}$, representing one of the most sensitive, quantitative, and characteristic means of measuring $O_2^{\bullet-}$.

1.1.3. Cytochrome C Reduction Assay

Traditionally, in the pre-ESR era, the cytochrome C reduction assay was considered the "gold standard" for detection of $O_2^{\bullet-}$. The rate constant for $O_2^{\bullet-}$ reduction of ferricytochrome C is approximately 1.5×10^5/M/s ***(8)***, allowing relatively sensitive detection of $O_2^{\bullet-}$.

1.1.4. Lucigenin Assay

Lucigenin at low concentrations ($< 5\,\mu$M) has been shown to detect $O_2^{\bullet-}$ without background noise derived from its own redox cycling ***(9)***. The experimental procedure, precautions, advantages, and potential caveats of this assay have been comprehensively reviewed previously by Munzel et al. ***(9)***. Lucigenin

remains a valid method for $O_2^{\bullet -}$ production, when appropriate experimental procedures are used, as clearly described in the abovementioned review.

1.2. Detection of Hydrogen Peroxide

Emerging evidence has demonstrated a critical role of H_2O_2 in vascular signaling (***19–20***). Different from the very short-lived $O_2^{\bullet -}$, H_2O_2 is more stable and freely diffusible among adjacent cells. Its production can be rapidly increased through agonist-provoked activation of vascular NAD(P)H oxidases. Uniquely, H_2O_2 also amplifies its own production and modulates endothelial function through complex mechanisms ***(19,20)***. These properties of H_2O_2 make it an ideal signaling molecule. Hydrogen peroxide levels in vascular cells and tissues can be monitored by the following assays.

1.2.1. Dichlorofluorescein Fluorescent Assay

The chemical basis of the assay is that upon entry into cells, 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) is cleaved by intracellular esterases to form DCFH, which is then oxidized by peroxides to highly fluorescent DCF.

1.2.2. Amplex Red Assay

Amplex Red (chemical name *N*-acetyl-3,7-dihydroxypgenoxazine) is a commercial compound from Molecular Probes. It reacts with extracellular H_2O_2 in the presence of peroxidase to form the highly fluorescent substance resorufin.

1.2.3. Hydrogen Peroxide Electrode

Liu and Zweier ***(21)*** previously reported that a *o*-phenylenediamine dihydrochloride (*o*-PD)-coated platinum microelectrode is capable of detecting H_2O_2 specifically and quantitatively from activated neutrophils. Although the specificity of the electrode is well characterized in the study and it detected 6–8 μmol/l H_2O_2 released from activated neutrophils, it remains unclear whether it is able to detect the small fraction of H_2O_2 released from vascular cells that have much lower production of H_2O_2.

1.3. Detection of Nitric Oxide Radical

It has been challenging to detect nitric oxide radical ($NO^{\bullet}$) directly from biological samples. Earlier studies mostly assessed $NO^{\bullet}$ "production" through indirect measurements of $NO^{\bullet}$ synthase activity using the L-arginine conversion assay or $NO^{\bullet}$ metabolites nitrite and nitrate using the Griess reagent. These assays, however, only reflect the enzyme activity or cumulative $NO^{\bullet}$ production, providing no information on actual bioavailable $NO^{\bullet}$. Studies in the past few

years have shown that a $NO^{\bullet}$-selective electrode and ESR represent specific and quantitative assays for detection of functional $NO^{\bullet}$.

1.3.1. $NO^{\bullet}$-Specific Microelectrode

Friedemann and colleagues ***(22)*** previously showed that nafion and *o*-PD-coated carbon electrode can directly detect $NO^{\bullet}$. We adapted this method for direct $NO^{\bullet}$ detection from porcine endocardium with and without atrial fibrillation ***(23)***. These studies showed that the $NO^{\bullet}$-selective electrode is able to detect $NO^{\bullet}$ in the low micromolar range.

1.3.2. ESR with $NO^{\bullet}$-Specific Spin Traps

Several iron compounds have been used to specifically trap $NO^{\bullet}$, including dithiocarbamate (DTC), N-methylglucamine dithiocarbamate (MGD), and diethyldithiocarbamate (DETC) ***(24)***. Although MGD is useful for extracellular detection of $NO^{\bullet}$ ***(25–27)***, iron-DETC is particularly useful for detection of $NO^{\bullet}$ in the cellular lipid membrane ***(28,29)***. The following protocols are described for both iron-MGD and iron-DETC.

2. Materials

2.1. Detection of Superoxide

2.1.1. DHE Staining—Microscopic Method

1. Endothelial cells, vascular smooth muscle cells, or isolated vascular segments.
2. Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC, USA).
3. Cover slips and slides (Fisher Scientific, Pittsburgh, PA).
4. DHE (Molecular Probes) stock solution: 2–5 mM dissolved in dimethylsulfoxide, prepare fresh in dark.
5. Phosphate-buffered saline (PBS).
6. Mounting media: Prolong anti-fade (Molecular Probes).
7. Fluorescent microscope.
8. Tissue Freezing Medium (Triangle Biomedical Sciences).

2.1.2. DHE Staining—HPLC Method

1. Endothelial cells, vascular smooth muscle cells, or isolated vascular segments.
2. Modified Kreb's/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer: 99.0 mM NaCl, 4.69 mM KCl, 1.87 mM $CaCl_2$, 1.20 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.03 mM K_2HPO_4, 20 mM sodium-HEPES, and 11.1 mM D-glucose (pH 7.35) containing 25 or 50 μM DHE Tissue Freezing Medium (Triangle Biomedical Sciences).
3. Acetonitrile (37–47%).
4. Trifluoroacetic acid (0.1%).

5. Xanthine oxidase (5 mU/ml).
6. Xanthine (10–100 μM).
7. Tissue Grinder (Fisher Scientific).
8. Syringe filter, 0.22 μm.
9. HPLC equipped with a sensitive fluorescent detector (Beckman Coulter, Fullerton, CA; Schimadzu by Fisher Scientific, Pittsburgh, PA).
10. C-18 reverse phase column (Necleosil 250, 4.5 mm; Sigma-Aldrich, St. Louis, MO, USA).

2.1.3. ESR

1. Endothelial or vascular smooth muscle cells.
2. Modified Krebs/HEPES buffer (*see* **Subheading 2.1.1.2.**).
3. Cyclic hydroxylamine CPH or 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH) stock solution (10 mM) (Alexis Biochemicals, San Diego, CA, USA) in modified Kreb's/HEPES buffer containing metal chelator, 25–50 μM deferoximine, and 3.5 μM DETC. This stock solution should be de-oxygenated by nitrogen gas continuously to maintain low background oxidation of the spin traps.
4. Lysis buffer containing protease inhibitors: 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM phenylmethyl sulfonyl fluoride, 2 μM bestatin, 1 μM pepstatin, and 2 μM leupeptin.
5. NADPH (0.2 mM).
6. Xanthine (0.1 mM).

2.1.4. Cytochrome C Reduction Assay

1. Endothelial cells or vascular cells.
2. Acetylated cytochrome C: 50 μM.
3. Potassium phosphate buffer pH 7.4 (KPi buffer): 50 mM K_2HPO_4 and 50 mM KH_2PO_4, pH 7.8.
4. Catalase 1 U/μl.
5. SOD (1 U/μl).
6. Fluorescent plate reader.

2.2. Detection of Hydrogen Peroxide

2.2.1. Dichlorofluorescein Fluorescent Assay

1. Cultured cells.
2. Dichlorofluorescein (DCF): 30 μM.
3. Fluorescent plate reader.

2.2.2. Amplex Red Assay

1. Amplex Red Assay kit from Molecular Probes.

2.3. Detection of Nitric Oxide Radical

2.3.1. $NO^{\bullet}$-Specific Microelectrode

1. Carbon fiber electrodes (100 μm length and 30 μm outer diameter; Word Precision Instruments, Sarasota, FL, USA).
2. *o*-PD solution (in 0.1 M PBS with 100 μM ascorbic acid).
3. Nafion (5% in aliphatic alcohols; Sigma-Aldrich).
4. Modified Kreb's/HEPES buffer.
5. Axopatch 200B amplifier (Axon Instruments, Union City, CA, USA).
6. Silver/silver chloride reference electrode.

2.3.2. Iron-DETC for Trapping of $NO^{\bullet}$

1. Culture endothelial cells.
2. Saline (0.9% NaCl).
3. $Fe^{2+}(DETC)_2$: $FeSO_4 \cdot 7H_2O$, 4.45 mg/10 ml for 1.6 mmol/l stock and DETC, 7.21 mg/10 ml for 3.2 mmol/l stock.
4. PBS.
5. Modified Kreb's/HEPES buffer.
6. Ferrous sulfate (4 mM).
7. *N*-methyl-D-glucamine dithiocarbamate MGD (20 mM).

3. Methods

3.1. Detection of Superoxide Anion

3.1.1. DHE Assay—Microscopic Method (See **Notes 1 and 2**)

1. Culture endothelial cells or vascular smooth muscle cells on glass cover slips in six-well plates. Alternatively, embed freshly isolated vascular segments (2 mm) in tissue freezing medium, section to 30 μm, and mount on cover slips.
2. Dilute DHE in PBS to final concentration of 2–5 μmol/l. Add 200 μl drops of DHE–PBS solution on cell monolayer or tissue section and incubate at 37 °C in dark for 30 min.
3. Rinse off excess DHE with PBS twice, drip off excess liquid, and mount cover slips to microscopic slides using mounting media.
4. Capture images immediately with a fluorescent microscope at excitation and emission wavelengths of 520 and 610 nm, respectively.
5. If drug treatment is desired, pre-incubate with cells or tissue sections prior to addition of DHE.

3.1.2. DHE Assay—HPLC Method (See **Notes 3 and 4**)

1. Treat endothelial cells or 2-mm vessel segments (*see* **Note 4**) with desired agonists and incubate in modified Kreb's/HEPEs buffer at 37 °C in dark for 15 min–1 h.

2. At end of the incubation, collect cells by gentle scraping, homogenize cells or vessel segments in chilled methanol using a Tissue Grinder, and filter suspensions through a 0.22-μm syringe filter.
3. Analyze the supernatant using a HPLC equipped with a C-18 reverse phase column and a sensitive fluorescent detector, at excitation and emission wavelengths of 480 and 580 nm, respectively. A gradient of acetonitrile from 37 to 47% over 23 min at a flow rate of 0.5 ml/min mixed with 0.1% trifluoroacetic acid is used as solvent to separate DHE, ethidium, and oxyethidium.
4. Calibration of oxyethidium formation from DHE is obtained by incubation of DHE with xanthine oxidase (5 mU/ml) and increasing concentrations of xanthine (10–100 μM).
5. A representative HPLC–DHE assay of $O_2^{\bullet -}$ production from angiotensin II-stimulated vascular smooth muscle cells is presented in **Fig. 3**.

*3.1.3. ESR with Superoxide-Specific Spin Traps (*See ***Notes 5 and 6***)

1. Culture endothelial or vascular smooth muscle cells to confluence on 100-mm Petri dishes. Wash off media and collect cells in modified Kreb's/HEPES buffer.
2. Gently scrape cells off culture dish and collect in ice-cold Kreb's/HEPEs buffer. After centrifuging at 500 *g* for 10 min at 4 °C, re-suspend cell pellets in freshly prepared deferoxamine-containing Kreb's/HEPEs buffer. Usually a density of 1×10^6 cells/10 μl is desired.
3. Mix in an Eppendorf tube 10 μl cell suspension, 80 μl of Kreb's/HEPEs buffer, and 10 μl CPH or CMH stock solution (final concentration 1 mM).
4. Immediately transfer sample into glass capillaries and load capillaries into appropriate ESR cavity for 10-min time scan.

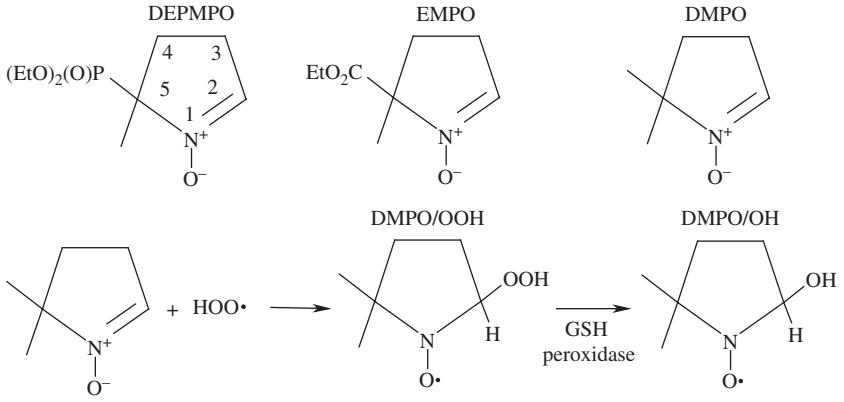


Fig. 3. Nitrone spin traps and formation of $O_2^{\bullet -}$-derived radical adducts.

3.1.3.1. Analysis of $O_2^{\bullet-}$ in Membrane Preparations of Cells or Tissues to Assess NADPH Oxidase and Xanthine Oxidase Activity

1. Homogenize cultured vascular endothelial cells, smooth muscle cells, or vessel segments in lysis buffer using a Tissue Grinder.
2. Centrifuge samples at 750 *g* at 4 °C for 10 min. Remove supernatant and centrifuge at 30, 000 *g* at 4 °C for 30 min.
3. Resuspend membrane pellet in 150 μl lysis buffer and measure protein concentration using the Bradford method.
4. Prepare cyclic hydroxylamine CPH or CMH stock solution as described above.
5. Mix in an Eppendorf tube with 10 μl membrane preparation: 80 μl of Kreb's/HEPEs buffer and 10 μl of the CPH stock solution (1 mM). Activity of NADPH oxidase can be determined by adding NADPH (0.2 mM) as the substrate, whereas activity of xanthine oxidase is determined by adding xanthine (0.1 mM).
6. Immediately transfer membrane-spin trap mix into glass capillaries and load capillaries into appropriate ESR cavity for 10-min time scan. Use the following ESR settings described below:

Bruker EMX. Field sweep, 50 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 2 G; conversion time, 1312 ms; time constant, 656 ms; 512 points resolution; and receiver gain, 1×10^5.

Miniscope 200. Biofield, 3350 G; field sweep, 40 G; microwave frequency, 9.78 GHz; microwave power, 20 mW; modulation amplitude, 3 G; 4096 points resolution; and receiver gain, 500.

*3.1.4. Cytochrome C Reduction Assay (*See ***Notes 7 and 8***)

1. Culture endothelial cells or vascular smooth cells in 6-well or 12-well plates or prepare vessel segments (3×2 mm rings). Wash off media or blood residues using warm PBS.
2. Incubate cells or tissues with acetylated cytochrome C (50 μM) in KPI buffer containing 1 U/μl catalase (to prevent re-oxidation of reduced cytochrome C by H_2O_2) in the dark at 37 °C for 1 h. An identical set of samples is incubated in the presence of SOD (1 U/μl) for subtraction of the SOD-inhibitable signal.
3. Transfer 200 μl post-incubation supernatant to a fresh 96-well plate and read the plate with a fluorescent plate reader at 540, 550, and 560 nm.
4. Calculate the optical density (OD) of reduced cytochrome C using the path-length-corrected values: $OD_{550nm} - (OD_{540nm} + OD_{560nm})/2$.
5. Subtract OD from that of identical samples containing SOD. Covert to $O_2^{\bullet-}$ concentration using the extinction coefficient equation of $Em_{550nm} = 2.1 \times 10^4$ M/cm.

3.2. Detection of Hydrogen Peroxide

*3.2.1. DCF Fluorescent Assay (*See ***Note 9***)

1. Culture endothelial cells or vascular smooth muscle cells on 100-mm Petri dishes.
2. Aspirate media, rinse cells with PBS, and load cells or vessel segments with freshly prepared DCFH-DA (30 μM) for 15 min to allow intracellular conversion of DHCF prior to stimulation with desired agonists.
3. At the end of incubation, gently scrape cells off dish in 1 ml ice-cold PBS, load 200 μl cell suspensions ($\sim 2 \times 10^5$ cells) into 96-well plate, and read with a fluorescent plate reader at excitation and emission wavelengths of 475 and 525 nm, respectively.

*3.2.2. Amplex Red Assay (*See ***Note 10***)

1. Culture endothelial cells or vascular smooth muscle cells on P100 Petri dishes or prepare three vessel segments of 2 mm size.
2. Incubate cells or vessel segments in Krebs Ringer phosphate-glucose buffer (see manufacturer's manual) containing 100 μM Amplex Red and 1 U/ml horseradish peroxidase at 37 °C for 30 min, with or without desired agonists.
3. At end of incubation, transfer 200 μl post-incubation buffers into 96-well plate and read with a fluorescent plate reader at excitation and emission wavelengths of 530 and 580 nm, respectively.
4. Generate calibration curve using resorufin standard supplied by the manufacturer.

3.3. Detection of Nitric Oxide Radical

*3.3.1. $NO^{\bullet}$-Specific Microelectrode (*See ***Notes 11 and 12***)

1. Coat bare carbon fiber electrodes (100 μm length and 30 μm outer diameter; Word Precision Instruments) with nafion and *o*-PD. Coat with freshly made *o*-PD solution at constant potential (+0.9 V vs. Ag/AgCl reference electrode) for 45 min. Dip in nafion solution for 3 s and dry for 5 min at 85 °C. The nafion-coating cycle should be repeated 10–15 times.
2. Culture endothelial cells on 35-mm dishes or prepare fresh tissue samples in freshly made modified Kreb's/HEPES buffer.
3. Place the electrode tip at the surface of an individual cell, endocardium, or lumen of blood vessels, and then withdraw precisely 5 μm.
4. Record $NO^{\bullet}$-dependent oxidation currents (voltage clamp mode, hold at 0.65 V, approximately the voltage for peak $NO^{\bullet}$ oxidation) immediately after addition of agonists using an Axopatch 200B amplifier (Axon Instruments). A silver/silver chloride reference electrode is used. Use pCLAMP 7.0 program (Axon Instruments) for delivery of voltage protocols and data acquisition and analysis.

5. Calculate $NO^{\bullet}$ concentrations from a standard curve obtained using dilutions of de-oxygenated, saturated $NO^{\bullet}$ gas solutions.

3.3.2. Iron-DETC Protocol for Intracellular Trapping of $NO^{\bullet}$ (See ***Notes 13 and 14***)

1. Culture endothelial cells on 100-mm Petri dishes or prepare vascular segments (6–12 2-mm vessel segments).
2. Bubble freshly prepared saline (0.9% NaCl) with nitrogen gas to remove oxygen.
3. Aspirate media and rinse cells with warm PBS once, add 1.5 ml modified Kreb's/HEPES buffer with or without desired agonists, then mix $Fe^{2+}(DETC)_2$, and immediately add to culture dish (500 µl of each solution, final volume 2.0 ml).
4. Incubate in cell culture incubator for desired period for cumulative trapping of $NO^{\bullet}$.
5. Aspirate buffer, gently collect cells into a 1-ml insulin syringe, snap freeze in liquid nitrogen, then transfer sample column into a finger dewer, and capture $Fe^{2+}(DETC)_2$–$NO^{\bullet}$ signal using ESR at the following settings:

Bruker EMX: Field sweep, 160 G; microwave frequency, 9.39 GHz; microwave power, 10 MW; modulation amplitude, 3 G; conversion time, 2621 ms; time constant, 328 ms; modulation amplitude, 3 G; receiver gain, 1×10^4; and four scans.

Miniscope 200: Biofield, 3267; field sweep, 100 G; microwave frequency, 9.78 GHz; microwave power, 40 mW; modulation amplitude, 10 G; 4096 points resolution; and receiver gain, 900.

3.3.2.1. Iron-MGD Protocol for Extracellular Trapping of $NO^{\bullet}$ (See ***Notes 15 and 16***)

1. Culture endothelial cells on 100-mm Petri dishes or prepare vascular segments (6–12 2-mm vessel segments).
2. Bubble freshly prepared saline (0.9% NaCl) with nitrogen gas to remove oxygen, and then make stock solutions of $FeSO_4 \cdot 7H_2O$, 4 mM, and MGD, 20 mM.
3. Prepare stock solutions of Fe^{2+}MGD by mixing $FeSO_4$ and MGD at the ratio of 1:5 or 1:10 (final Fe concentration: 0.5 mM).
4. Aspirate media and rinse cells with warm PBS once, add 1.5 ml modified Kreb's/HEPES buffer with or without desired agonists, then mix $Fe^{2+}(MGD)_2$, and immediately add to culture dish.
5. Incubate in cell culture incubator for desired period for cumulative trapping of $NO^{\bullet}$.
6. Collect 1 ml of post-incubation supernatant into a 1-ml insulin syringe and snap freeze in liquid nitrogen, then transfer sample column into a finger dewer, and capture Fe^{2+}MGD–$NO^{\bullet}$ signal using ESR settings as described above for $Fe^{2+}(DETC)_2$.

4. Notes

1. DHE is cell permeable, which allows intracellular detection of $O_2^{\bullet-}$. It is relatively specific with minimal potential of being oxidized by H_2O_2 or other reactive oxygen species ***(30)***. It is very sensitive (fluorescent detection with HPLC identifies as low as 1 pmol/mg protein $O_2^{\bullet-}$, see protocol below) ***(11)*** and generally reproducible. The procedure is convenient to use as an initial screening for $O_2^{\bullet-}$ production from both cells and tissues. Results from this widely used $O_2^{\bullet-}$ -detection method have been confirmed by recent ESR studies that unequivocally identify and quantify individual reactive oxygen species. For example, aortic $O_2^{\bullet-}$ production was found to be increased in angiotensin II-induced hypertension using Lucigenin ***(31)*** or DHE assays ***(32)***, and this was recently confirmed by ESR $O_2^{\bullet-}$ trapping with the $O_2^{\bullet-}$-specific spin trap cyclic hydroxylamine ***(16)***.
2. DHE staining is semi-quantitative. It was demonstrated that DHE can be oxidized by excessive cytochrome C (when the molar ratio of cytochrome C to DHE is >10) ***(30,33)*** or other heme-containing proteins such as hemoglobin ***(33)***. Thus, caution should be used when interpreting DHE data in cases where apoptosis or mitochondrial damage is involved or post-hemorrhage endothelium is studied. To minimize influences of this non-specific reaction on data analysis, it is recommended to use cell permeable SOD such as polyethylene glycol-conjugated SOD (Sigma-Aldrich) in parallel and only compare responses that are SOD inhibitable. Another caution is *not* to use lower wavelengths of 490–495/580–600 nm ***(34)***. Recent studies demonstrated that peroxidase-catalyzed oxidation of DHE by H_2O_2 produced fluorescent products detectable at the abovementioned wavelengths ***(34)***. Using 520/610 nm could potentially avoid these non-specific overlapping fluorescent signals caused by H_2O_2. In many cases, researchers have to use the wavelengths with which the microscope is equipped, and this may have caused variations in data reporting.
3. DHE–HPLC provides quantitative, sensitive, and specific detection of $O_2^{\bullet-}$ from vascular cells and tissues. Fink and colleagues ***(11)*** have shown that the specific HPLC peak for oxyethidium is completely preventable by SOD but is not formed in response to H_2O_2 or peroxynitrite.
4. Modestly larger amounts of biological materials are needed compared to fluorescent imaging of DHE (i.e., three 2-mm aortic rings are required for HPLC assay of DHE compared to only one 2-mm ring for fluorescent microscope). This is, however, a minimal disadvantage considering the quantitative nature of the HPLC-based assay.
5. ESR spin trapping of $O_2^{\bullet-}$ has been proven to selectively detect $O_2^{\bullet-}$ from cultured vascular cells or tissue homogenates in the low nanomolar range ***(16,18)***. Our unpublished data suggest that this method is also applicable to intact, isolated blood vessels, but a slightly larger capillary and fitting holder is required to accommodate vessel segments inside the ESR cavity. In addition, cyclic hydroxylamine compounds can be injected into mice for in vivo detection of $O_2^{\bullet-}$, but the preferential tissue distribution patterns of these compounds remains unclear ***(35,36)***.

6. CMH is cell permeable and more suitable for detection of intracellular $O_2^{\bullet-}$. CPH, however, is used for extracellular detection of $O_2^{\bullet-}$. The advantage of the spin traps is that the ESR spectrum of the radical spin traps works as a "finger print" of the trapped radical. The problem, however, deals with the limited stability of the radical adducts. Even relatively stable $O_2^{\bullet-}$ adducts of DMPO and DEPMPO can be decomposed by intracellular peroxidases into the corresponding hydroxyl adducts (**Fig. 3**). In case of cyclic hydroxylamines, though very stable when forming adducts with $O_2^{\bullet-}$, they can also trap peroxynitrite to form identical nitroxides ***(15)***, limiting specificity. Thus, it is highly recommended that only the SOD-inhibitable fraction of the ESR signals is compared between experimental groups.
7. Because cytochorome C is cell impermeant, this assay provides quantitative detection of *extracellular* $O_2^{\bullet-}$.
8. Reduced cytochrome C can be oxidized by peroxidases or oxidants such as H_2O_2 and peroxynitrite ***(8,37)***. This will underestimate the rate of production of $O_2^{\bullet-}$. Our own experience suggests that the reproducibility is not great, and the assay is not sensitive enough to detect the low levels of $O_2^{\bullet-}$ found in vascular cells. As cytochrome C is also strictly cell *im*permeable, it is more applicable to activated neutrophils where $O_2^{\bullet-}$ is produced in large quantities extracellularly. It is likely that some intracellular $O_2^{\bullet-}$ can be transported through membrane pores to extracellular space. However, as regulation of this process is poorly understood, it is difficult to predict the intracellular $O_2^{\bullet-}$ production rate based on cytochrome C data. Nevertheless, the cytochrome C assay can be used as a secondary method for $O_2^{\bullet-}$ detection in conjunction with another more sensitive method for intracellular $O_2^{\bullet-}$ to estimate overall bioactive $O_2^{\bullet-}$.
9. This assay can be used for intracellular detection of H_2O_2 and is relatively specific. DCF assay is only semi-quantitative, because it is difficult to generate a standard curve that mimics the intracellular situation. In the past, we attempted to add esterases to ex vivo incubation of DCFH-DA, but this was not sufficient to produce optimal concentration-absorbance curves when serial dilutions of standard were used. It is unclear whether other enzymes besides esterases are involved in converting DCFH-DA intracellularly. Earlier work by LeBel et al. ***(38)*** demonstrated that DCF is not oxidized by hydroxyl radical or $O_2^{\bullet-}$ directly, although it can also be oxidized by peroxynitrite ***(39)***. Similar to $O_2^{\bullet-}$ assays, it is always recommended to use cell-permeable catalase (i.e., polyethylene glycol-conjugated catalase from Sigma-Aldrich) and compare only the catalase-inhibitable signal, because DCF reacts with other cellular peroxides and can redox cycle ***(40)***. Nevertheless, the DCF assay can be used for relative comparison among different experimental groups.
10. The Amplex Red assay is good for specific, extracellular detection of H_2O_2 and is also quantitative. Recent studies using this method have demonstrated basal endothelial H_2O_2 production at the rate of 30 pmol/mg protein/min ***(17,27)***. However, this assay may not accurately reflect intracellular formation of H_2O_2

although H_2O_2 is expected to diffuse to the outside of the cell to reach equilibrium. It is unclear how much H_2O_2 is able to diffuse out of the cell, considering presence of intracellular catalase and small thiols that are capable of "trapping" H_2O_2 intracellularly. The activities of these H_2O_2-consuming enzymes or small molecules may change with agonist treatment to compound the capability of the assay to reflect actual H_2O_2 production rate.

11. Control experiments showed that nafion and *o*-PD coatings effectively eliminated electrode responsiveness to other oxidizable species, including nitrate, nitrite, and H_2O_2. This method is not only specific and quantitative, it also offers "real-time" monitoring of $NO^{\bullet}$ production over a time course that has particular utility for signal transduction studies examining temporal and spatial activation of $NO^{\bullet}$-producing pathways.
12. This assay requires an entire patch-clamp electrophysiology setup. It only detects $NO^{\bullet}$ from released from single endothelial cell or the most surrounding few endothelial cells. This is because $NO^{\bullet}$ has limited diffusion ability to reach the electrode in the in vitro system. It may not reflect overall $NO^{\bullet}$ production very accurately.
13. Several advantages exist for $NO^{\bullet}$ detection using ESR and $Fe^{2+}(DETC)_2$. $Fe^{2+}(DETC)_2$ is specific for bioactive $NO^{\bullet}$ and does not detect nitrite or nitrate. In addition, the stability of the $Fe^{2+}(DETC)_2$–$NO^{\bullet}$ complex allows for measurement of the cumulative amount of bioactive $NO^{\bullet}$ produced over time.
14. Limitations of this technique include the special handling required for $Fe^{2+}(DETC)_2$ colloid to prevent oxidation. In addition, $Fe^{2+}(DETC)_2$–$NO^{\bullet}$ can be oxidized by extracellular H_2O_2 or $O_2^{\bullet-}$ to form the ESR silent, $Fe^{3+}(DETC)_2$–$NO^{\bullet}$. However, our data showed that bolus addition of 100 μmol/l H_2O_2 or the same amount of $O_2^{\bullet-}$ generated by xanthine oxidase decreased the $Fe^{2+}(DETC)_2$–$NO^{\bullet}$ signal by 20%. Both H_2O_2 and $O_2^{\bullet-}$ caused line broadening of the ESR spectra due to accumulation of Fe^{3+} in the samples. The effect of the line broadening on the quantification of $NO^{\bullet}$ can be avoided by double integration of the ESR signal of $Fe^{2+}(DETC)_2$–$NO^{\bullet}$.
15. Fe^{2+}MGD stays in the extracellular compartment and thus allows detection of $NO^{\bullet}$ that is diffused out the of endothelial cells. It may thus reflect amount of $NO^{\bullet}$ that is available to travel within intracellular space. However, how much $NO^{\bullet}$ eventually reaches adjacent vascular smooth muscle cells also depends on extracellular SOD activity in vivo.
16. It has been previously reported that autoxidation of MGD may cause formation of $O_2^{\bullet-}$ and H_2O_2, which may interfere with quantitative detection of $NO^{\bullet}$ ***(41)***. It was also shown that anaerobic solutions of Fe^{2+}MGD can reduce nitrite to $NO^{\bullet}$ ***(42)***. However, this reaction is negligible under normoxic conditions.

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