ROS

ROS PROTOCOLS

Assays for Detecting Biological Superoxide

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ABSTRACT | Superoxide is produced from various endogenous and exogenous sources and has been shown to play an important role in diverse physiological and pathophysiological processes. Thus, detection and measurement of this ubiquitous reactive oxygen species (ROS) are instrumental in understanding its role in biology and medicine. This ROS protocol article describes two well-established assays for detecting superoxide, namely, superoxide dismutase-inhibitable ferricytochrome c reduction and electron paramagnetic resonance (EPR) spectrometry in conjunction with 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO)-spin trapping. The article provides a step-by-step, recipe-type protocol for each of the two assays and discusses their advantages and limitations.

KEYWORDS | 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; Electron paramagnetic resonance; Ferricytochrone c reduction; Reactive oxygen species; Spin trapping; Superoxide; Superoxide dismutase

ABBREVIATIONS | CPBS, phosphate-buffered saline; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DEPMPO-OOH, DEPMPO-superoxide adduct; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance; HASMCs, human aortic smooth muscle cells; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TPA, 12-*O*-tetradeconylphorbol-13-acetate; XO, xanthine oxidase

CONTENTS

1. OVERVIEW

Superoxide, among the different forms of ROS, has received the most attention due to its unique biochemical properties, including: (i) its reactivity towards iron-sulfur cluster-containing enzymes; (ii) its rapid reaction with nitric oxide, forming peroxynitrite, a potent oxidant; and, (iii) more importantly, its

acting as a precursor for many other reactive oxygen and related species. Superoxide production has been implicated in diverse physiological and pathophysiological processes. Therefore, detection and quantification of fluxes of superoxide in cells and tissues are of critical importance for further investigating the biological effects of this oxygen free radical.

Over the past several decades, a variety of assays have been developed for the detection and/or measurement of superoxide in biological systems. These include assays based on ultraviolet/visible (UV/Vis) spectrophotometry (e.g., the superoxide dismutase-inhibitable ferricytochrome c reduction assay); chemiluminescence-based methods (e.g., the lucigenin-derived chemiluminescence assay); electron paramagnetic resonance (EPR) spectrometry in conjunction with spin trapping technique (e.g., the 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide-spin trapping assay); and fluorescence-based spectrometry, microscopy, and flow cytometry (e.g., the dihydroethidium oxidation and MitoSox assays), among many others [1–5].

In this ROS protocol article, we describe two well-established methods for detecting biological superoxide. They are the superoxide dismutase (SOD)-inhibitable ferricytochrome c reduction and the 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO)-spin trapping assays.

2. METHOD PRINCIPLES

As mentioned above, the SOD-inhibitable ferricytochrome c reduction and the EPR DEPMPO-spin trapping assays are among the most commonly used and firmly established methods for detecting biological superoxide. Understanding of the method principles is imperative for the appropriate use of these two methods as well as the correct interpretation of the data produced.

2.1. Method Principle of the SOD-Inhibitable Ferricytochrome c Reduction Assay

Reduction of ferricytochrome c is measured spectrophotometrically. This necessitates a brief introduction to the basic theory and principle of UV/Vis spectrophotometry. This section focuses on describing the assay principle behind using SOD-inhibitable ferricytochrome c reduction to detect biological superoxide formation. This assay is one of the earliest methods developed for detecting superoxide.

2.1.1. General Principle of UV/Vis Spectrophotometry

UV/Vis spectrophotometry is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample solution and measuring the intensity of light reaching a detector. The Beer-Lambert law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length (typically 1 cm), UV/Vis spectrophotometry can be used to determine the concentration of the absorber in a solution. For detecting ROS, including superoxide, by UV/Vis spectrophotometry, the detecting probe is incubated with the ROS-generating system (e.g., enzymes, cells, tissues), and the reaction product is detected at a specific absorbance wavelength. The ideal ROS-detecting UV/Vis spectrophotometric probes are substances with optical properties that change markedly after reaction with ROS and show high specificity for different ROS [5]. Simplicity is a major advantage of the UV/Vis spectrophotometry-based ROS-detecting assays. The principal disadvantage is the limited sensitivity as compared with other methods, such as fluorescence- and chemiluminescence-based assays.

2.1.2. Assay Principle of SOD-Inhibitable Ferricytochrome c Reduction

Ferricytochrome c reduction assay is a classical method for detecting the rates of superoxide formation by enzymes, cells, and tissues. Superoxide reduces ferricytochrome c (Fe^{3+}-cytochrome c) to ferrocytochorme c (Fe^{2+}-cytochrome c), and the reaction rate constant is estimated to be ~3×10^5 $M^{-1}s^{-1}$ at a physiologically relevant pH and room temperature [6]. The reaction is monitored spectrophotometrically at 550 nm (Reaction 1) (**Figure 1**). The extinction coefficient for ferricytochrome c is 0.89×10^4 $M^{-1}cm^{-1}$and for ferrocytochrome c is 2.99×10^4 $M^{-1}cm^{-1}$. Hence, $\Delta E_{m550} = 2.1 \times 10^4$ $M^{-1}cm^{-1}$ [7]. ΔE_{m550} is used to calculate the amount of ferrocytochrome c formation in the reaction. Reduction of ferricytochrome c is nonspecific for superoxide. Hence, exogenous SOD is included in the assay to determine

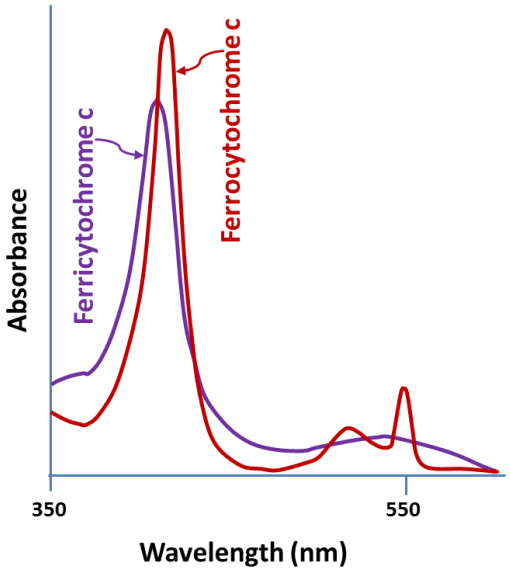

FIGURE 1. Absorbance spectra of ferricytochrome c and ferrocytochrome c. Ferrocytochrome c has a stronger absorbance at 550 nm compared with ferricytochrome c.

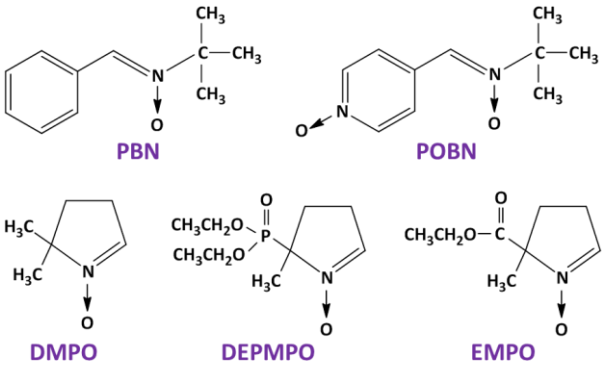

FIGURE 2. Structures of commonly used nitrone spin traps for detecting biological free radicals. PBN, α-phenyl-*tert*-butylnitrone; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; EMPO, 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide.

the SOD-inhibitable ferricytochrome c reduction, which is a selective indicator of biological superoxide generation.

$$Fe^{3+}\text{-cytochrome c} + O_2^{\bullet -} \rightarrow Fe^{2+}\text{-cytochrome c} + O_2 \quad (1)$$

2.2. Method Principle of the DEPMPO-Spin Trapping Assay

The DEPMPO-spin trapping assay involves the use of EPR spectrometry. This necessitates a brief introduction to EPR and EPR-spin trapping before description of the principle of DEPMPO-spin trapping detection of biological superoxide.

2.2.1. General Principle of EPR Spectrometry and Spin Trapping

Free radicals have unpaired electrons and as such are paramagnetic and detectable by EPR. EPR techniques are considered the only analytic approach that permits the direct detection of free radicals. EPR methods are also most specific for detecting free radicals in biological systems. Due to the relatively low sensitivity of EPR detection and the fact that biologically relevant free radicals, such as superoxide, hydroxyl radical, and nitric oxide, are in low concentrations and short-lived, these free radical species cannot be directly detected by EPR spectrometry. This dilemma can be circumvented by EPR detection of more stable secondary radical adducts formed by adding exogenous spin traps. Spin traps are molecules that react with primary free radical species to give rise to longer lasting free radical adducts with characteristic EPR signatures that can accumulate to levels permitting detection [5].

Nitrones have been the most widely used spin traps for detecting free radicals including oxygen radicals and carbon-centered radical species in biological systems. There are two classes of nitrone spin traps: the linear and the pyrroline-based cyclic nitrones. The linear nitrone spin traps include α-phenyl-*tert*-butylnitrone (PBN) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN). The pyrroline-based cyclic nitrone spin traps consist of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), DEPMPO, and 5-ethoxycarbonyl-5-methyl-1-pyrroline-*N*-oxide (EMPO) [8] (structures shown in **Figure 2**). These spin traps differ with respect to their affinity to free radicals and the stability of the radical adducts formed. In ad-

dition, the toxicity also varies with different spin traps. In this context, PBN and POBN have been shown to be well-tolerated in experimental animals, permitting their use for in vivo detection of free radical generation.

The nitrone chemical structure in its simplest form can be written as X-CH=NO-Y. The nitrone trapping of a free radical intermediate is represented in the following simple reaction in which the free radical intermediate (R˙) is trapped by the nitrone to form a nitroxyl free radical spin adduct (X-CHR-NO˙-Y) (Reaction 2) [9].

$$X\text{-}CH{=}NO\text{-}Y + R^{\cdot} \rightarrow X\text{-}CHR\text{-}NO^{\cdot}\text{-}Y \quad (2)$$

The nitroxyl free radical spin adduct is usually much more stable than the original free radical intermediate, therefore making it possible in principle to detect and characterize the original free radical intermediate using EPR spectrometry.

2.2.2. Assay Principle of DEPMPO-Spin Trapping

When conjugated to specific spin traps (here spin traps refer to compounds that react with superoxide to form relatively stable adducts), superoxide-spin trap adducts exhibit characteristic signature spectra that can be quantitatively analyzed by EPR spectrometry. **Figure 3** shows that the reaction between superoxide and the spin trap DEPMPO results in the formation of DEPMPO-superoxide adduct (DEPMPO-OOH) with a characteristic signature spectrum [10]. By measuring the magnitude of the spectrum, the relative amounts of superoxide formation in biological systems can be estimated. As mentioned above, certain EPR spin traps, such as PBN (**Figure 2**), can also be administered to experimental animals to determine the in vivo formation of free radicals, including superoxide [5].

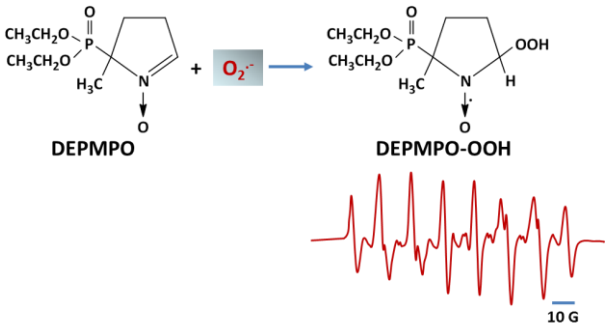

FIGURE 3. Specific detection of biological superoxide generation by EPR spectrometry/spin trapping using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) as a spin trap. As shown, reaction of superoxide with DEPMPO forms the DEPMPO-superoxide (DEPMPO-OOH) adduct with a characteristic signature EPR spectrum. The magnitude of the spectrum is proportional to the amounts of biological superoxide formation.

3. MATERIALS AND INSTRUMENTS

3.1. Materials and Instruments for the SOD-Inhibitable Ferricytochrome c Reduction Assay

This article describes the SOD-inhibitable ferricytochrome c reduction assay for detecting superoxide generated by two systems: an enzymatic system of xanthine/xanthine oxidase (XO) and a cell culture system of normal human aortic smooth muscle cells (HASMCs).

3.1.1. Materials for the SOD-Inhibitable Ferricytochrome c Reduction Assay

The major materials for the SOD-inhibitable ferricytochrome c reduction assay are listed alphabetically in **Table 1**.

3.1.2. Major Instruments for the SOD-Inhibitable Ferricytochrome c Reduction Assay

(1) Beckman Coulter DU-800 UV/Vis spectrophotometer with a 6-cuvette holder
(2) Water bath or dry bath
(3) Cell culture equipment (CO_2 incubator, tissue culture hood, centrifuge, microscope, etc.)

3.2. Materials and Instruments for the DEPMPO-Spin Trapping Assay

This article describes DEPMPO-spin trapping assay for detecting superoxide generated by two systems: an enzymatic system of xanthine/XO and a cell culture system of mouse macrophages stimulated with 12-*O*-tetradeconylphorbol-13-acetate (TPA).

TABLE 1. Major materials for the SOD-inhibitable ferricytochrome c reduction assay

Major Material	Vendor and Catalog Number	Note
Complete phosphate-buffered saline (CPBS)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, 2.67 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose, pH 7.4
Cu,Zn superoxide dismutase	Sigma-Aldrich; S5395	Source: bovine erythrocytes
Ferricytochrome c	Sigma-Aldrich; C7752	Source: horse heart; molecular mass: 12,384 Da
Human aortic smooth muscle cells (HASMCs)	ATCC; PCS-100-012	HASMCs are normal primary cells.
Phosphate-buffered saline (PBS)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, 2.67 mM KCl, pH 7.4
Xanthine	Sigma-Aldrich; X3627	Sodium salt
Xanthine oxidase	Sigma-Aldrich; X4500	Source: bovine milk

3.2.1. Materials for the DEPMPO-Spin Trapping Assay

The major materials for the DEPMPO-spin trapping assay are listed alphabetically in **Table 2**.

3.2.2. Major Instruments for the DEPMPO-Spin Trapping Assay

(1) Bruker eScan EPR spectrometer or Bruker EMXplus EPR spectrometer
(2) Water bath or dry bath
(3) Cell culture equipment (CO_2 incubator, tissue culture hood, centrifuge, microscope, etc.)

4. PROTOCOLS AND STEPS

This section describes the detailed protocols and steps involved in the use of the SOD-inhibitable ferricytochrome c reduction and DEPMPO-spin trapping assays to detect superoxide generated from the xanthine/XO system as well cultured cells.

4.1. Protocols and Steps for the SOD-Inhibitable Ferricytochrome c Reduction Assay

The protocol and steps for the SOD-inhibitable ferricytochrome c reduction assay cover the following 6 aspects: assay layout, assay description, preparation of reagents, individual steps, calculation, and other considerations.

4.1.1. Xanthine/XO

4.1.1.1. Assay Layout

Figure 4 illustrates the assay layout for the determination of SOD-inhibitable ferricytochrome c reduction resulting from superoxide generated by xanthine (0.5 mM) and XO (5 mU/ml) in CPBS during a 30 min of incubation at 37°C.

4.1.1.2. Assay Description

The SOD-inhibitable ferricytochrome c reduction assay is used to detect superoxide formation from the xanthine/XO system. Briefly, xanthine (final concentration of 0.25 mM) and XO (final concentration/activity of 5 mU/ml) are added to tubes containcontaining, in 1 ml CPBS, 60 μM ferricytochrome c in the presence or absence of 100 units/ml of Cu,ZnSOD. The reaction mixtures are then incubated at 37°C for 30 min. Immediately after this incubation, the tubes are placed on ice to stop the reaction, and then the reaction mixtures are transferred into cuvettes for measuring absorbance at 550 nm in a spectrophotometer using deionized water as the blank. Reduction of ferricytochrome c in the presence of Cu,ZnSOD is subtracted from the values without Cu,ZnSOD to specifically account for superoxide-mediated reduction. The SOD-inhibitable ferricytochrome c reduction is calculated using the molecular extinction coefficient for ferrocytochrome c of 21 $mM^{-1}cm^{-1}$, and expressed as nanomoles of ferricyto-

TABLE 2. Major materials for DEPMPO-spin trapping assay

Major Material	Vendor and Catalog Number	Note
12-*O*-Tetradeconylphorbol-13-acetate (TPA)	Sigma-Aldrich; P8139	TPA elicits respiratory burst in phagocytic cells via activating NAD(P)H oxidase.
5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO)	Enzo Life Sciences; ALX-430-093	Molecular mass: 235.22; purity: ≥99%; DEPMPO is in liquid form at room temperature; density: 1.14 g/ml at 25°C
Complete phosphate-buffered saline (CPBS) (also see Table 1)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, 2.67 mM KCl, 0.5 mM $MgCl_2$, 0.7 mM $CaCl_2$, 0.1% glucose, pH 7.4
Cu,Zn superoxide dismutase (also see Table 1)	Sigma-Aldrich; S5395	Source: bovine erythrocytes
Ferricytochrome c (also see Table 1)	Sigma-Aldrich; C7752	Source: horse heart; molecular mass: 12,384 Da
Macrophages	Investigator-prepared	Macrophages are isolated from the peritonea of mice.
Phosphate-buffered saline (PBS) (also see Table 1)	Investigator-prepared	Components: 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 138 mM NaCl, 2.67 mM KCl, pH 7.4
Xanthine (also see Table 1)	Sigma-Aldrich; X3627	Sodium salt
Xanthine oxidase (also see Table 1)	Sigma-Aldrich; X1875	Source: bovine milk

chrome c reduced (or ferrocytochrome c formed) per 30 min.

4.1.1.3. Preparation of Reagents

(1) Ferricytochrome c (60 μM in CPBS): 60 μM equals 0.743 mg/ml CPBS. For the above assay layout (**Figure 4**), 6 ml are needed. It is suggested that 7 ml be prepared to ensue 1 ml for each of the 6 tubes. This solution should be prepared freshly at room temperature immediately prior to the experiment.

(2) XO solution (1 unit/ml in CPBS): This XO solution is 200 times concentrated (1 unit/ml versus 5 mU/ml). For the above assay layout, 30 μl are needed. It is suggested that 35 μl be prepared to ensue 5 μl for each of the 6 tubes. The concentration/activity of the XO solution from Sigma-Aldrich (X1875) is typically ~20 units/ml. Immediately before use, prepare the 1 unit/ml of XO solution by diluting the above stock solution (~20 units/ml) in cold CPBS.

(3) Xanthine sodium solution (50 mM in water): 8.7 mg xanthine sodium (molecular mass = 174.09) dissolved in 1 ml deionized water.

(4) Cu,ZnSOD solution (20,000 units/ml in CPBS): 4 mg Cu,ZnSOD (5000 units/mg) dissolved in 1 ml cold CPBS (aliquot into microfuge tubes and store at −20°C).

4.1.1.4. Steps

(1) Add 0.985 ml of the ferricytochrome c solution (60 μM) to each of the 6 tubes.

(2) Add 5 μl of the Cu,ZnSOD solution (20,000 units/ml) to tubes 1, 2, and 3 (final concentration/activity of Cu,ZnSOD = 100 units/ml), and 5 μl of CPBS to tubes 4, 5, and 6.

(3) Add 5 μl of the xanthine solution (50 mM) to each of the 6 tubes (final concentration of xanthine = 0.25 mM).

(4) Add 5 μl of the XO solution (1 unit/ml) to each of the 6 tubes to start the reaction (final concentration/activity of XO = 5 mU/ml).

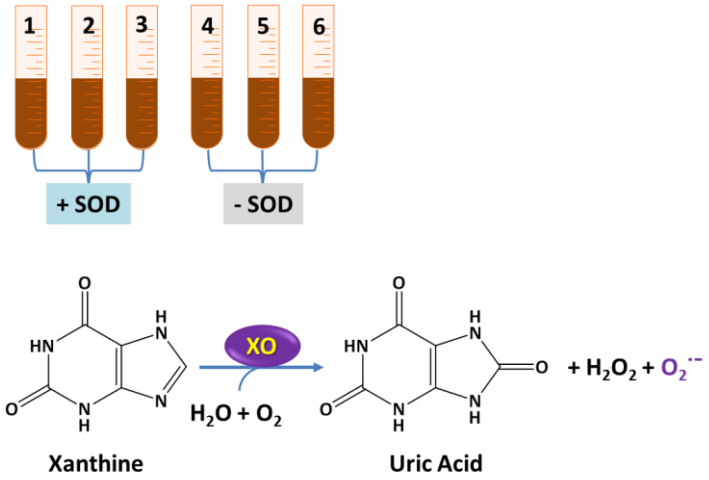

FIGURE 4. Assay layout for SOD-inhibitable ferricytochrome c reduction by the xanthine/XO system. In addition to generating superoxide, the xanthine/XO system also causes 2-electron reduction of oxygen to form hydrogen peroxide. As shown, each sample is run in triplicate with or without SOD.

(5) Transfer the tubes to a 37°C water bath (or a dry bath) and incubate for 30 min.
(6) Put tubes on ice to stop the reaction immediately after the above 30 min incubation.
(7) Transfer the reaction mixtures to cuvettes and measure the absorbance at 550 nm (A_{550}) in a DU800 UV/Vis spectrophotometer using deionized water as the blank.

4.1.1.5. CALCULATION

(1) Subtract A_{550} with Cu,ZnSOD from A_{550} without Cu,ZnSOD to obtain A_{550} specifically due to superoxide-mediated reduction of ferricytochrome c. This is designated as $A_{550\text{-superoxide}}$.
(2) According to the Beer-Lambert law, $A_{550\text{-superoxide}} = \varepsilon \times b \times c$, where $\varepsilon = 21\ mM^{-1}cm^{-1}$, b = 1 cm, and c denotes the concentration of ferrocytochrome c.
(3) $C_{ferrocytochrome\ c} = A_{550\text{-superoxide}} \div [\varepsilon \times b] = A_{550\text{-superoxide}} \div [21\ mM^{-1}cm^{-1} \times 1\ cm] = [A_{550\text{-superoxide}} \div 21] \times 1\ mM = [A_{550\text{-superoxide}} \div 21] \times [1\ mmole/1000\ ml] = [A_{550\text{-superoxide}} \div 21] \times [1\ \mu mole/1\ ml]$
(4) Since the reaction volume = 1 ml, the amount of ferrocytochrome c formed in this 1 ml volume = $[A_{550\text{-superoxide}} \div 21]$ (μmoles).
(5) The amount of ferrocytochrome c formed during the 30 min incubation = $[A_{550\text{-superoxide}} \div 21]$ (μmoles) = $A_{550\text{-superoxide}} \times 0.0476$ (μmoles) = $A_{550\text{-superoxide}} \times 47.6$ (nmoles)
(6) If, for example, $A_{550\text{-superoxide}} = 0.5$, then the amount of ferrocytochrome c = 0.5 × 47.6 (nmoles) = 23.8 nmoles. The result is thus expressed as 23.8 nmoles of ferrocytochrome c formed per 30 min.

4.1.1.6. OTHER CONSIDERATIONS

Due to the low solubility of xanthine, xanthine sodium (Sigma-Aldrich; X3627) is used to prepare the stock solution of 50 mM in deionized water instead of CPBS. This stock solution is stable at −20°C for at least one month. Alternatively, a working solution of 0.25 mM xanthine can be prepared in CPBS freshly for the experiment. Although XO (5 mM/ml) and xanthine (0.25 mM) are typically used to achieve a good rate of superoxide generation, other concentrations of xanthine and activities of XO as well as various incubation times and temperatures could be used depending on the experimental requirement. One way to determine the optimal concentration/activity of the xanthine/XO system is to monitor oxygen consumption resulting from the reaction. An optimal condition allows a relatively stable rate of oxygen

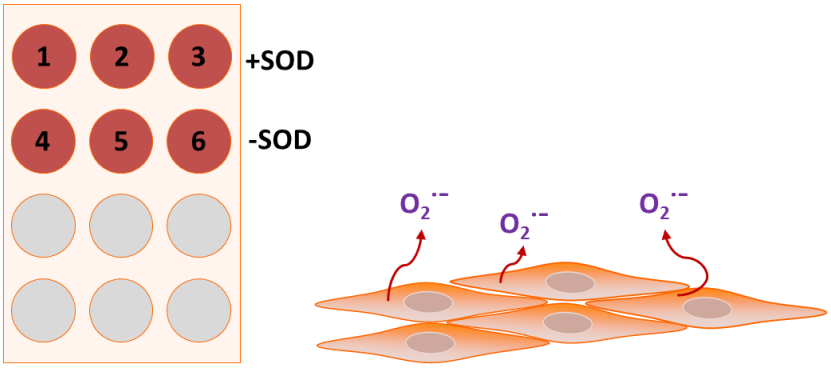

FIGURE 5. Assay layout for SOD-inhibitable ferricytochrome c reduction by superoxide released from HASMCs. HASMCs are known to release superoxide extracellularly, possibly through an NAD(P)H oxidase-dependent mechanism. Each sample is run in triplicate with or without SOD. Illustrated is a 12-well cell culture plate, and only the top 6 wells are used and the bottom 6 wells are empty.

consumption over a period of 30–60 min. If the XO is too much, xanthine will be consumed quickly within a couple of minutes, giving rise to only a burst of superoxide generation, rather than a relatively long-lasting stable rate.

The xanthine/XO system described above in combination with the SOD-inhibitable ferricytochrome c reduction assay may be used to study the effects of various conditions or factors (e.g., temperature, pH, concentration/activity of xanthine/XO, novel inhibitors/activators of XO, etc.) on the production of superoxide. The same protocol may also be applied to determine the potential formation of superoxide by other novel enzymatic and non-enzymatic systems.

4.1.2. HASMCs

4.1.2.1. Assay Layout

Figure 5 illustrates the assay layout for the determination of SOD-inhibitable ferricytochrome c reduction resulting from the superoxide released from cultured HASMCs in CPBS during a 30 min of incubation at 37°C.

4.1.2.2. Assay Description

The SOD-inhibitable ferricytochrome c reduction assay is used to detect extracellular superoxide released from cultured HASMCs. Briefly, HASMCs are cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (FBS), 0.01 mg/ml of insulin, 0.01 mg/ml of transferin, 10 ng/ml of sodium selenite, 0.03 mg/ml of endothelial cell growth supplement (ECGS), 100 units/ml of penicillin, and 100 µg/ml of streptomycin in 150 cm^2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO_2 [11]. The cells are fed every 2–3 days, and subcultured once they reached 80–90% confluence.

For detecting extracellular superoxide by The SOD-inhibitable ferricytochrome c reduction assay, HASMCs are plated into a 12-well culture plate at 1 × 10^6 cells/well in 1 ml culture medium. After 12 hr (or overnight), the culture medium is discarded and the cells are washed once with CPBS followed by addition of 1 ml CPBS containing 60 µM ferricytochrome c in the presence or absence of 100 units/ml of Cu,ZnSOD. Immediately after incubation at 37°C for 30 min, the culture plate is placed on ice to stop the reaction. The culture medium from each of the 6 wells is carefully collected for the measurement of absorbance at 550 nm using a spectrophotometer with deionized water as the blank. Reduction of ferricytochrome c in the presence of Cu,ZnSOD is subtracted from the values without Cu,ZnSOD to account for superoxide-mediated reduction. The SOD-inhibitable ferricytochrome c reduction is calculated using the molecular extinction coefficient for ferrocytochrome c of 21 $mM^{-1}cm^{-1}$, and expressed as nanomoles of ferricytochrome c reduced (or ferrocytochrome c formed) per 30 min per 1 × 10^6 cells.

4.1.2.3. PREPARATION OF REAGENTS

(1) Ferricytochrome c (60 μM in CPBS): 60 μM equals 0.743 mg/ml CPBS. For the assay layout in **Figure 5**, 6 ml are needed. It is suggested that 7 ml be prepared to ensue 1 ml for each of the 6 wells. This solution should be prepared freshly at room temperature each time immediately prior to experiment. This 7 ml is equally divided into two tubes. To one tube containing 3.5 ml ferricytochrome c, 17.5 μl of Cu,ZnSOD (20,000 units/ml; see below) is added to give a final concentration/activity of 100 units/ml; to the other tube, 17.5 μl of CPBS is added.
(2) Cu,ZnSOD solution (20,000 units/ml in CPBS): see Section 4.1.1.3 for preparation.

4.1.2.4. STEPS

(1) Harvest HASMCs from culture flasks and plate the cells into 6 wells of a 12-well plate at 1×10^6 cells per well in 1 ml of culture medium. Incubate the plate at 37°C overnight in a humidified atmosphere of 5% CO_2 to allow cell attachment to the bottom of the wells.
(2) Immediately before carrying out the SOD-inhibitable ferricytochrome c reduction assay, the culture medium from each of the 6 wells is discarded and the wells are gently washed once with CPBS (1 ml per well).
(3) Add 1 ml of the ferricytochrome c solution (60 μM) containing 100 units/ml of Cu,ZnSOD to wells 1, 2, and 3, and 1 ml of the ferricytochrome c solution (60 μM) containing no Cu,ZnSOD to wells 4, 5, and 6.
(4) Incubate the culture plate at 37°C for 30 min in a humidified atmosphere of 5% CO_2.
(5) Immediately after the above 30 min incubation, place the culture plate on ice to stop the reaction.
(6) Transfer the supernatants into cuvettes and measure the absorbance at 550 nm (A_{550}) in a DU800 UV/Vis spectrophotometer using deionized water as the blank.

4.1.2.5. CALCULATION

(1) Subtract A_{550} with Cu,ZnSOD from A_{550} without Cu,ZnSOD to obtain A_{550} specifically due to superoxide-mediated reduction of ferricytochrome c. This is designated as $A_{550\text{-superoxide}}$.

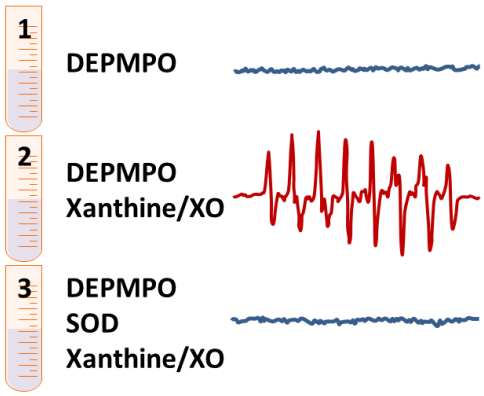

FIGURE 6. Assay layout for the DEPMPO-spin trapping detection of superoxide generated by the xanthine/XO system. The spectra are included to illustrate the typical responses.

(2) According to the Beer-Lambert law, $A_{550\text{-superoxide}} = \varepsilon \times b \times c$, where $\varepsilon = 21\ mM^{-1}cm^{-1}$, b = 1 cm, and c denotes the concentration of ferrocytochrome c.
(3) $C_{ferrocytochrome\ c} = A_{550\text{-superoxide}} \div [\varepsilon \times b] = A_{550\text{-superoxide}} \div [21\ mM^{-1}cm^{-1} \times 1\ cm] = [A_{550\text{-superoxide}} \div 21] \times 1\ mM = [A_{550\text{-superoxide}} \div 21] \times [1\ mmole/1000\ ml] = [A_{550\text{-superoxide}} \div 21] \times [1\ \mu mole/1\ ml]$
(4) Since the reaction volume = 1 ml, the amount of reduced cytochrome c in this 1 ml volume = $[A_{550\text{-superoxide}} \div 21]$ (μmoles).
(5) The amount of ferrocytochrome c formed during the 30 min incubation = $[A_{550\text{-superoxide}} \div 21]$ (μmoles) = $A_{550\text{-superoxide}} \times 0.0476$ (μmoles) = $A_{550\text{-superoxide}} \times 47.6$ (nmoles)
(6) If, for example, $A_{550\text{-superoxide}} = 0.2$, then the amount of ferrocytochrome c = 0.2 × 47.6 (nmoles) = 9.52 nmoles. Because the above amount of ferrocytochrome c is formed during a 30 min incubation of 1×10^6 cells, the amount of extracellular superoxide released from HASMCs is expressed as 9.52 nmoles of ferrocytochrome c formed per 30 min per 1×10^6 cells.

4.1.2.6. OTHER CONSIDERATIONS

The SOD-inhibitable ferricytochrome c reduction assay can also be used to detect superoxide generation in cell suspension [2]. To this end, the cells are

suspended in CPBS containing ferricytochrome c and other reagents and incubated at 37°C in a shaking water bath. After incubation, the cells are pelleted by centrifugation, and the supernatants collected for measuring absorbance at 550 nm.

4.2. Protocols and Steps for the DEPMPO-Spin Trapping Assay

The protocols and steps for the DEPMPO-spin trapping assay cover the following 6 aspects: assay layout, assay description, preparation of reagents, individual steps, spectrum identification and quantification, and other considerations.

4.2.1. Xanthine/XO

4.2.1.1. ASSAY LAYOUT

Figure 6 depicts the assay layout for DEPMPO-spin trapping detection of superoxide generated by xanthine (0.5 mM) and XO (5 mU/ml) in CPBS at 37°C.

4.2.1.2. ASSAY DESCRIPTION

The DEPMPO-spin trapping assay is used to detect superoxide formation from the xanthine/XO system. Briefly, xanthine (final concentration of 0.25 mM) and XO (final concentration/activity of 5 mU/ml) are added to a tube containing, in 1 ml CPBS, 10 mM DEPMPO in the presence or absence of 100 units /ml of Cu,ZnSOD. The reaction mix is then incubated at 37°C for 15 min. Immediately after this incubation, the reaction mix is transferred into an EPR flat cell, and spectra are recorded at room temperature with a Bruker EMXplus spectrometer, operating at X-band with a TM cavity. The spectrometer settings are: modulation frequency, 100 kHz; modulation amplitude, 0.5 G; microwave power, 20 mW; microwave frequency, 9.78 GHz; and scan time, 30 s. Spectral simulations are performed on the EPR data by matching directly to the spectra reported in the literature and/or databases. Reaction of DEPMPO with superoxide forms a spin adduct, known as DEPMPO-OOH with a characteristic EPR spectrum. The spectrum of DEPMPO-OOH adduct corresponds to an exchange between two conformers *X* and *Y* of the DEPMPO-OOH adduct with the following parameters: *X* (43%): a_N = 13.13 G; a_P = 55.61 G; a_H^β = 13.11 G; a_H^γ = 0.71, 0.42, 0.7, 0.25, and 0.6 G. *Y* (57%): a_N = 13.08 G; a_P = 45.85 G; a_H^β = 9.53 G; a_H^γ = 1.05, 0.42, 0.7, 0.25, and 0.6 G. On the other hand, the DEPMPO-hydroxyl adduct (DEPMPO-OH) has the following parameters: a_N = 14.05 G; a_P = 47.29 G; a_H^β = 13.40 G; a_H^γ = 0.6 (×3) G [2]. The intensity of the EPR spectrum is determined by measuring the height of the spectrum, which is proportional to the amount of DEPMPO-OOH adduct formed, that in turn correlates with the amount of superoxide generated by the xanthine/XO system.

4.2.1.3. PREPARATION OF REAGENTS

(1) DEPMPO (4 M in deionized water): For a 50 mg (0.05 g) size from the vendor (**Table 2**), the volume = weight ÷ density = 0.05 g ÷ 1.14 g/ml = 0.0439 ml; the molar concentration of DEPMPO = [weight (g) ÷ molecular mass] ÷ volume (L) = [0.05 g ÷ 235.22] ÷ [0.0000439 L] = 4.84 M; To prepare 4 M solution, add 0.0092 ml deionized water to the vial containing 50 mg DEPMPO (0.0439 ml), and store this 4 M solution in aliquots at −80°C.
(2) XO solution (1 unit/ml in CPBS): see Section 4.1.1.3 for preparation.
(3) Xanthine sodium solution (50 mM in water): see Section 4.1.1.3 for preparation.
(4) Cu,ZnSOD solution (20,000 units/ml in CPBS): see Section 4.1.1.3 for preparation.

4.2.1.4. STEPS

(1) To tube 1, add 0.9975 ml CPBS followed by adding 2.5 µl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the reaction mix to an EPR flat cell for acquisition of the EPR spectra under the conditions described above (Section 4.2.1.2). This serves as a control. DEPMPO of high purity alone in CPBS under the above conditions should not give rise to any significant EPR signal (**Figure 6**).
(2) To tube 2, add 0.9875 ml CPBS followed by adding 2.5 µl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM), 5 µl of 50 mM xanthine (final concentration of xanthine = 0.25 mM), and 5 µl of 1 unit/ml of XO (final concentration/activity of XO = 5 mU/ml). Incubate the tube at 37°C for 15 min. Immediately after this

incubation, transfer the reaction mix to an EPR flat cell for acquisition of the EPR spectra under the conditions described above. This generates an EPR spectrum characteristic of DEPMPO-OOH adduct (**Figure 6**).

(3) To tube 3, add 0.9825 ml CPBS followed by adding 2.5 µl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM), 5 µl of 20,000 units/ml of Cu,ZnSOD (final concentration/activity of Cu,ZnSOD = 100 units/ml), 5 µl of 50 mM xanthine (final concentration of xanthine = 0.25 mM), and 5 µl of 1 unit/ml of XO (final concentration/activity of XO = 5 mU/ml). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the reaction mix to an EPR flat cell for acquisition of the EPR spectra under the conditions described above. This tube should show a complete abolishment of the DEPMPO-OOH adduct (**Figure 6**).

4.2.1.5. Spectrum Identification and Quantification

As aforementioned, the DEPMPO-OOH adduct exhibits a characteristic EPR spectrum. By directly comparing the spectrum derived from the experiment with that reported in the literature/databases, one can draw a conclusion about the identity of the spin adduct. This is particularly true when superoxide is known to be produced in the experimental system and the focus of the experiment is to determine the relative amount of the superoxide formation under different conditions. Definitive identification of the free radical species, especially in an experimental system where multiple free radical adducts are formed, may require spectral simulation. In this regard, the public EPR software tools, such as WinSim 2002 are available through the U.S. National Institute of Environmental Health Sciences (NIEHS) (http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/ accessed on December 18, 2015).

With regard to quantification, the height of the EPR spectrum is proportional to the amount of the spin-adduct formed which in turn is related to the amount of the radical species formed in a system. Due to the high reactivity and short half-life of free radicals including superoxide, absolute quantification of these reactive species in biological systems or samples is not practical. Hence, EPR spin trapping is typically used to identify free radical species and determine their relative amounts generated in a biological system under specific conditions. Accordingly, the height of the spectrum of DEPMPO-OOH is determined and used to indicate the relative amount of superoxide formed by the xanthine/XO system during the period of the experiment (15 min incubation at 37°C plus the time taken for spectrum acquisition at room temperature).

4.2.1.6. Other Considerations

Trace amounts of transition metal ions, such as copper and iron ions in CPBS might interact with the spin traps to give rise to EPR signals. To eliminate this effect, 0.1 mM diethylenetriamine pentaacetic acid (DTPA) can be added to CPBS to chelate the transition metal ions. Depending on the experimental conditions and particular requirements, concentrations of DEPMPO higher than 10 mM may be used. Due to the high cost of the spin traps, it is suggested that a pilot experiment be carried out to determine the optical concentration of a spin trap for detecting superoxide in a specific biological system. In this regard, DEPMPO is commonly used in the concentration range of 10−25 mM. On the other hand, DMPO is typically used in the range of 40−80 mM.

4.2.2. Macrophages

4.2.2.1. Assay layout

Figure 7 illustrates the assay layout for DEPMPO-spin trapping detection of superoxide released from TPA-stimulated macrophages in CPBS at 37°C.

4.2.2.2. Assay Description

The DEPMPO-spin trapping assay is used to detect superoxide released from TPA-stimulated macrophages isolated from the peritonea of mice. Briefly, each of the 10 male C57BL/6 mice (25–30 g) is intraperitoneally injected 1 ml of 4% thioglycollate medium [12]. Four days later, total peritoneal cells are harvested by washing the peritoneal cavity with serum-free RPMI-1640 medium. Following centrifugation, the cells are resuspended in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml of penicillin and 100 µg/ml of streptomycin, and incubated in tissue culture flasks at 37°C for 1 h. Then, the non-adherent cells are removed by vigorous

washing of the cultures three times with serum-free RPMI-1640 medium. The resultant macrophages (>98% purity) are continuously cultured at 37°C in a humidified atmosphere of 5% CO_2 [12]. The macrophages are used for experiments within three passages following the initial isolation. For DEPMPO-spin trapping, macrophages are harvested from the cultures and washed once in CPBS. Exactly 1×10^6 cells are suspended in 1 ml CPBS containing 10 mM DEPMPO in the presence or absence of 100 units/ml of Cu,ZnSOD. After adding TPA (final concentration of 1 μg/ml), the cell suspension is then incubated at 37°C for 15 min. Immediately after this incubation, the cell suspension is transferred to an EPR flat cell, and spectra are recorded at room temperature with a Bruker EMXplus spectrometer, operating at X-band with a TM cavity. The spectrometer settings and the characyeristics of the EPR spectra are the same as described in Section 4.2.1.2. The intensity of the EPR spectrum is determined by measuring the relative height of the spectrum, which is proportional to the amount of DEPMPO-OOH adduct formed, that in turn correlates with the amount of superoxide released extracellularly by TPA-stimulated macrophages. TPA activates NAD(P)H oxidase in macrophages, leading to extracellular release of superoxide [2].

4.2.2.3. Preparation of Reagents

(1) DEPMPO (4 M in deionized water): see Section 4.2.1.3 for preparation.
(2) Cu,ZnSOD solution (20,000 units/ml in CPBS): see Section 4.1.1.3 for preparation.
(3) TPA (1 mg/ml in dimethyl sulfoxide): Add 1 ml of dimethyl sulfoxide (DMSO) to a vial containing 1 mg of TPA (Sigma-Aldrich; P8139) and store this stock solution in aliquots at −20°C.

4.2.2.4. Steps

(1) To tube 1 containing no cells, add 0.9975 ml CPBS followed by adding 2.5 μl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the reaction mix to an EPR flat cell for acquisition of the EPR spectra under the conditions described above (Section 4.2.2.2). This serves as a control. DEPMPO of high purity alone in CPBS under the above condition should not give rise to any significant EPR signal (**Figure 7**).

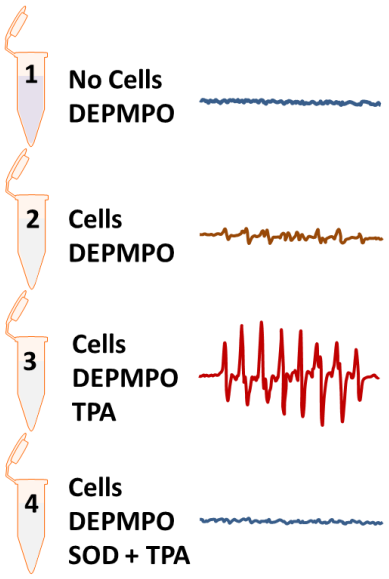

FIGURE 7. Assay layout for the DEPMPO-spin trapping detection of superoxide released from TPA-stimulated macrophages. The spectra are included to illustrate the typical responses.

(2) To tube 2 with a pellet of 1×10^6 cells (the volume of the cell pellet ≈ 5 μl), add 0.9925 ml CPBS to resuspend the cells followed by adding 2.5 μl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the reaction suspension to an EPR flat cell for acquisition of the EPR spectra under the conditions described above. As resting macrophages release a small amount of superoxide extracellularly, this may give rise to a weak, if any, signal of DEPMPO-OOH adduct (**Figure 7**).
(3) To tube 3 with a pellet of 1×10^6 cells (the volume of the cell pellet ≈ 5 μl), add 0.9915 ml CPBS to resuspend the cells followed by adding 2.5 μl of 4 M DEPMPO (final concentration of DEPMPO = 10 mM) and 1 μl of 1 mg/ml of TPA (final concentration of TPA = 1 μg/ml). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the cell suspension to an EPR flat cell for acquisition of the EPR spectra under the conditions described above. As

TPA-stimulated macrophages release a large amount of superoxide extracellularly, this typically gives rise to a strong signal of DEPMPO-OOH adduct (**Figure 7**).

(4) To tube 4 with a pellet of 1×10^6 cells (the volume of the cell pellet ≈ 5 µl), add 0.9865 ml CPBS to resuspend the cells followed by adding 5 µl of 20,000 units/ml of Cu,ZnSOD (final concentration = 100 units /ml), 2.5 µl of 4 M DEPMPO (final concentration = 10 mM) and 1 µl of 1 mg/ml of TPA (final concentration of TPA = 1 µg/ml). Incubate the tube at 37°C for 15 min. Immediately after this incubation, transfer the reaction suspension to an EPR flat cell for acquisition of the EPR spectra under the conditions described above. Because of the presence of Cu,ZnSOD, this tube should show a complete abolishment of the signal of DEPMPO-OOH adduct (**Figure 7**).

4.2.2.5. SPECTRUM IDENTIFICATION AND QUANTIFICATION

Similar to what described above for the xanthine/XO system (Section 4.2.1.5), the height of the spectrum of DEPMPO-OOH is determined and used to indicate the relative amount of superoxide released by macrophages during the period of the experiment (15 min incubation at 37°C plus the time taken for spectrum acquisition at room temperature).

4.2.2.6. OTHER CONSIDERATIONS

As discussed below, one disadvantage of the EPR spin trapping technique is its relatively low sensitivity. The sensitivity issue may become a major limiting factor when biological superoxide is generated at a relatively low rate. This represents a major drawback of EPR spectrometry, particularly for a low-grade EPR spectrometer. In this context, due to recent improvement in instrumentation, research grade EPR spectrometers, such as the Bruker EMXplus spectrometer allow sensitive detection of biological free radicals. It is estimated that DEPMPO-spin trapping detection of superoxide with the Bruker EMXplus spectrometer may be at least 5–10 times more sensitive than that with the Bruker eScan spectrometer. It should be noted also that the EMXplus spectrometer system is much more costly than the eScan spectrometer and requires more intensive care.

5. DISCUSSION OF ADVANTAGES AND LIMITATIONS

No methods are perfect with regard to detecting biological free radicals. Each method has its own advantages and limitations. This notion applies to both the SOD-inhibitable ferricytochrome c reduction assay and the DEPMPO-spin trapping technique. Knowing the capabilities and limitations of a particular assay would allow its appropriate use as well as the correct interpretation of the data generated.

5.1. Advantages and Limitations of the SOD-Inhibitable Ferricytochrome c Reduction Assay

5.1.1. Advantages

Simplicity is the biggest advantage of the SOD-inhibitable ferricytochrome c reduction assay. The major instrumental requirement is a visible spectrophotometer. Indeed, this assay is among the most widely used methods for detecting superoxide generation in both enzymatic and cellular systems, partly due to its simplicity and low cost.

5.1.2. Limitations

The major disadvantages of the SOD-inhibitable ferricytochrome c reduction assay include its limited sensitivity and inability to detect superoxide generated intracellularly (due to the inability of ferricytochrome c to cross cell membranes). Moreover, potent reduction of ferricytochrome c by other molecules, such as thiols and phenolic compounds may make the accurate measurement of SOD-inhibitable ferricytochrome c reduction impossible. Nevertheless, this assay is currently considered a well-established method for detecting biological superoxide.

5.2. Advantages and Limitations of the DEPMPO-Spin Trapping Assay

5.2.1. Advantages

EPR spin trapping is the most specific method for identifying and detecting biological superoxide production in both enzymatic systems and cells/tissues. Although various spin traps are available for detecting superoxide, DEPMPO and DMPO appear to be more commonly used due to the relatively long half-

life of the DEPMPO-OOH adduct, and the more research experience with DMPO, respectively. Recent studies also suggested that DEPMPO and DMPO may cross cell membranes and be able to also detect intracellular superoxide and other free radicals [13].

5.2.2. Limitations

Despite its high specificity for identifying and detecting superoxide, EPR spin trapping is also associated with limitations. A notable disadvantage is the high cost of the EPR spectrometer as well as the nitrone spin traps. Although the spin adducts are much longer-lived than the initial free radicals, these adducts are generally unstable with half-lives in the range of a few minutes. The nitrone spin traps and spin adducts are also metabolized by tissues and may exert tissue and cell toxicity. These issues make EPR spin trapping less than ideal for the in vivo determination of free radicals and also limit the detection sensitivity of the technique. In this context, recent studies have reported that cyclic hydroxylamines, such as 1-hydroxy-3-carboxy-pyrrolidine (CPH) react with superoxide to form much more stable spin adducst than the nitrone spin traps (e.g., DEPMPO). CPH has been shown to specifically detect superoxide with high sensitivity in biological systems [14, 15].

Another notion regarding EPR spin trapping detection of superoxide is the decomposition of the spin adducts to form products that also possess characteristic signature EPR spectra [5]. For example, the DEPMPO-superoxide spin adduct (DEPMPO-OOH) can decompose to form DEPMPO-hydroxyl radical (DEPMPO-OH) adduct. Therefore, the detection of DEPMPO-OH adduct in a biological system does not necessarily indicate the formation of hydroxyl radical, and it may be instead derived from the initially formed DEPMPO-OOH adduct. To prove the initial involvement of superoxide in the formation of the DEPMPO-OH adduct, exogenous SOD or an SOD mimetic can be included in the assay. If the formation of DEPMPO-OH adduct is prevented by SOD, it suggests the initial involvement of superoxide, rather than hydroxyl radical (**Figure 8**).

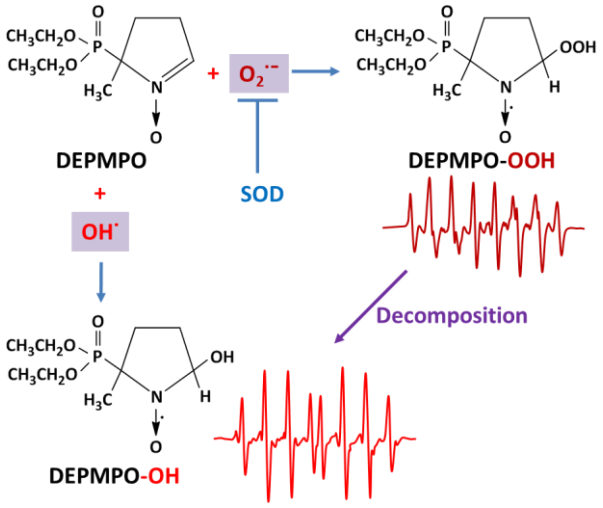

FIGURE 8. Decomposition of a DEPMPO-OOH adduct to form DEPMPO-OH. Addition of SOD to the reaction is used to determine the initial involvement of superoxide in the eventual formation of the DEPMPO-OH adduct.

6. CONCLUSION

Various techniques and methods have been developed over the past several decades for sensitive and specific detection of biological superoxide generation. These include assays based on UV/Vis spectrophotometry, fluorescence and chemiluminescent techniques, and EPR spectrometry/spin trapping. Among the various methods, the SOD-inhibitable ferricytochrome c reduction assay is commonly used for detecting superoxide formation from both enzymatic and cellular systems due to its simplicity and low cost. However, this assay lacks high sensitivity and is unable to detect intracellular superoxide formation. Although sensitivity is also an issue with EPR spectrometry, EPR spectrometry/spin trapping represents the most specific technique for identifying and detecting biological free radicals, including superoxide. The improvement in EPR instrumentation in recent years along with the development of new spin traps, such as DEPMPO has greatly augmented the sensitivity of spin trapping detection of superoxide and other free radical species in biological systems. It is hoped that this ROS protocol article would provide the readers detailed instructions with regard to the appropriate use of the SOD-inhibitable ferricytochrome c reduction assay and the DEPMPO-spin trapping technique for reliably detecting biological superoxide generation.

REFERENCES

1. Barbacanne MA, Souchard JP, Darblade B, Iliou JP, Nepveu F, Pipy B, et al. Detection of superoxide anion released extracellularly by endothelial cells using cytochrome c reduction, ESR, fluorescence and lucigenin-enhanced chemiluminescence techniques. *Free Radic Biol Med* 2000; 29(5):388–96.
2. Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA. Validation of lucigenin (bis-*N*-methylacridinium) as a chemilumigenic probe for detecting superoxide anion radical production by enzymatic and cellular systems. *J Biol Chem* 1998; 273(4):2015–23.
3. Li Y, Zhu H, Trush MA. Detection of mitochondria-derived reactive oxygen species production by the chemilumigenic probes lucigenin and luminol. *Biochim Biophys Acta* 1999; 1428(1):1–12.
4. Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, et al. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide. *Free Radic Biol Med* 2003; 34(11):1359–68.
5. Li YR. Free Radical Biomedicine: Principles, Clinical Correlations, and Methodologies. Dubai, UAE: Bentham Science Publishers; 2012.
6. Butler J, Koppenol WH, Margoliash E. Kinetics and mechanism of the reduction of ferricytochrome c by the superoxide anion. *J Biol Chem* 1982; 257(18):10747–50.
7. van GB, Slater EC. The extinction coefficient of cytochrome c. *Biochim Biophys Acta* 1962; 58:593–5.
8. Villamena FA, Zweier JL. Detection of reactive oxygen and nitrogen species by EPR spin trapping. *Antioxid Redox Signal* 2004; 6(3):619–29. doi: 10.1089/152308604773934387.
9. Floyd RA, Kopke RD, Choi CH, Foster SB, Doblas S, Towner RA. Nitrones as therapeutics. *Free Radic Biol Med* 2008; 45(10):1361–74. doi: S0891-5849(08)00473-5 [pii] 10.1016/j.freeradbiomed.2008.08.017.
10. Zhu H, Jia Z, Mahaney JE, Ross D, Misra HP, Trush MA, et al. The highly expressed and inducible endogenous NAD(P)H:quinone oxidoreductase 1 in cardiovascular cells acts as a potential superoxide scavenger. *Cardiovasc Toxicol* 2007; 7(3):202–11. doi: 10.1007/s12012-007-9001-z.
11. Zhu H, Cao Z, Zhang L, Trush MA, Li Y. Glutathione and glutathione-linked enzymes in normal human aortic smooth muscle cells: chemical inducibility and protection against reactive oxygen and nitrogen species-induced injury. *Mol Cell Biochem* 2007; 301(1–2):47–59. doi: 10.1007/s11010-006-9396-z.
12. Zhu H, Jia Z, Zhang L, Yamamoto M, Misra HP, Trush MA, et al. Antioxidants and phase 2 enzymes in macrophages: regulation by Nrf2 signaling and protection against oxidative and electrophilic stress. *Exp Biol Med (Maywood)* 2008; 233(4):463–74. doi: 10.3181/0711-RM-304.
13. Anzai K, Aikawa T, Furukawa Y, Matsushima Y, Urano S, Ozawa T. ESR measurement of rapid penetration of DMPO and DEPMPO spin traps through lipid bilayer membranes. *Arch Biochem Biophys* 2003; 415(2):251–6.
14. Landmesser U, Cai H, Dikalov S, McCann L, Hwang J, Jo H, et al. Role of $p47^{phox}$ in vascular oxidative stress and hypertension caused by angiotensin II. *Hypertension* 2002; 40(4):511–5.
15. Spiekermann S, Landmesser U, Dikalov S, Bredt M, Gamez G, Tatge H, et al. Electron spin resonance characterization of vascular xanthine and NAD(P)H oxidase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. *Circulation* 2003; 107(10):1383–9.