Video Article

Detection of Nitric Oxide and Superoxide Radical Anion by Electron Paramagnetic Resonance Spectroscopy from Cells using Spin Traps

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Abstract

Reactive nitrogen/oxygen species (ROS/RNS) at low concentrations play an important role in regulating cell function, signaling, and immune response but in unregulated concentrations are detrimental to cell viability[1, 2]. While living systems have evolved with endogenous and dietary antioxidant defense mechanisms to regulate ROS generation, ROS are produced continuously as natural by-products of normal metabolism of oxygen and can cause oxidative damage to biomolecules resulting in loss of protein function, DNA cleavage, or lipid peroxidation[3], and ultimately to oxidative stress leading to cell injury or death[4].

Superoxide radical anion (O_2•-) is the major precursor of some of the most highly oxidizing species known to exist in biological systems such as peroxynitrite and hydroxyl radical. The generation of O_2•- signals the first sign of oxidative burst, and therefore, its detection and/or sequestration in biological systems is important. In this demonstration, O_2•- was generated from polymorphonuclear neutrophils (PMNs). Through chemotactic stimulation with phorbol-12-myristate-13-acetate (PMA), PMN generates O_2•- via activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase[5].

Nitric oxide (NO) synthase which comes in three isoforms, as inducible-, neuronal- and endothelial-NOS, or iNOS, nNOS or eNOS, respectively, catalyzes the conversion of L- arginine to L-citrulline, using NADPH to produce NO[6]. Here, we generated NO from endothelial cells. Under oxidative stress conditions, eNOS for example can switch from producing NO to O_2•- in a process called uncoupling, which is believed to be caused by oxidation of heme[7] or the co-factor, tetrahydrobiopterin (BH_4)[8].

There are only few reliable methods for the detection of free radicals in biological systems but are limited by specificity and sensitivity. Spin trapping is commonly used for the identification of free radicals and involves the addition reaction of a radical to a spin trap forming a persistent spin adduct which can be detected by electron paramagnetic resonance (EPR) spectroscopy. The various radical adducts exhibit distinctive spectrum which can be used to identify the radicals being generated and can provide a wealth of information about the nature and kinetics of radical production[9].

The cyclic nitrones, 5,5-dimethyl-pyrroline-*N*-oxide, DMPO[10], the phosphoryl-substituted DEPMPO[11], and the ester-substituted, EMPO[12] and BMPO[13], have been widely employed as spin traps--the latter spin traps exhibiting longer half-lives for O_2•- adduct. Iron (II)-N-methyl-D-glucamine dithiocarbamate, $Fe(MGD)_2$ is commonly used to trap NO due to high rate of adduct formation and the high stability of the spin adduct[14].

Video Link

The video component of this article can be found at https://www.jove.com/video/2810/

Protocol

1. Culture of Bovine Aortic Endothelial Cells (BAEC)

1. Proper aseptic techniques were followed.
2. In a water bath, warm medium without antibiotics at 37 °C.

Note: The medium consists of phenol free Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L D-glucose, 4 mM L-glutamine, 1% non-essential amino acids, supplemented with 10% fetal bovine serum (FBS) and 2.5 mg/L endothelial growth factor.

3. Remove the T75 flask containing cells from the incubator and clean the surface of the flask with 70% ethanol before placing it inside the hood.
4. Remove the old medium using an aspirator and wash twice with 5 ml of Dulbecco's phosphate buffer saline (DPBS).

5. Add 2 ml of trypsin and wait for 4-5 min for cells to detach while periodically inspecting under the microscope.
6. Add 3 ml of the medium and repeatedly mix using a pipette to separate the cells and create an even suspension.
7. Transfer 5 ml of the medium with trypsin to a 15 ml tube and centrifuge at 121 g for 5 min.
8. Remove the supernatant using an aspirator. Add 5 ml of DPBS and mix thoroughly. Centrifuge at 121g for 5 min.
9. Aspirate the supernatant and re-suspend the cell pellet by adding 6 ml of the medium.
10. On a 6-well plate, add 1 ml of the cell suspension to each well then add 1 ml of the medium. Mix the suspension using a pipette.
11. Label the plate and incubate overnight at 37 °C and 5% CO_2.

2A. Detection of NO with BAEC Cell

1. Proper aseptic techniques were followed.
2. Remove the 6-well plate from the incubator and aspirate the medium from the first well. Wash the cells twice with 1 ml DPBS.
3. Add 210 µl of 1.9 mM iron(II)sulfate heptahydrate ($FeSO_4.7H_2O$, prepare freshly by dissolving 0.8 mg in 1 ml DPBS with $CaCl_2$ and $MgCl_2$) and 210 µl of ammonium N-methyl-D-glucamine dithiocarbamate (MGD, prepare freshly by dissolving 2.7 mg in 500 µl DPBS with $CaCl_2$ and $MgCl_2$) using a ratio of 1:7. *(Note: This ratio where excess MGD is used has been conventionally employed for the preparation of Fe^{2+}-MGD complex due to the fact that the yield for Fe^{3+}-MGD is maximized in the presence of excess MGD. The addition of ascorbate in solution to stabilize the Fe^{2+}-MGD is not necessary since the NO-Fe^{3+}-MGD formed is endogenously reduced to the EPR detectable NO-Fe^{2+}-MGD by ascorbate, hydroquinone, or cysteine with conversion efficiency of up to 99.9%.. The low spin diamagnetic state of Fe^{2+} allows the detection of NO using flat cell or capillary tube without the need of a low temperature device)*[15].
4. Swirl the resulting suspension well and add 4.6 µl of calcium ionophore (CaI) (prepared from a stock solution of 1.9 mM by dissolving 1 mg in 1 ml DMSO).
5. Swirl the solution again and incubate at 37 °C for 36 min in order to further allow reduction of NO-Fe^{3+}-MGD to the EPR detectable NO-Fe^{2+}-MGD.
6. Collect the supernatant (425 µl) in an Eppendorf tube and transfer to an EPR flat cell (or to a 50 µl capillary tube).
7. EPR acquisition parameters are: microwave frequency: 9.8 GHz; center field: 3427 G; modulation amplitude: 6.0 G; sweep width: 100 G; receiver gain: 1 x 10^5; microwave power: 10 mW; total number of scans: 121; sweep time: 10 s; and time constant: 20 ms. *(Note: Since parameters will vary from one instrument and experimental conditions to another, therefore, only the center field, frequency and modulation amplitude are the most important parameters to consider.)*
8. Record the spectra at room temperature and the 2-D spectra were integrated to reduce the background noise and baseline corrected using Bruker WinEPR Data Processing software or other data processing software. For quantification of adduct formation, standard plots of concentration versus signal intensity (or area) can be constructed using an NO donor SNAP.

2B. eNOS Uncoupling Experiment

1. Remove the 6-well plate from the incubator and aspirate the medium from the second well and wash twice with 1 ml DPBS.
2. A peroxynitrite donor, 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1) was used to uncouple eNOS[16]. Add 100 µl of 0.5 mM SIN-1 (M_r 206.6 g/mol, from 10 mM stock solution freshly prepared by dissolving 1 mg SIN-1 in 500 µl of PBS with no Ca/Mg ions) and dilute to 2 ml with DPBS and 10% FBS.
3. Incubate for 2 h at 37 °C and 5% CO_2.
4. Remove the plate from incubator and wash twice with DPBS.
5. Add 210 µl of 2.8 mM $FeSO_4.7H_2O$ and 210 µl of 19.6 mM MGD freshly prepared according to the procedure mentioned above.
6. Swirl the solution and add 4.6 µl of 1.9 mM CaI.
7. Swirl the solution again and incubate at 37 °C for 36 min.
8. Collect the supernatant (425 µl) in an Eppendorf tube and transfer to an EPR flat cell.
9. EPR acquisition parameters are: microwave frequency: 9.8 GHz; center field: 3427 G; modulation amplitude: 6 G; sweep width: 100 G; receiver gain: 1 x 10^5; microwave power: 10 mW; total number of scans: 121; sweep time: 10 s; and time constant: 20 ms.
10. Record the spectra and the 2-D spectra were integrated to reduce the background noise and baseline corrected as mentioned above.

3. Detection of O_2•- from Polymorphonuclear Neutrophils (PMNs)

1. Neutrophils were isolated from human blood sample as previously described[17].
2. Make a stock solution of 1 M DMPO* in PBS containing 0.1 mM diethylenetriamine-pentaacetic acid (DTPA). DMPO has a melting point of 25-29 °C so it is more convenient to pipette liquid DMPO (density ~ 1.02 g/ml at 25 °C) in to a glass vial *(Note: do not use plastic vials for weighing since pure DMPO reacts with plastic)*. Frozen DMPO can be melted by running luke warm water on to the vial *(Note: do not run hot water as DMPO may decompose)*.
3. It is important to use high purity DMPO (> 99%) since some of the commercially available spin traps contain paramagnetic impurities, and therefore, it is imperative to run EPR spectrum of just the DMPO solution alone (10 mM in this case). It is critical that no background signal is evident (see **Figure 3A**).
4. Prepare stock solution (1 mg/ml) of phorbol-12-myristate-13-acetate (PMA) in DMSO. Make aliquots by diluting the solution to 10 µg/ml in PBS.
5. In a 1.5 ml Eppendorf tube, prepare a solution with a total volume of 0.6 ml by following the sequence of addition: ~10^6 cells per ml of PMN, D-glucose (1 mg/ml) and albumin (1 mg/ml), 10 mM DMPO and 0.2 µg/ml of PMA. *(Note: PMA is the radical activator and should be added last)*.
6. Transfer the solution to an EPR flat cell.
7. EPR acquisition parameters conditions are: microwave frequency: 9.8 GHz; center field: 3486 G; modulation amplitude: 0.5 G; sweep width: 100 G; receiver gain: 5 x 10^5; total number of scans: 10; sweep time: 30 s; microwave power: 20 mW; and time constant: 81 ms.

For quantification of adduct formation, standard plots of concentration versus signal intensity (or area) can be constructed using the stable nitroxides such as TEMPO or 3-carboxylic acid-PROXYL.

** Important note on the use of DMPO: By using a flat cell, one can increase the cell density and thereby increasing the signal intensity of the spin adduct but the half-life of $O_2^{\bullet-}$ adduct of DMPO is short ($t_{1/2}$ ~ 1 min) which decomposes to DMPO-OH. DMPO can be substituted using the same concentrations of EMPO, BMPO or DEPMPO which are available commercially for increased adduct stability with $t_{1/2}$ ~ 8 and 14 min, respectively.*

4. Representative Results

Spin trapping of NO radical was performed using Fe^{2+}-MGD. **Figure 2A and 2B** show no EPR signal from Fe^{2+}-MGD or mixture of Fe^{2+}-MGD with CaI, indicating that no NO background signal originates from these reagents. BAEC upon stimulation with CaI releases NO which reacts with Fe^{2+}-MGD to form the spin adduct, NO-Fe^{2+}-MGD, and shows a characteristic triplet signal with hyperfine splitting constant (hfsc) value of a_N = 12.66 G and g-factor of g= 2.040. (**Figure 2C**). The hfsc value was determined using the WINSIM simulation program that can be downloaded from NIEHS EPR Software Database website. The experimental hfsc is consistent with the literature value of a_N=12.70 G and g = 2.041[18] for NO-Fe^{2+}-MGD adduct. Similarly, the effect of SIN-1 on BAEC lowered the NO production due to eNOS uncoupling as shown in **Figure 2D**.

DMPO spin trap was used for $O_2^{\bullet-}$ detection. DMPO alone did not give a signal as shown in the **Figure 3A** confirming that the spin trap is free from paramagnetic impurities. **Figure 3B** is the spectrum of DMPO and PMNs only, and similarly, there is no detectable signal suggesting that the DMPO does not cause activation of the enzyme NADPH oxidase. **Figure 3C** shows the observed EPR signal upon stimulation of PMN by PMA. The hfsc values for this signal were determined to be a_N =14.71 G, $a_{\beta\text{-}H}$ =11.40 G and $a_{\gamma\text{-}H}$ =1.25 G, and are consistent with the literature values of a_N =14.3 G, $a_{\beta\text{-}H}$ =11.7 G and $a_{\gamma\text{-}H}$ =1.3 G [19] for DMPO-O_2H adduct.

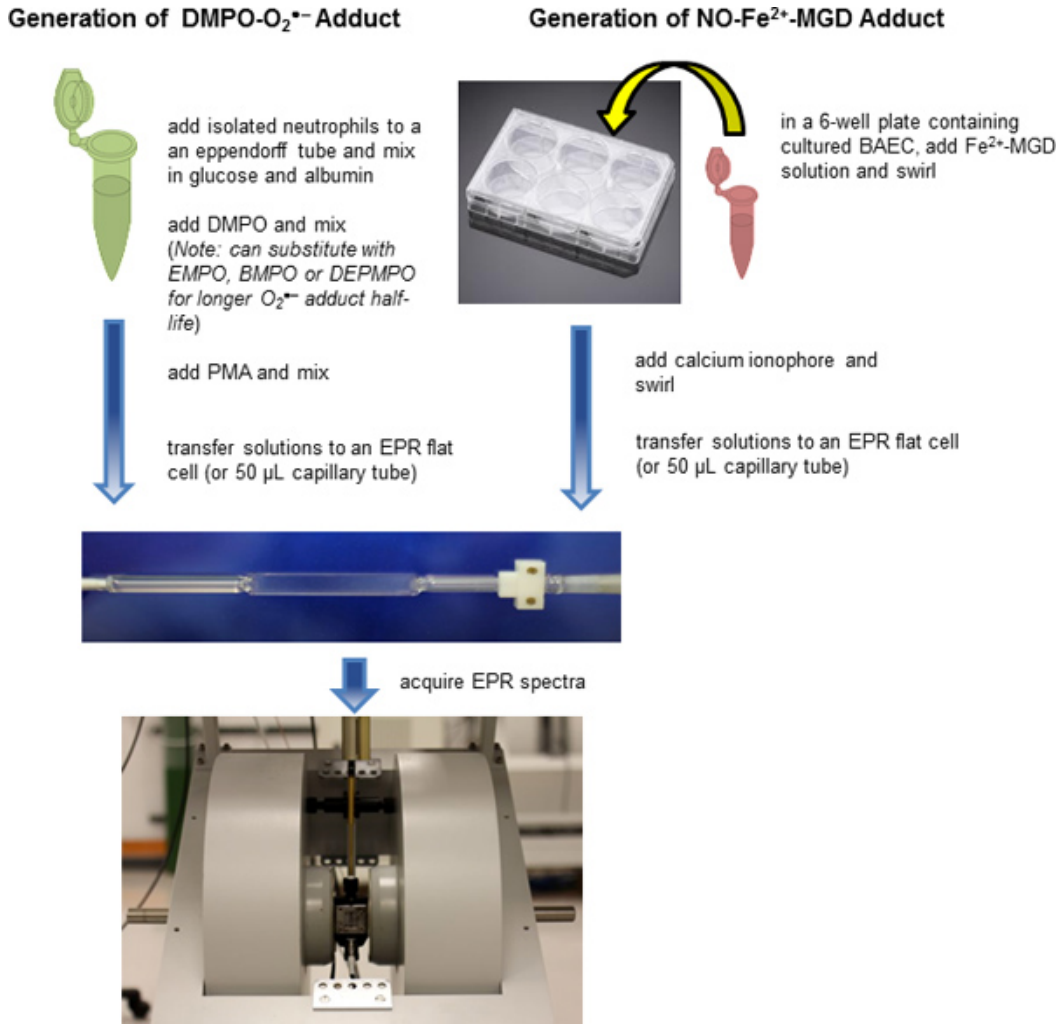

Figure 1. Flow chart for the detection of radicals from neutrophils and BAEC using EPR spin trapping. (**A**) PMNs were mixed with DMPO and PMA, and the resulting mixture transferred to an EPR flat cell for data acquisition. (**B**) BAEC were grown on a plate, and washed with DPBS. The spin trap $Fe(MGD)_2$ was added along with CaI. The solution was mixed thoroughly and incubated. The mixture was transferred to an EPR flat cell for EPR data acquisition.

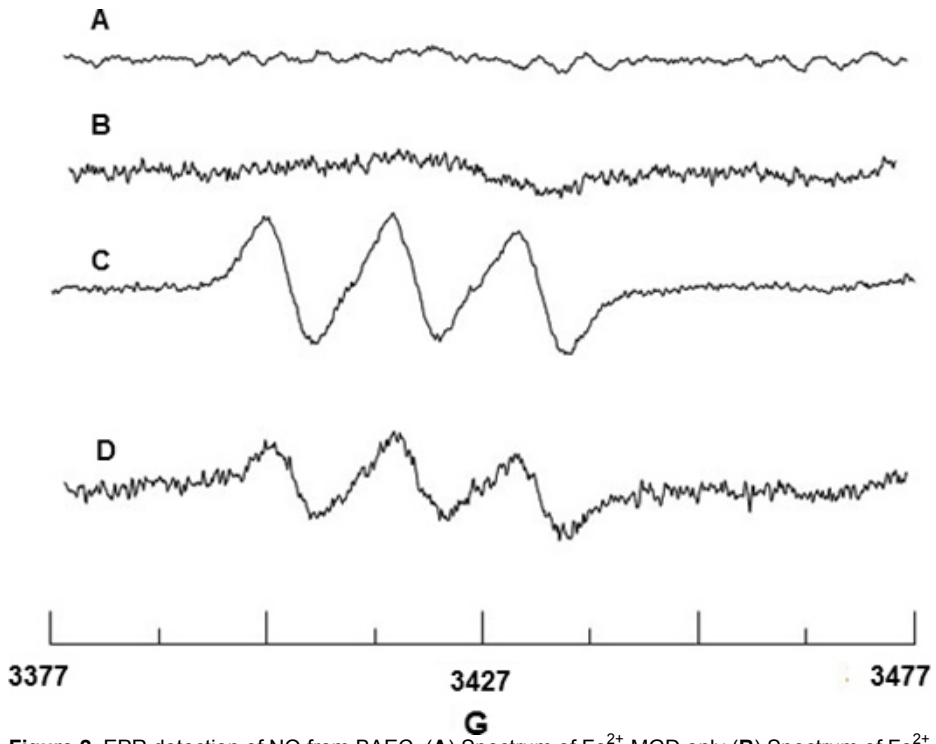

Figure 2. EPR detection of NO from BAEC. (**A**) Spectrum of Fe^{2+}-MGD only (**B**) Spectrum of Fe^{2+}-MGD + CaI only. (**C**) Triplet spectrum resulting from NO trapping by Fe^{2+}-MGD using CaI-stimulated cells. (**D**) Spectrum showing decrease in NO production due to 0.5 mM SIN-1 treatment of cells.

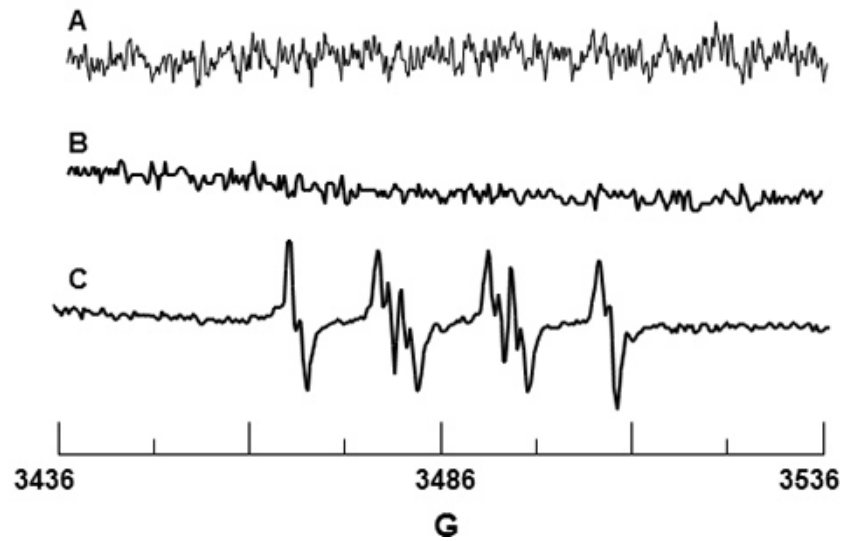

Figure 3. EPR detection of DMPO-O_2H from activated neutrophils. (**A**) Spectrum of 10 mM DMPO only. (**B**) Spectrum of PMN alone in the presence of 10 mM DMPO. (**C**) Spectrum of PMN activated by PMA in the presence of 10 mM DMPO.

Discussion

EPR spin trapping has been employed in a wide range of biomedical applications for quantifying and identifying free radicals. Spin trapping is highly sensitive, capable of detecting radicals at concentrations ranging from nM to μM thus making it suitable for application in biological systems. The formation of the paramagnetic adduct, NO-Fe^{2+}-MGD, is the basis of NO detection *via* EPR. Fe^{2+}-MGD reacts with NO rapidly[18] at a rate of ~ 10^6 M^{-1} s^{-1}. NO-Fe^{2+}-MGD adduct has a long half-life and is highly stabile. In fact, one can acquire EPR spectra within 24 hr after the samples have been collected by freezing the solutions at -80 °C containing the NO-Fe^{2+}-MGD without significant lose in signal intensity (unpublished results). The disadvantage of Fe^{2+}-MGD is that it is air sensitive and usually requires mixing 5 or more molar equivalents of the ligand to Fe(II). Oxidation of Fe^{2+}-MGD to Fe^{3+}-MGD by air is inevitable and difficult to control, however, in *in vitro* and *in vivo* systems, the NO-Fe^{3+}-MGD adduct formed is reduced by biological reductants to the EPR detectable adduct NO-Fe^{2+}-MGD[15], hence the need for ascorbate addition can be omitted.

EPR can also be used to detect O_2•- in biological systems using nitrone spin traps. As an example, **Figure 3C** shows the spin trapping using DMPO of O_2•- generated from PMA- activated PMN. One disadvantage of using DMPO as spin trap is that the rate of O_2•-/HO_2• addition to DMPO is very slow (< 1 M^{-1} s^{-1}) at neutral pH which requires the use of high concentrations of DMPO (10-100 mM) for biological spin trapping applications. Moreover, DMPO-O_2H half life is short (<1 min)[20] which decomposes to DMPO-OH due to the presence of β-hydrogen, and lacks target specificity making the determination of the site of radical production from the cell ambiguous. Success in O_2•- detection depends on the type of cells being used. For example, human neutrophils upon stimulation generates considerable flux of O_2•- which can be easily detected using DMPO but caution should be made when dealing with other types of cells where radical production is significantly less robust (*e.g.*, endothelial or epithelial cells). Several nitrones have been developed to increase the half-life of the of O_2•- adduct such as the use of EMPO and BMPO with half-lives of 7-8 min[12, 13], and DEPMPO which gives the longest half-life of 14 min[21]. However, the rates of spin trapping with O_2•- of these spin traps still remain slow compared to DMPO, and therefore still requires the use of high concentration of the spin traps. The use of randomly methylated-β-cyclodextrin (Me-β-CD) as a co-reagent has also been employed to enhance spin trapping ability of nitrones for O_2•-. For example using isolated thylakoid membrane and photosystem II particles, Snyrychova [22] showed higher signal intensity and adduct stability for EMPO-O_2H in the presence of Me-β-CD compared to EMPO alone. It is strongly recommended to compare signal intensities generated from nitrone-O_2H in the presence and absence of Me-β-CD. Moreover, the target specificity of spin traps has also been a limitation. Efforts to develop more improved spin traps that address their poor reactivity to O_2•-, short adduct half-life and target specificity in one molecular design are now underway[23-26].

Disclosures

No conflicts of interest declared.

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