^{14}N-Spin trapping of free radicals in the presence of ^{15}N-spin labeled *Neisseria gonorrhoeae*

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One of the difficult tasks confronting the study of free radicals in biology is the inability to measure "on line" injury to a biological target, while characterizing the reactive species responsible for the toxic event. This is particularly relevant in light of the fact that specific free radicals play a critical role in host immune response. An approach towards addressing this important issue draws upon the unique EPR spectral properties of ^{14}N/^{15}N-labeled compounds. In particular, *Neisseria gonorrhoeae* has been covalently labeled with ^{15}N-deuterium$_{17}$-containing 4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxyl (^{15}N-D$_{17}$-4-MAL-TEMPO). The EPR spectrum from bacteria so labeled exhibited two low-field peaks: (a) a broad, strongly immobilized species classified as "S"; (b) a more narrow, weakly immobilized component termed "W". The W/S ratio is an indicator of changes in membrane organization. In the presence of the superoxide-generating system, hypoxanthine/xanthine oxidase, an increase in the W/S ratio from 3.3 for control to 6.4 was observed, which was only partially inhibited by superoxide dismutase (W/S ratio of 4.4). When the spin trap 5,5-dimethyl-1-pyrroline ^{14}*N*-oxide (DMPO) was included in the above reaction mixture, an EPR spectrum was recorded, which was a composite of 2,2,-dimethyl-5-hydroperoxypyrrolidin-1-yl-^{14}*N*-oxyl (DMPO-OOH) and ^{15}N-D$_{17}$-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae*. With the use of computer subtraction procedures, the W/S ratio was found to be 6.4. The experiments demonstrate the utility of ^{14}N/^{15}N-labeled aminoxyls as a valuable tool in accessing the effects of specific free radicals on the fluidity of cell membranes.

Introduction

Neutrophils are a class of leukocytes that play a critical role in host immune response.[1] In doing so, these cells control bacterial and fungal infections by ingesting the microbe. Intracellular killing of these micro-organisms takes place by means of free radical-dependent and/or free radical-independent mechanisms.[2] Yet the importance of each to the control of microbial growth remains difficult to estimate, since it is arduous to assess each pathway in one general method.

One approach that may address this obvious limitation comes from the studies of Butterfield and co-workers, who found that 4-maleimide-2,2,6,6-tetramethylpiperidin-1-yloxyl, MAL-6 could covalently bind to proteins.[3] The corresponding EPR spectrum was a composite of Weakly (W) and Strongly (S) immobilized aminoxyls.† Based on a series of experiments, they were able to correlate the ratio of W/S to an on-line EPR spectral measure of membrane fluidity.[3] Since the initial series of experiments,[3a] we have expanded these studies by demonstrating that the W/S ratio of MAL-6 labeled membranes could be drastically altered by free radicals, such as superoxide and hydroxyl radical.[4] More recently, we have extended this approach to viable functioning cells.[5] Unlike earlier studies with isolated membrane fractions, we needed to determine the locale to which MAL-6 was covalently bound to cell proteins. Our approach was straightforward: develop an antibody to MAL-6, fractionate the cell and measure the localization of MAL-6.[5] When such experiments were completed, it was found that the plasma membrane of the endothelial cell was the primary target for MAL-6.[5] This, therefore, gave us the opportunity to study one of the more perplexing problems in free radical biology—measure the effects of extracellularly-generated free radicals on the integrity of intact cells in real time.[6]

Central to the investigation of the role of free radicals in cell biology is the ability to detect and characterize these reactive species. Of the available methods for the identification of free radicals, spin trapping/EPR spectroscopy has received considerable attention. This technique, which was initially developed by Iwamura and Inamoto[7] and later refined by Janzen and Blackburn,[8] consists of using a nitrone or nitroso compound to "trap" the initial unstable free radical as a long-lived aminoxyl, which can be observed by EPR spectroscopy at ambient temperature.[9] Spin trapping offers the opportunity to simultaneously measure and distinguish among a variety of important biologically generated free radicals.[10]

Various ring and substituent aliphatic hydrogens contribute

† Formerly known as nitroxides.

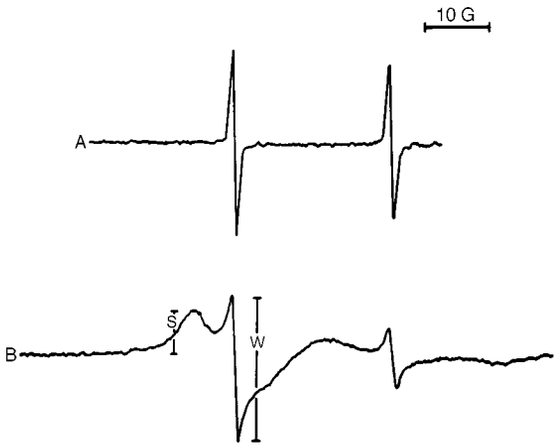

Fig. 1 (A) EPR spectrum of rapidly tumbling ^{15}N-D$_{17}$-4-MAL-TEMPO (1 μM) in HBSS (pH 7.4) at room temperature. (B) EPR spectrum of ^{15}N-D$_{17}$-4-MAL-TEMPO covalently bound to *Neisseria gonorrhoeae*. Each of the two peaks split into two subpopulations of aminoxyl, designated "W" weakly and "S" strongly immobilized. The ratio (3.3) is a measure of membrane fluidity in the bacterium. Microwave power was 20 mW, modulation frequency was 100 kHz with an amplitude of 1.0 G, sweep time was 12.5 G min^{-1}, and the receiver gain of 5×10^3 with a response time 1.0 s.

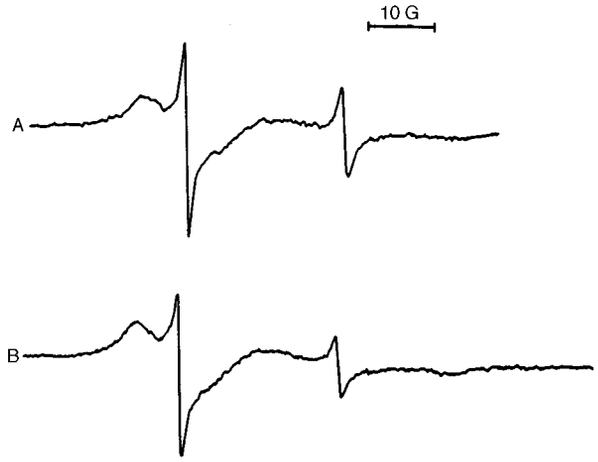

Fig. 2 (A) EPR spectrum of ^{15}N-D$_{17}$-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* in the presence of a hypoxanthine/xanthine oxidase superoxide-generating system. The calculated W/S was found to be 6.4. (B) The same experimental conditions noted in (A) except SOD (10 μg mL^{-1}) was included in the mixture. The W/S ratio was only partially inhibited, suggesting a non-free radical pathway also contributed to the enhanced W/S ratio. Microwave power was 20 mW, modulation frequency was 100 kHz with an amplitude of 1.0 G, sweep time was 12.5 G min^{-1}, and the receiver gain of 5×10^3 with a response time 1.0 s.

to the unresolved broadening of the EPR spectral lines. Deuterium has a magnetic moment approximately 1/7 that of hydrogen, but higher multiplicity, giving an overall linewidth reduction by a factor of four. Further reduction in the hyperfine splitting from 3 to 2 by replacing ^{14}N with ^{15}N results in an additional enhancement of sensitivity by a factor of 1.5. Thus, it was not surprising to discover that the EPR spectrum obtained from a protein covalently labeled with ^{15}N-deuterium$_{17}$-containing 4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxyl (^{15}N-D$_{17}$-4-MAL-TEMPO) was remarkably enhanced as compared to identical studies using ^{14}N-hydrogen-containing 4-MAL-TEMPO.[11]

These and similar observations by Radner *et al.*[12] suggested that increased sensitivity afforded by using ^{15}N-D$_{17}$-4-MAL-TEMPO might allow more accurate measurements of free radical-mediated changes in membrane fluidity. In addition as the hyperfine splitting constants for ^{14}N- and ^{15}N-containing aminoxyls are significantly different, it might be possible in one EPR spectrum to measure changes in membrane integrity using ^{15}N-D$_{17}$-4-MAL-TEMPO and to identify the free radical responsible for the observed alteration employing a ^{14}N-containing spin trap. In this communication, we report on the use of 5,5-dimethylpyrroline ^{14}N-oxide (DMPO) and ^{15}N-D$_{17}$-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* to address "on line" changes in the integrity of this bacterium, while we monitor the contribution of superoxide to bacterial injury. Even though this double isotope study has been applied to differentiate between free radical-dependent and free radical-independent mechanisms of bacterial killing, one can envision other experimental designs in which ^{14}N- and ^{15}N-containing aminoxyls might be of benefit in resolving issues pertaining to the role of free radicals in specific biologic phenomena.[3b,3c]

Results and discussion

Incubation of *Neisseria gonorrhoeae* (1×10^8 cells mL^{-1}) with ^{15}N-D$_{17}$-4-MAL-TEMPO (0.1 mM) (Fig. 1A) for 2 h at 37 °C followed by extensive washing gave the EPR spectrum depicted in Fig. 1B. As is the case with 4-MAL-TEMPO-labeled endothelial cells,[5] the recorded spectrum demonstrated the presence of two dominant classes of binding sites, covalently bound onto the outer membrane of this Gram-negative bacterium. One class exhibited a high degree of immobilization, represented by the broad low-field peak labeled "S" and a second less immobilized class was characterized by the relatively narrow peak labeled "W" (Fig. 1B). The ratio of the populations of these two classes of bound aminoxyls, typified by the relative peak heights and referred to as the W/S ratio, is an indicator of membrane fluidity.[3–5,13] In the studies presented herein, the changes in the W/S ratio reflect the independent effects of superoxide and xanthine oxidase on the organization of the outer membrane of *Neisseria gonorrhoeae*.

Analysis of the EPR spectrum obtained from ^{15}N-D$_{17}$-4-MAL-TEMPO labeled intact *Neisseria gonorrhoeae* yielded a W/S ratio of 3.3, which was unchanged over a 1 h period of time (Fig. 1B). Despite the possibility that cellular reductants such as ascorbic acid have been reported to reduce aminoxyls,[14] there is no evidence that such a reaction is operative with MAL-6-labeled *Neisseria gonorrhoeae.* With the inclusion of hypoxanthine/xanthine oxidase, generating a continued flux of superoxide at a rate of 10 μM min^{-1}, the W/S ratio at equilibrium increased to 6.3 (Fig. 2A). In the presence of SOD (30 U mL^{-1}), the W/S ratio decreased at equilibrium to 4.5 (Fig. 2B), suggesting that only a portion of the observed increase in the fluidity of the bacterial outer membrane was directly associated with superoxide. This observation parallels earlier findings with xanthine oxidase as a source of superoxide in which a direct attack on cellular membranes by this enzyme was proposed to account for the additional change in the W/S ratio.[4]

As superoxide and/or hydroxyl radical appeared to be partially responsible for the increase in the W/S ratio shown in Fig. 2A, we decided to explore the possibility of simultaneously monitoring changes in the W/S ratio while we spin trapped the free radical responsible for the injury to the bacterial membrane of *Neisseria gonorrhoeae*. First, however, it was important to determine whether there were any overlapping peaks in the EPR spectral lines of ^{15}N-D$_{17}$-4-MAL-TEMPO and ^{14}N-containing spin trapped adducts of DMPO. For these studies, xanthine oxidase was added to a sodium phosphate buffer (previously passed through a Chelex-100 ion exchange resin, 50 mM, pH 7.8) containing hypoxanthine (400 μM), DTPA (1 mM), ^{14}N-DMPO (100 mM) and ^{15}N-D$_{17}$-4-MAL-TEMPO (1 μM). As shown in Fig. 3A, there appears to be sufficient discrimination between the ^{14}N- and ^{15}N-containing aminoxyls to test the underlying hypothesis. Substituting ^{15}N-D$_{17}$-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* for ^{15}N-D$_{17}$-4-MAL-TEMPO in the above reaction mixture gave the composite EPR spectrum depicted in Fig. 4A. This spectrum clearly

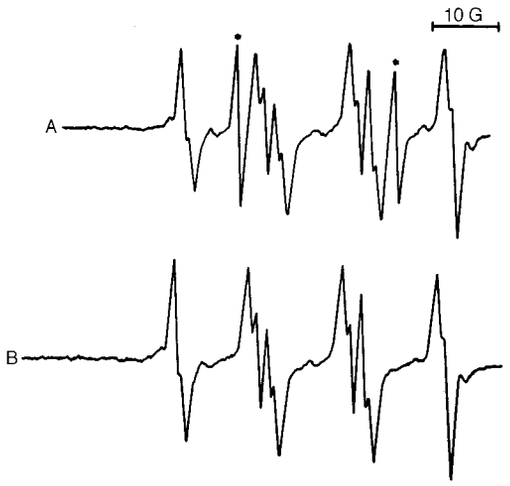

Fig. 3 (A) A composite EPR spectrum consisting of ^{14}N-DMPO-OOH and ^{15}N-D_{17}-4-MAL-TEMPO (designated with a *). Superoxide was generated using a hypoxanthine/xanthine oxidase in sodium phosphate buffer (0.1 M, pH 7.8), as a source of this free radical. (B) The same conditions as in (A) except ^{15}N-D_{17}-4-MAL-TEMPO was not present in the reaction mixture. Microwave power was 20 mW, modulation frequency was 100 kHz with an amplitude of 1.0 G, sweep time was 12.5 G min^{-1}, and the receiver gain of 5×10^3 with a response time 1.0 s.

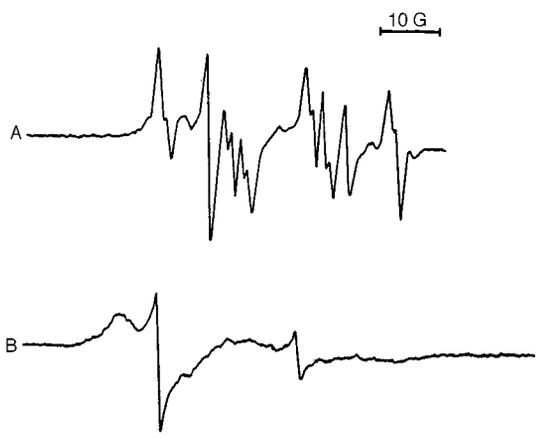

Fig. 4 (A) A composite EPR spectrum consisting of ^{14}N-DMPO-OOH and ^{15}N-D_{17}-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae*. Superoxide was generated using a hypoxanthine/xanthine oxidase in sodium phosphate buffer (0.1 M, pH 7.8). (B) The same experimental conditions as in (A) except SOD (10 μg mL^{-1}) was added to the reaction mixture. Microwave power was 20 mW, modulation frequency was 100 kHz with an amplitude of 1.0 G, sweep time was 12.5 G min^{-1}, and the receiver gain of 5×10^3 with a response time 1.0 s.

demonstrated a marked increase in the W/S ratio, even though determination of the number was not possible due to the overlap of the negative portion of the low-field peak of DMPO-OOH with the "S" of the ^{15}N-D_{17}-4-MAL-TEMPO-labeled bacteria. The addition of SOD (30 U mL^{-1}), but not catalase (300 U mL^{-1}), to the reaction mixture resulted in an EPR spectrum characteristic of ^{15}N-D_{17}-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* with a W/S ratio of 4.4 (Fig. 4B). These experiments demonstrate that the EPR spectrum depicted in Fig. 4A was due to the spin trapping of superoxide, but not hydroxyl radical, by DMPO and that the observed increase in the W/S ratio shown in Fig. 2A was, in part, due to superoxide and not hydroxyl radical.

Through the use of a computer subtraction program, it was possible to derive the EPR spectrum of ^{15}N-D_{17}-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* under experimental conditions where hypoxanthine/xanthine oxidase are present in the reaction mixture. Spectra were initially normalized to minimize the residual spectral signal of DMPO-OOH, resulting

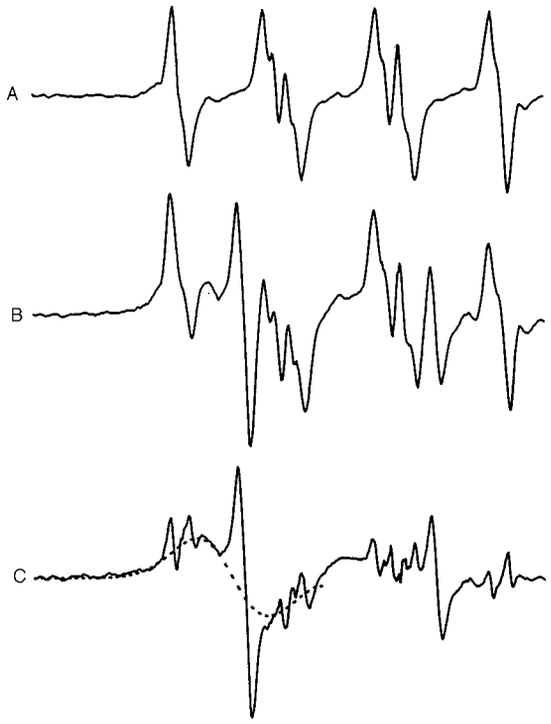

Fig. 5 Spectral subtraction (C) to determine the W/S ratio from Fig. 4A. The EPR spectrum of ^{14}N-DMPO-OOH (A, taken from Fig. 3B) is subtracted from the composite EPR spectrum of ^{14}N-DMPO-OOH and ^{15}N-D_{17}-4-MAL-TEMPO-labeled *Neisseria gonorrhoeae* (B, taken from Fig. 4A). The solid line in C represents the raw subtraction data, with the dotted line as the "best fit" to calculate the W/S ratio of 6.4. See Experimental section for details of data manipulation.

from the digital subtraction of the EPR spectrum depicted in Fig. 3B from the EPR spectrum represented in Fig. 4A. Due to subtraction artifacts, the "W" parameter could not be accurately measured from the subsequent subtraction method. For this reason, the subtracted data were numerically integrated, baseline corrected and fitted to two Gaussian functions. The Gaussian fit for the "S" parameter was then numerically derivatized to obtain an estimation of this variable (dotted line in Fig. 5C). The resulting scan indicated that the peak heights in Figs. 3B and 4A are not completely identical, resulting in a small residual spectrum of DMPO-OOH. Based on the computerized spectral subtraction procedure described above, the calculated W/S ratio shown in Fig. 5C becomes 6.4, in good agreement with experimental findings (Fig. 2A).

The results described in this communication suggest a new and novel approach to the assessment of free radical events at a cellular level. Prior to this report, typical experimental designs required disparate methods to initially characterize a specific free radical and secondarily to evaluate the cellular consequences of its formation. Here, we have drawn upon the unique magnetic properties of ^{14}N/^{15}N-labeled aminoxyls to identify a biologically-generated free radical and to measure its impact on the membrane of a viable organism using a single spectroscopic method—EPR spectroscopy. Although this technique is of particular interest in host immune response as recent studies in our[15] and other laboratories[16] have found that endothelial cells can phagocytosis and selectively kill bacteria, its general application to a wider audience interested in the critical role of free radicals in biology should catalyze considerable interest in those on the periphery of this field.

Experimental

Reagents

Hypoxanthine, cytochrome c, *N*,*N*-bis{2-[bis(carboxymethyl)-amino]ethyl}glycine (DTPA) were purchased from Sigma

Chemical Company (St. Louis, MO). Superoxide dismutase (SOD), catalase and xanthine oxide were obtained from Boehringer Mannheim (Indianapolis, IN). A sample of ^{15}N-deuterium-containing 4-maleimido-2,2,6,6-tetramethylpiperidin-1-yloxyl (^{15}N-D$_{17}$-4-MAL-TEMPO) was generously provided by Dr Alan Beth (Vanderbilt University, Nashville, TN). Additionally, ^{15}N-D$_{17}$-4-MAL-TEMPO was purchased from MSD (Montreal, Canada) through a contract with Vanderbilt University. The spin trap, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was prepared according to synthetic procedures described in the literature[17] and distilled just prior to use. When such a procedure is employed, there were no errant EPR spectra in a chelexed buffer containing DMPO (100 mM) in the absence of a superoxide-generating system. All buffers are of reagent grade and were passed through a Chelex-100 (Bio-Rad, Richmond, CA) ion exchange column to remove trace metal ion impurities prior to use.

Bacterium

Neisseria gonorrhoeae strain FA 1090 was provided by Dr P. F. Sparling (University of North Carolina, Chapel Hill, NC). The organism was subcultured daily on GC agar (GCB medium base, Difco Laboratories, Detroit, MI) containing 1% and 2% (vol/vol) Kellogg[18] defined supplements I and II and maintained at 37 °C in an atmosphere of 95% air–5% CO_2.

Bacterial labeling with maleimide-containing aminoxyl

Neisseria gonorrhoeae are removed from the plates by scraping and placed into Hank's balanced salt solution (HBSS, GIBCO, Laboratories, Life Technologies, Inc., Grand Island, NY), pH 7.4 to a final concentration of 1×10^9 cells mL^{-1}. Bacterial concentrations were estimated spectrophotometrically. Viable cell numbers were determined by dilution plating on GCB agar and by counting colony forming units after 48 h of incubation at 37 °C in an atmosphere of 95% air–5% CO_2. To the suspension of bacteria (diluted to a final concentration of 1×10^8 cells mL^{-1}) was added ^{15}N-D$_{17}$-4-MAL-TEMPO (0.1 mM final concentration dissolved in DMSO, 0.14 M) and incubated at 37 °C for 2 h. At this point, the flask was gently centrifuged at 2,500 × *g* and the solution was discarded. The remaining pellet of bacteria was gently resuspended in HBSS and centrifuged as described above. This procedure was repeated several more times until the supernatant was void of the aminoxyl signal in the EPR spectrum. At this point, the bacterial pellet was suspended to a final concentration of 1×10^9 cells mL^{-1}.

Superoxide detection

Superoxide was generated by the action of xanthine oxidase, dialyzed against deferoxamine (1 mM), on hypoxanthine (400 μM), 1 mM DTPA, 50 mM sodium phosphate, pH 7.8, such that the rate of forming this free radical was 10 μM min^{-1} at 25 °C. Measurement[19] of superoxide production was estimated optically by monitoring the reduction of ferricytochrome c at 550 nm, in the absence and presence of SOD (30 U mL^{-1}), using an extinction coefficient of 20 mM^{-1} cm^{-1}.

Spin trapping of superoxide and other EPR spectral acquisition data

The spin trapping of superoxide was accomplished by including DMPO (100 mM) with sufficient buffer to a final volume of 0.5 mL. For studies involving ^{15}N-D$_{17}$-4-MAL-TEMPO-labeled bacteria (1×10^8 cells mL^{-1}, final concentration), cells were added to the reaction mixture such that the final volume remained at 0.5 mL, transferred to a quartz EPR flat cell (Wilmad Glass Co., Buena, NJ) open to air, and placed into the cavity of an EPR spectrometer (Varian Associates, model E-109, Palo Alto, CA). Spectra were recorded at 25 °C. Instrumentation settings were microwave power, 20 mW; modulation frequently, 100 kHz; modulation amplitude 1.0 G; sweep time, 12.5 G min^{-1}; receiver gain, 5×10^3 and response time 1 s.

A computer subtraction program allowed the acquisition of the EPR spectrum shown in Fig. 5C. This required EPR spectra depicted in Figs. 3B and 4A to be stored digitally with the help of Data Thief (version 1.07, written by Kees Huyser and Jan van der Laan). Spectra were then loaded into data analyses package Origin (version 4.1, Microcal Software, Inc., Northampton, MA) for computation.

Acknowledgements

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