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SPIN TRAPPING ISOTOPICALLY-LABELLED NITRIC OXIDE PRODUCED FROM [^{15}N]L-ARGININE AND [^{17}O]DIOXYGEN BY ACTIVATED MACROPHAGES USING A WATER SOLUBLE Fe^{++}-DITHIOCARBAMATE SPIN TRAP

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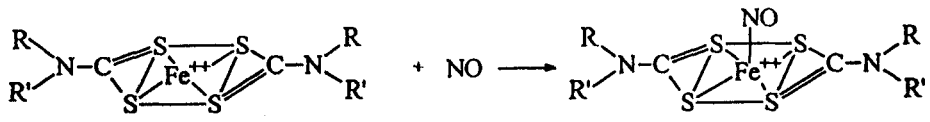
The unique capabilities of EPR spin trapping of nitric oxide based on a ferrous-dithiocarbamate spin trap have been demonstrated in a study verifying the source of the nitrogen and oxygen atoms in nitric oxide produced from activated macrophages. Spin trapping experiments were performed during nitric oxide generation from activated mouse peritoneal macrophages using the ferrous complex of N-methyl D-glucamine dithiocarbamate as a spin trap. When ^{15}N-substituted arginine was given to the activated macrophages in the presence of the spin trap, a characteristic EPR spectrum of the nitric oxide spin adduct was obtained, which indicates the presence of the ^{15}N atom in the nitric oxide molecule. The hyperfine splitting (hfs) constant of the ^{15}N nucleus was 17.6 gauss. When ^{17}O-containing dioxygen (55%) was supplied to the medium, an EPR spectrum consistent with the ^{17}O-substituted nitric oxide spin adduct was observed in the composite spectrum. The hfs of ^{17}O was estimated to be 2.5 gauss. The ^{14}NO spin adduct observed after prolonged incubation in the medium which contains [^{15}N]L-arginine as the only extracellular source of arginine demonstrates that arginine is recycled through its metabolite in activated macrophages.

KEY WORDS: nitric oxide, spin trapping, macrophages, EPR, ferrous-dithiocarbamate,

INTRODUCTION

Research on the roles of nitric oxide (NO) in biological systems has revealed its multiple physiological functions (1–5). Naturally, the development of improved methods of detecting NO have attracted considerable interest. All methods of NO detection except the gas-specific electrode detection (6,7) involve chemical modification of NO followed by spectroscopic identification. The spin trapping method is one of these chemical methods, however it is unique in the sense that the resultant electron paramagnetic resonance (EPR) spectrum depicts the presence of NO by way of the hyperfine splitting (hfs) of the nitrogen nucleus (8–11). Organic spin traps and ferrous complex-based spin traps have been used in the spin trapping studies of NO, however, use of organic spin traps in biological NO generating systems is rare (9). When NO is spin-trapped using a lipid-soluble (11–13) or a water-soluble (14) ferrous complex of dithiocarbamate according to the following reaction, the NO molecule is 'visible' in the EPR spectrum:

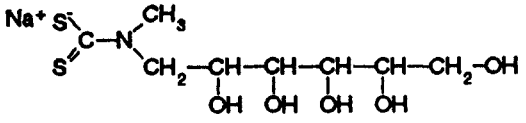


L-Arginine is the substrate of NO synthase for the generation of NO in biological systems. Compelling evidence exists that supports the proposal that NO synthase produces NO from L-arginine, NADPH and dioxygen. Previously, the source of the nitrogen and oxygen atoms in NO has been demonstrated by means of isotope incorporation into the NO molecule from [^{15}N]L-arginine and [^{18}O]dioxygen, using gas chromatography-mass spectrometry (GC-MS) technique (15–19). Results indicated that the source of the nitrogen atom in NO is one of the two guanidinyl nitrogens in L-arginine, and the source of the oxygen atom is dissolved dioxygen in the medium. Experimental techniques used in those studies involved procedures such as chemical derivatization of NO before GC-MS analyses. In the present study, in order to demonstrate that spin trapping is a fast and unambiguous technique to verify the origin of the nitrogen and oxygen atoms in NO, we have performed experiments using isotopically-labelled L-arginine and dioxygen as substrates for NO synthase in activated macrophages. Spin trapping seems to be a suitable method to study the recycling process of L-arginine in activated macrophages since the ^{14}NO spin adduct was detected after prolonged incubation in the medium which contained [^{15}N]L-arginine as the only source of extracellular L-arginine.

MATERIALS AND METHODS

Materials

Sodium N-methyl-D-glucamine dithiocarbamate (MGD) was synthesized from D-glucamine and carbon disulfide following the method described by Shinobu *et al.* (20) and purified with recrystallization in 10% acetone/water. The RPMI-1640 medium was prepared from each component using Gibco (Grand Island NY) RPMI-1640 Select-Amine Kit. In some experiments, L-arginine in the medium was replaced with arginine in which both guanidinyl nitrogen atoms were substituted with the ^{15}N atoms ([^{15}N]L-arginine, Cambridge Isotope, Andover MA). Lipopolysaccharide (LPS, from *E. coli 0111.B4*, Sigma, St. Louis MO), mouse recombinant γ-interferon (γINF, Boehringer-Mannheim, Indianapolis IN) and fetal calf serum (FCS, Sigma) were used as received. NO gas was obtained from Matheson (Secaucus NJ), and ^{15}NO (99%) and $^{17}O_2$ (55%) were purchased from Isotec (Miamisburg OH). All other chemicals were obtained from Aldrich (Milwaukee WI) in the highest available grade.



MGD

Macrophage isolation and activation

Intraperitoneal macrophages obtained from mice were used in this study (21). Macrophages were taken from adult male BALB/c mice five days after intraperitoneal injection

of brewer thioglycollate medium (DIFCO LAB., Detroit MI). Macrophages were collected by infusing the peritoneal cavity with ice cold RPMI-1640 medium. After centrifugation at 1200 × g for 10 min, cells were resuspended in RPMI-1640 with 5% FCS to make a cell suspension at 6×10^6 cells/ml. The cell suspension was placed in a flat EPR sample cuvette (quartz, 0.3 mm thick, 200 μl in volume, Wilmad), and was kept in a horizontal position at 37°C for 20 min to promote cell adhesion to the flat surface, and then a new cell suspension was introduced to attach cells on the other face of the cuvette. Total number of attached cells in the cuvette was 2.4×10^6 cells. The medium in the cuvette was gently replaced with a solution which contained LPS (100 ng/ml) and γINF (500 units/ml) for activation and cells were incubated for 3 hrs in this medium. Subsequently, the medium was replaced with the solution without activation agent and incubation ensued for 10 hrs. During the 10 hr incubation fresh medium was infused to the cuvette at the rate of 0.2 ml/hr using a syringe infusion/withdrawal pump (Harvard Apparatus Model 942). These incubations were carried out at 37 ± 1°C in a warm room.

Preparation of Fe^{++}-MGD solution and in situ spin trapping measurements

To the RPMI-1640 medium (1 ml) which had been deoxygenated by bubbling with nitrogen for 30 min, MGD (20 mM) and $FeSO_4$ (2 mM) were added. Because Fe^{++}-MGD spin trap is slowly autooxidized under high concentration of dioxygen the spin trap solution must be prepared in anaerobic conditions. In order to mix a known amount of dioxygen to the spin trap solution, 1 ml air-saturated RPMI-1640 medium was added to the spin trap solution in a syringe. To the cells in the cuvette which had been incubated for 10 hrs, the spin trap solution was infused using a syringe without exposure to the atmosphere, and both ends of the cuvette were closed with rubber septa. The sample cuvette was set horizontally in the EPR cavity which was temperature-controlled by water circulation in the jacket (22). EPR spectra were recorded at 37°C over the period of 20 hours.

The same procedure was followed to detect ^{15}NO adduct except that RPMI-1640 which contains [^{15}N]L-arginine was used in place of normal RPMI-1640 for incubations including that with activation agents. For $N^{17}O$ adduct detection, dioxygen ($^{17}O_2$-content: 55%)-nitrogen gas mixture (1:4. v/v) was used to prepare 'air'-saturated medium. A Bruker ER300E EPR spectrometer was used with 100 kHz field-modulation for the recording of EPR spectra. Typical spectrometer settings are, modulation amplitude; 1.0 gauss, microwave power; 20 mw, time constant; 0.3 s, field sweep; 100 gauss/84 s.

EPR measurement of [NO-Fe^{++}-MGD] produced from NO gas

No gas was used as a source of NO to produce the NO adduct of Fe^{++}-MGD as 'control' spectra. A degassed solution of MGD (25 mM) and $FeSO_4$ (1 mM) was bubbled with either ^{14}NO gas or ^{15}NO gas and EPR spectra were recorded to obtain the hfs of the NO adduct.

RESULTS

^{15}NO trapping: When a solution of Fe^{++}-MGD was infused over adherent macrophages which had been activated by LPS and γINF in the horizontal EPR sample cuvette, a three-line EPR spectrum slowly developed and increased in intensity

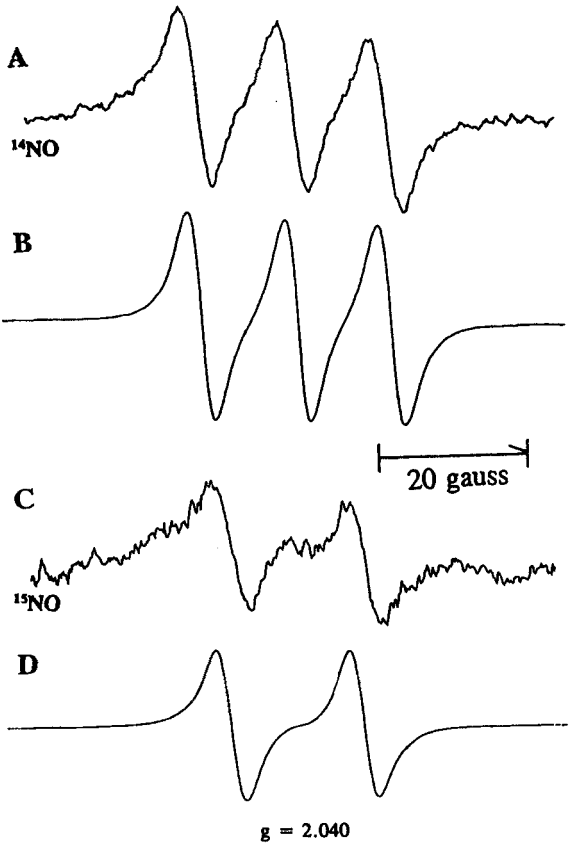


FIGURE 1 **A:** EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap from activated mouse peritoneal macrophages. Cells in the RPMI-1640 medium (with 5% FCS) were attached to the inside surface of an EPR cuvette, and activated by infusion with RPMI-1640 containing LPS (100 ng/ml) and γINF (500 unit/ml). After 3 hr activation fresh medium without activation agents was infused and incubated for 10 hrs at 37°C. Subsequently, the medium which contains oxygen (0.03 mM), $FeSO_4$ (1 mM) and MGD (10 mM) was infused to replace the old medium. The spectrum was recorded by employing the temperature-controlled (37°C) EPR cavity 1 hr after introduction of the spin trap solution. **B:** EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap obtained by bubbling NO gas through aqueous solution of $FeSO_4$ (1 mM) and MGD (10 mM). **C:** EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap from activated mouse peritoneal macrophages in the medium which contains [^{15}N]L-arginine. The same conditions were used as in **A** except L-arginine in RPMI-1640 was replaced with guanidinyl-[^{15}N]L-arginine. **D:** EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap obtained by bubbling ^{15}NO gas through aqueous solution of $FeSO_4$ (1 mM) and MGD (10 mM).

(Fig. 1A). It coincided with that obtained from an NO gas-bubbled Fe^{++}-MGD solution (Fig. 1B). When L-arginine in the incubation medium was replaced with [^{15}N]L-arginine, a two-line EPR spectrum (Fig. 1C) was obtained after infusion of the spin trap solution. The same spectrum was obtained when ^{15}NO gas was bubbled through the spin trap solution (Fig. 1D), indicating that the ^{15}NO adduct of Fe^{++}-MGD formed in the macrophage system. The ^{15}N-hfs in the ^{15}NO adduct was found to be 17.6 gauss.

$N^{17}O$ trapping: When the infused spin trap solution was previously treated with dioxygen which contained 55% ^{17}O, the EPR spectrum of the spin adduct revealed

TABLE I
EPR Parameters for NO Spin Adducts of Ferrous-MGD Spin Trap Produced from Macrophages in the RPMI-1640 Medium.

Adduct	Number of EPR lines	HFS[a] (gauss)			Line width (gauss)	g-value (field shift from DPPH[b])
		^{14}N	^{15}N	^{17}O		
^{14}NO	3	12.6	—	—	4.0	2.040 (–60 gauss)
^{15}NO	2	—	17.6	—	4.0	2.040 (–60 gauss)
$^{14}N^{17}O$	18[c]	12.6		2.5	4.0	2.040 (–60 gauss)

a. Error is ±0.1 gauss.
b. At X-band. The g-value of DPPH (diphenylpicrylhydrazyl radical) is 2.0036.
c. Unresolved.

deformations as compared to the one obtained in the presence of natural dioxygen (Fig. 2A). The difference in the spectrum in Fig. 2A as compared to Fig. 1A, is the presence of additional humps in the lower and higher field end of the spectrum and the weaker intensity in the center line. If one assumes that 50% [^{17}O]dioxygen provides the same concentrations for both $N^{16}O$ and $N^{17}O$ adducts, the intensity of any one line of the EPR spectrum from $N^{17}O$ adduct was expected to be 1/6 of that of $N^{16}O$ adduct, because

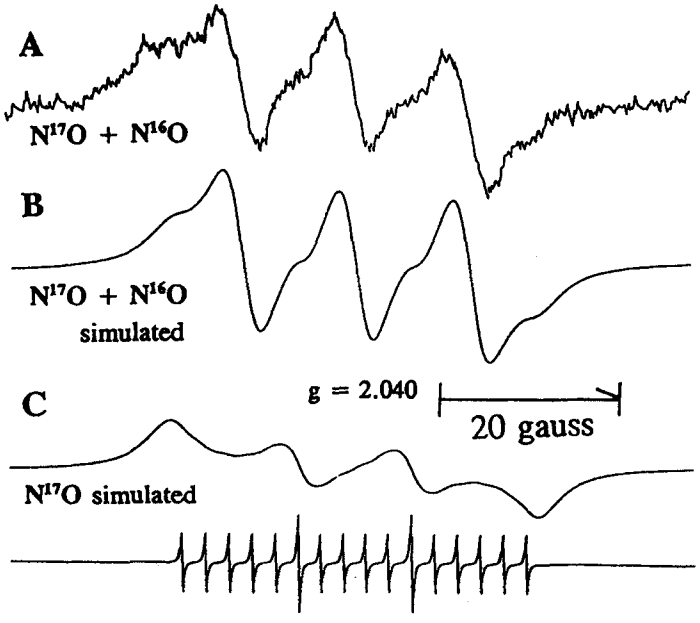


FIGURE 2 **A:** EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap from activated mouse peritoneal macrophages. RPMI-1640 medium which contains naturally abundant L-arginine was used with the same conditions in Fig. 1A except dioxygen in the spin trap solution was replaced with [^{17}O]dioxygen (atomic content of ^{17}O is 55.9%). **B:** Calculated EPR spectrum generated by superimposing spectra form NO adduct and $N^{17}O$ adduct. Spectral parameters used for the calculation are listed in Table 1 and the concentration ratio of NO adduct and $N^{17}O$ adduct was 45: 55. **C:** Calculated EPR spectrum for $N^{17}O$ adduct generated by computer spectrum simulation. The same parameters as in **B** were used (top). Computer-simulated spectrum of $N^{17}O$ adduct with the same parameters except for the reduced linewidth to show the line position of individual EPR line (bottom).

of the six-fold increase in the number of lines. Computer spectrum simulation was performed to reproduce the EPR spectrum by assuming that the spectrum contains spin adducts of both $N^{16}O$ and $N^{17}O$. Using the same linewidth, N-hfs and adjusting the abundance for the two species and including the ^{17}O-hfs in one species, the observed spectrum was reproduced (Fig. 2B). Thus, the ^{17}O-hfs in the NO adduct of Fe^{++}-MGD was estimated to be 2.5 gauss. EPR parameters obtained for the spin adducts are listed in Table 1.

Recycling: An EPR spectrum of nearly isotopically pure ^{15}NO adduct (Fig. 1C) was obtained by infusion of the spin trap solution over activated macrophages; however during prolonged incubation with the spin trap solution, increase in the amount of ^{14}NO adduct in the EPR spectrum was observed (Fig. 3A, 3B), indicating that the ^{14}N-containing L-arginine was being produced. After 20 hrs of *in situ* spin trapping, the EPR spectrum shows approximately equal amount of ^{14}NO and ^{15}NO adduct (Fig. 3B).

DISCUSSION

Enzymatic pathways of generation of NO have been established and well documented (1–5). Experimental evidence exists revealing that the source of the nitrogen atom in NO produced from the macrophage's NO synthase is one of the two guanidinyl

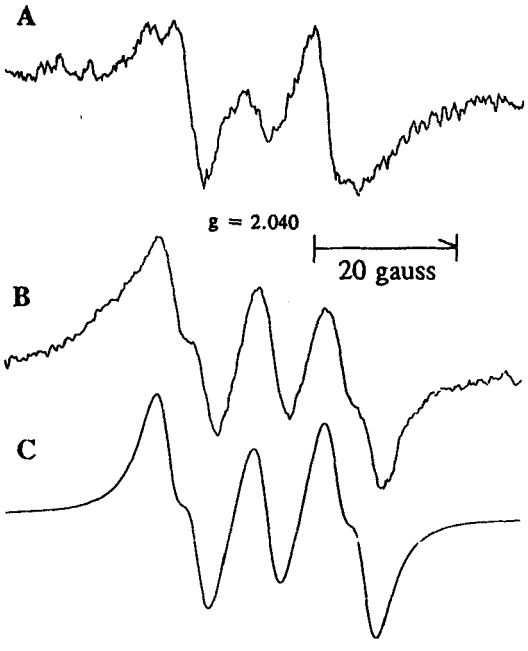
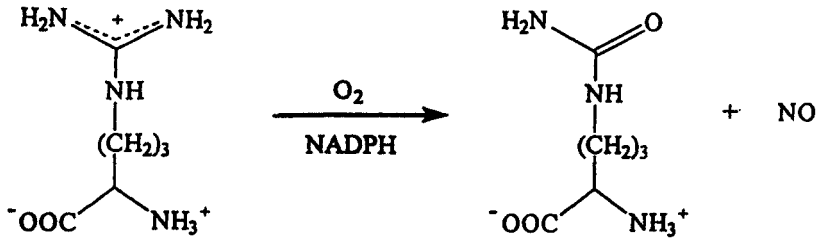


FIGURE 3 EPR spectrum of NO spin adduct of Fe^{++}-MGD spin trap from activated mouse peritoneal macrophages obtained 15 hrs (**A**), and 24 hrs (**B**) after *in situ* incubation with the spin trap solution which contains [^{15}N]L-arginine as the only source of arginine. Activation (3 hrs) and preincubation (10 hrs) were also carried out in the RPMI-1640 medium with [^{15}N]L-arginine. **C:** Computer simulated spectrum for **B** by superimposing the equal amount of ^{15}NO and ^{14}NO adduct.

nitrogen atoms and the source of the oxygen atom in NO is one of the oxygen atoms in the dioxygen molecule (15–19):



L-Arginine **L-Citrulline**

The present results demonstrate in a direct manner the incorporation of the N atom into NO from L-arginine and thus verifies the source of the nitrogen atom in NO. The EPR spectrum of the NO spin adduct obtained in the RPMI-1640 medium containing [^{15}N]L-arginine, clearly depicted the presence of ^{14}NO (Fig. 1C). It is evident that the electronic structures of ^{14}NO spin adduct and ^{15}NO spin adduct are the same because the ratio of the hfs (17.6 gauss/12.6 gauss = 1.40) coincides with the ratio of the magnetic dipole moments of the ^{15}N and ^{14}N nuclei (1.42). The EPR spectrum obtained using [^{17}O]dioxygen displayed the presence of the N^{17}O adduct, indicating that the dissolved dioxygen is incorporated into the NO molecule. Because the EPR spectrum of the mixture of N^{17}O and N^{16}O adduct (Fig. 2A) was reproduced by using the same ratio as for ^{17}O-content in dioxygen (55: 45), this result suggests that dissolved dioxygen is a sole source of oxygen in NO. However, explicit verification requires the use of pure ^{17}O^{17}O. In spin trapping experiments using 5,5-dimethyl pyrolline-N-oxide, ^{17}O labeling was also used to identify superoxide (O_2^-) and hydroxyl (OH) adduct (23). In the previous studies to pursue the source of the nitrogen and oxygen atoms in NO, similar procedures have been used but GC/MS was used as a detection method (15–19). In those studies, isotopically-labelled arginine or dioxygen were used as substrates and produced NO was derivatized to a molecule which is suitable for GC analysis and then the shifted molecular weight was detected by MS. Previous spin trapping studies using lipid soluble ferrous N-diethyl dithiocarbamate (DETC) as a spin trap have also revealed the formation of ^{15}NO from activated macrophages in the medium which contained [^{15}N]L-arginine (13). However, yeast membrane was used to solubilize DETC. The present study verified these results in intact macrophage system without isolating the product from the generating system. Since low toxicity of the Fe^{++}-MGD spin trap has been shown in spin trapping studies of the nitroprusside-injected mouse (14) and in the mouse sepsis model (24) and because Fe^{++}-MGD is water soluble, this spin trap is suitable for continuous monitoring of NO formation from viable biological systems.

In the present spin trapping experiment using [^{15}N]L-arginine as source of NO, although L-arginine in all RPMI-1640 media was replaced with 99% [^{15}N]L-arginine, the EPR spectrum of the ^{14}NO adduct was not completely removed especially in the later phase of spin trapping. The primary reason for this may be that the medium with [^{15}N]L-arginine contains naturally abundant L-arginine as a contaminant. However, we observed that the relative amount of the ^{14}NO adduct (3 lines) in the EPR spectrum was increased as a function of time after infusion of spin trap solution (Fig. 3A, 3B).

Although endothelial cells' capability of converting L-citrulline into L-arginine has been shown (25,26), Wu and Brosnan have observed that the same occurs in macrophages (27) using the formation of radioactive [^{14}C]L-arginine from [^{14}C]L-citrulline. In these processes, it is speculated that L-citrulline acquires the nitrogen atom from a naturally abundant aspartate to produce L-arginine by the function of argininosuccinate synthetase and argininosuccinate lyase in macrophages. Therefore, L-arginine with both guanidinyl nitrogens substituted with ^{15}N atoms may lose one of the ^{15}N atoms to form L-citrulline, and then gains one ^{14}N atom when it is recycled back to L-arginine. If this recycled L-arginine is used as a substrate for NO synthase to generate NO, formation of ^{14}N-containing NO will result. Increase in the amount of ^{14}NO adduct as a function of incubation time supports the presence of the recycling. After 16 hr incubation, the produced NO contained approximately equal amount of ^{14}NO and ^{15}NO (Fig. 3B), suggesting that the total amounts of original and recycled arginine in the medium were approximately the same at that time point. However, in the spin trapping experiment for an extended period of time, the concentration of dissolved dioxygen wil vary because of the consumption by intact cells, therefore, quantitive evaluation of the rate of recycling by spin trapping is difficult at present.

In conclusion, this study has demonstrated that the spin trapping method using ferrous dithiocarbamate as a spin trap is capable of directly and continuously identifying the source of the NO molecule produced in intact cellular systems. This is because the spectral shape of the EPR spectrum of the spin adduct directly depicts the presence of the NO molecule. In contrast, other methods require chromatographic separation of products before it is identified or analyzed. In addition, the spin trap is water soluble and is not cytotoxic.

Acknowledgements

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