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In vivo Nitric Oxide Detection in the Septic Rat Brain by Electron Paramagnetic Resonance

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To detect nitric oxide (NO) in the rat brain during lipopolysaccharide (LPS)-induced sepsis, electron paramagnetic resonance (EPR) was employed with the NO trapping technique, using an iron and *N,N*-diethyldithiocarbamate (DETC) complex. An X-band (about 9.5 GHz) EPR system detected a triplet signal ($g = 2.038$) derived from an NO-Fe-DETC complex being superimposed on the $g_{\perp}$ signal of Cu–DETC complex at liquid nitrogen temperature. The height of the triplet signal peaked seven hours after injection of 40 mg/kg of LPS, and over 25×10^4 U/kg of IFN-γ enhanced the LPS-induced NO formation. Pretreatment with N^G-monomethyl-L-arginine (NMMA), an NO synthase inhibitor, deleted only the triplet signal. A triplet signal ($g_{iso} = 2.040$, $a_N = 1.28$ mT) derived from the NO–Fe–DETC complex was also observed at ambient temperature. Then, a home-built 700 MHz EPR system was used to detect an NO signal in the septic rat brain *in vivo*. We successfully monitored the NO–Fe–DETC signal in the head region of a living rat under the condition that provided maximum height of the NO–Fe–DETC signal in the X-band EPR study. Pretreatment with NMMA again deleted the NO–Fe–DETC signal. This is the first EPR observation of endogenous NO in the brain of living rats.

Keywords: Diethyldithiocarbamate, electron paramagnetic resonance, lipopolysaccharide, nitric oxide, sepsis

INTRODUCTION

Septic shock is a lethal condition caused by infection with various bacteria.[1] It is clinically characterized by hypotension, a lack of response to vasoconstrictors, tissue damage, and multiple organ failure.[2] Nitric oxide (NO), a diatomic free radical and an endogenous vasodilator, is one of the mediators implicated in septic shock.[3,4] Intravenous administration of lipopolysaccharide (LPS), a bacterial endotoxin, produces a septic-shock like syndrome with significant induction of inducible NO synthase (iNOS) in animals and human.[5,6] In the brain, in addition to physiological roles,[7–10] NO may play cytotoxic roles under various pathological conditions, such as cerebral ischemia/reperfusion,[11,12] multiple sclerosis,[13,14] and acquired immunodeficiency

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syndrome.[15] Recently, the induction of NOS messenger RNA (mRNA) was observed in the rat brain during LPS-induced sepsis.[16] However, the pathophysiological roles of NO in the brain during sepsis remain to be clarified.

To elucidate various actions of endogenous NO, it is essential to know its quantities and distributions in the organisms. However, its extremely low concentration and short half-life make it difficult to measure NO formation in a living system directly. To overcome these difficulties, an NO-trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy has been applied to the detection of NO in biological systems, including the brain.[17]

For EPR measurement of free radicals, it is common to use X-band (about 9.5 GHz) microwave which provides a high sensitivity. However, X-band microwave only allows measurement of a small volume (less than 0.1 ml) of aqueous specimens because of the dielectric loss by water in the liquid phase. Thus, X-band EPR detection of NO has been used for *in vitro* or *ex vivo* studies. In contrast, in an EPR system operating at low microwave frequencies (S-band, about 3 GHz; L-band, about 1 GHz), the dielectric losses of aqueous samples are lower, and the system has a resonator with a larger volume. Hence, a low-frequency EPR system enables one to measure relatively larger aqueous specimens, although its sensitivity is lower than that of an X-band EPR system.

An X-band EPR system has been applied in several studies to determine the NO content, including the excised brain of rat which exposed to cerebral ischemia, epilepsy or intracranial infection, employing an Fe–*N*,*N*-diethyldithiocarbamate (DETC) complex as an NO trapping reagent.[18–20] Lai and Komarov attempted *in vivo* NO detection using low-frequency EPR system with an NO trapping technique and observed an endogenous NO-derived EPR signal in the tails of living mice suffering from septic shock, using an S-band EPR spectrometer with an Fe–*N*-methyl-D-glucamine dithiocarbamate (MGD) complex.[21] We have also reported on the endogenous NO distribution within the mouse abdomen during sepsis, employing a 700 MHz-EPR system with an Fe–*N*-(dithiocarboxy)sarcosine (DTCS) complex. The two-dimensional EPR image showed that NO adducts were accumulated in the liver.[22]

The purpose of this study is to detect NO formed *in vivo* in the brains of rats that suffered from LPS-induced sepsis, using an NO trapping technique with Fe–DETC. First, we detect the NO signal with the X-band EPR system in the excised brain tissue to establish the optimal condition to produce the maximum NO level in the septic rat brain. Second, under this optimal condition, a home-built 700 MHz EPR system is applied to monitor NO production in the heads of living rats.

MATERIALS AND METHODS

Female Sprague–Dawley rats weighing approximately 200 g were used in this study. Sepsis was induced by intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS, Serotype 055:B5, Sigma, St. Louis, MO, USA). Recombinant rat interferon-gamma (IFN-γ) was purchased from Biosource International (CA, USA). For NO-trapping, 400 mg/kg of sodium *N*,*N*-diethyldithiocarbamate trihydrate (DETC) (Wako, Osaka, Japan) and 20 mg/kg $FeSO_4 \cdot 7H_2O$ (Wako, Japan)–100 mg/kg sodium citrate (Wako, Japan) (a mixture of 40 mg/ml of $FeSO_4 \cdot 7H_2O$ + 200 mg/ml of Na citrate) were injected intraperitoneally and subcutaneously, respectively, 30 min prior to the measurement. For the inhibition experiment, 100 mg/kg of N^G-monomethyl-L-arginine (NMMA, Sigma, USA) was injected intraperitoneally into the animals 30 min prior to NO-trapping. All chemicals had been dissolved in sterile saline.

For the X-band study, the animals were sacrificed by decapitation under deep anesthesia with pentobarbital. The right cerebral hemisphere was immediately excised and placed in

a 5 ml plastic syringe that was attached to an infusion tube. The sample was extruded into an EPR quartz tube (o.d., 5 mm) and immediately frozen in liquid nitrogen.[20] X-band EPR spectra were recorded at both ambient and liquid nitrogen temperatures with an X-band spectrometer (TE-200, 9.2–9.5 GHz, JEOL, Tokyo, Japan). The instrument settings were: field scan, 20 mT; sweep time, 4 min; time constant, 0.3 s; modulation amplitude, 0.63 mT; modulation frequency, 100 kHz; microwave power, 10 mW at the liquid nitrogen temperature and 60 mW at the ambient temperature. All animals had been alive until X-band EPR measurement. Signal height of each group is presented as mean ± SEM ($n = 3$).

A home-built 700 MHz microwave EPR system was composed of the following: power supplies; a personal computer; a main electromagnet (air-core, water-cooled, two-coil Helmholtz designed) equipped with a pair of field gradient coils and field scan coils; and a 700 MHz microwave EPR unit that consisted of a two-gap loop-gap resonator (41 mm in diameter; 10 mm in axial length) and modulation coils. The system was used to measure *in vivo* EPR spectra.[22] The head of a rat was held in the resonator that was adjusted by a thermostat at 37°C. Instrument settings were: field scan, 10 mT; sweep time, 1 s; time constant, 0.001 s; modulation amplitude, 0.2 mT; microwave power, 40 mW. An average spectrum was calculated from 256 scans.

RESULTS AND DISCUSSION

EPR NO-Trapping Technique

Iron complexes with dithiocarbamate derivatives such as DETC, MGD, and DTCS have a high affinity for NO, and form stable NO complexes that can be readily detected by EPR spectroscopy.[17,23,24] Fe–MGD and Fe–DTCS are water-soluble and are therefore suitable for NO trapping in aqueous specimens, including living bodies. To the contrary, DETC reacts with Fe in an aqueous solution and the complex precipitates immediately (therefore Fe and DETC have to be given separately to animals). The Fe–DETC complex is distributed predominantly within the cell membranes.[25] It has been presumed that endothelial cells are the site of NO trapping with Fe–DETC, although this assumption has never been verified. Some reports indicate that DETC has an advantage over MGD and DTCS in its permeability to brain tissue across the blood-brain barrier.[26,27] Therefore Fe and DETC were used to trap NO in this study. It has been reported that an excessive amount of DETC causes systemic hypotension with a resultant reduction in cerebral blood flow; and an excessive amount of Fe itself elicits NO formation.[20,28] To avoid those effects, we used 400 mg/kg of DETC (200 mg/ml, 2 ml/kg, i.p.) and 20 mg/kg of $FeSO_4$–100 mg/kg of sodium citrate (a mixture of 40 mg/ml of $FeSO_4$ + 200 mg/ml of Na citrate, 0.5 ml/kg, subcutaneously), which may produce only insignificant adverse effects.[20]

X-Band EPR Spectroscopy

The rats that had been treated with LPS exhibited tufted fur, lethargy, and somnolence that corresponded to the symptoms of rats and mice with sepsis.

When a rat received a vehicle (0.25 ml of sterile saline) alone prior to the trapping reagents, a four-line signal was observed in the excised brain tissue on a cryogenic X-band EPR spectrum (Figure 1(a)). This signal was recently assigned to a $g_\perp$ signal ($g = 2.025$) of a copper(II) complex with DETC [$Cu(DETC)_2$; Cu–DETC complex].[26] LPS treatment resulted in the development of a triplet signal ($g = 2.038$) being superimposed on the $g_\perp$ signal of the Cu–DETC complex (Figure 1(b)). This signal is characteristic of $g_\perp$ component of a nitrosyliron(II) complex with DETC [NO–$Fe(DETC)_2$; NO–Fe–DETC complex]. Pretreatment with NMMA (100 mg/kg, i.p.), an NOS inhibitor, eliminated the triplet signal only,

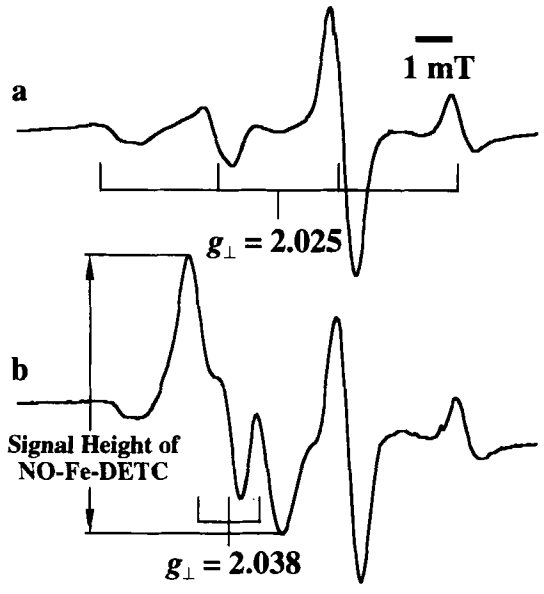

FIGURE 1 (a): X-band EPR spectrum at liquid nitrogen temperature observed in the brain tissues of vehicle-treated rat. The rat was received a saline vehicle following DETC and Fe citrate. The signal was assigned to a $g_\perp$ signal of a Cu–DETC complex.[26] (b): X-band EPR spectrum of an NO–Fe–DETC complex at liquid nitrogen temperature detected in the brain tissue of LPS-treated rat. Fe and DETC were administered 6.5 h after the injection of LPS. EPR spectrum was recorded at 7 h after the LPS-treatment. The NO–Fe–DETC signal was superimposed on the $g_\perp$ signal of Cu–DETC complex.

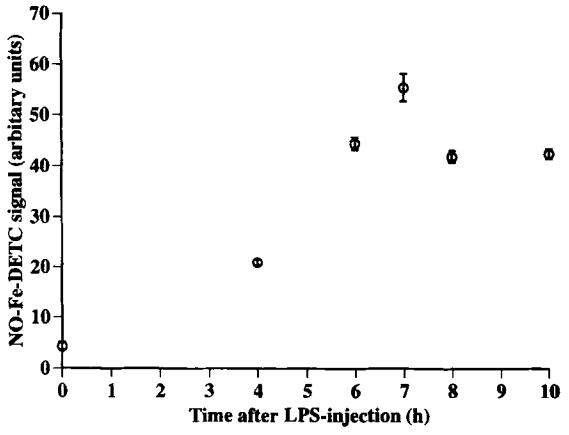

FIGURE 2 Sequential changes of the NO–Fe–DETC signal height in the rat brain tissue. X-band EPR spectra were recorded at 0, 4, 6, 7, 8 and 10 h after the injection of 10 mg/kg of LPS. The NO–Fe–DETC signal appeared 4 h and its signal height peaked 7 h after the LPS-treatment. ($n = 3$, mean ± SEM).

indicating that this NO had been synthesized with NOS induced by LPS. Because the height of the NO–Fe–DETC signal is proportional to the amount of NO generated *in vivo*,[17,20] the sequential changes in NO formation after LPS administration and the dose-responsiveness of NO with LPS and IFN-γ were studied, using the NO–Fe–DETC signal height as an index of the NO level. The EPR spectra obtained from all rats under the same experimental conditions were identical in spectral features, although minor individual variations in signal intensity were observed.

LPS-elicited NO formation in the brain was evaluated with EPR signals at 0, 4, 6, 7, 8 and 10 h after the injection of 10 mg/kg of LPS. The NO–Fe–DETC signal appeared at 4 h and its signal height peaked 7 h after the injection (Figure 2). According to Westenberger *et al.* nitrosyl hemoglobin was detected by cryogenic X-band EPR spectra in the blood of septic rats 2–3 h after the LPS injection; and its signal height reached a maximum level at 6–8 h, with a subsequent decline.[29] Further, it was reported that iNOS mRNA was expressed in a murine primary glial culture 6 h after incubation with LPS and IFN-γ.[30] These observations correspond well to our results (LPS-elicited NO reaching a maximum level around 7 h after LPS injection).

In the dose–response study, the NO–Fe–DETC signal height increased in proportion to the increase in the dose of LPS, while the signal height of 60 mg/kg of LPS corresponded to that of 40 mg/kg of LPS (Figure 3). Therefore NO formation in the LPS-treated rat brain is dependent on the amount of LPS and levels off at 40 mg/kg. It has been reported that IFN-γ increases the level of NO hemoglobin in rat blood following LPS treatment.[31] Here, an effect of combined administration of IFN-γ (0, 5×10^4, 25×10^4, 50×10^4 and 100×10^4 U/kg) with 40 mg/kg of LPS on NO formation was examined. An increase of the signal height was observed in rats that received 25×10^4, 50×10^4 and

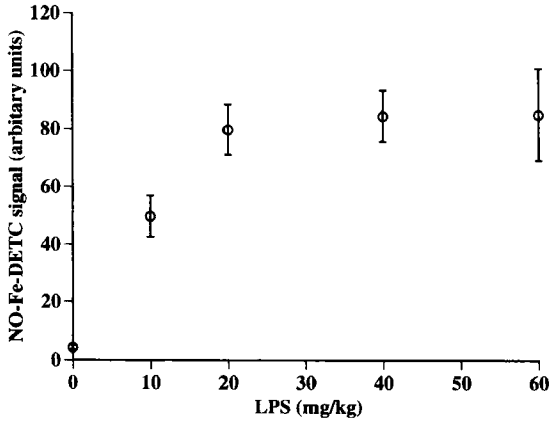

FIGURE 3 The relationship between the intensity of the NO–Fe–DETC signal and the amount of LPS. Rats were administered 0, 10, 20, 40 and 60 mg/kg of LPS. NO formation in the LPS-treated rat brain was dependent on the amount of LPS and leveled off at 40 mg/kg. ($n = 3$, mean ± SEM).

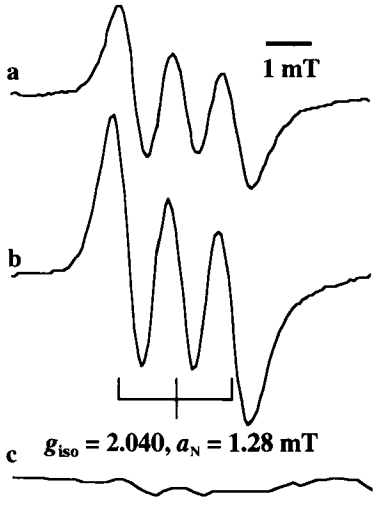

FIGURE 4 X-band EPR spectra of the NO–Fe–DETC complex at ambient temperature observed in the rat brain tissue. EPR spectra were recorded at 7 h after the administration of 40 mg/kg of LPS (a) and 40 mg/kg of LPS plus 100×10^4 U/kg of IFN-γ (b). The Cu–DETC signal was not recognized at ambient temperature. Pretreatment with NMMA (100 mg/kg, intraperitoneally) completely inhibited the LPS-elicited NO formation (c).

100×10^4 U/kg, but not 5×10^4 U/kg IFN-γ (data not shown). The enhancement of NO formation by IFN-γ supports that LPS and IFN-γ stimulate different pathways of iNOS induction and that they act synergistically.[32]

X-band EPR spectra were also recorded at ambient temperature. Trapping reagents were administered 6.5 h after the administration of 40 mg/kg of LPS. Thirty minutes after the injections of the NO trap, the brain was excised and extruded into a glass capillary tube (75 mm in length, 46 µl inner volume) with the same device described above. A triplet signal characteristic of an NO–Fe–DETC complex was observed in the LPS-treated brain tissue (Figure 4(a), $g_{iso} = 2.040$, $a_N = 1.28$ mT). Concurrent injection of 100×10^4 U/kg of IFN-γ with 40 mg/kg of LPS again enhanced the intensity of the NO–Fe–DETC signal (Figure 4(b)). The Cu–DETC signal was not recognized at ambient temperature. Pretreatment with NMMA (100 mg/kg) also completely inhibited the NO generation under this experimental condition (Figure 4(c)). This X-band EPR study at ambient temperature indicated that NO–Fe–DETC formation is not temperature dependent.

In vivo EPR Spectroscopy

In Vivo NO measurement with 700 MHz microwave was performed under the experimental conditions that gave the maximum NO signal in the X-band EPR study. Seven hours after the injection of 40 mg/kg of LPS and 30 min after the NO trap, the head of an anesthetized rat was placed in the center of the resonator of the 700 MHz microwave EPR system. Thirty minutes after Fe and DETC injections, a weak triplet signal was observed in the head region of the animal. The height of the triplet signal increased with time, peaked at 1.0–1.5 h after the injection of trapping reagents (Figure 5(a), $g_{iso} = 2.040$, $a_N = 1.25$ mT), then decreased. This NO–Fe–DETC signal was identical to the one observed in the X-band EPR study on the excised brain tissue at ambient temperature. Combined injection of IFN-γ enhanced the signal intensity (Figure 5(b)) and rendered the triplet signal even more definable. NMMA that had been injected 30 min prior to the trap also eliminated the

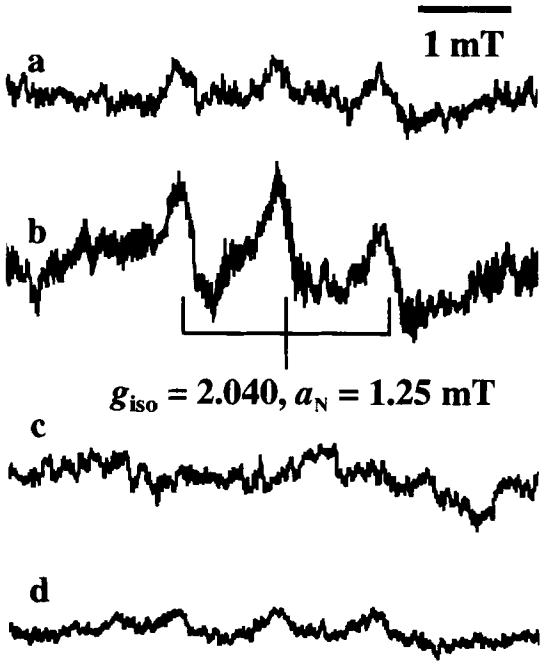

FIGURE 5 700 MHz EPR spectra of the NO–Fe–DETC complex detected in the head regions of septic rats. *In vivo* EPR spectra were recorded at 7 h after the injection of 40 mg/kg of LPS (a) and 40 mg/kg of LPS plus 100×10^4 U/kg of IFN-γ (b). NMMA (100 mg/kg, intraperitoneally, 30 min prior to the NO trap) eliminated the NO–Fe–DETC signal (c). When EPR spectrum from the head without brain, of which rat was treated in a manner similar to (b), was measured by using 700 MHz EPR system, an NO–Fe–DETC signal that was rather weak in intensity was observed (d).

NO–Fe–DETC signal (Figure 5(c)), as seen in the X-band study. These results indicate that the LPS-elicited NO that was detected in the *in vivo* EPR study had been generated by iNOS. To our knowledge, this is the first report describing the NO–Fe–DETC signal in a living organism.

The results of the X-band study on an excised brain support that the NO–Fe–DETC signal observed in the head region with a 700 MHz EPR system was originated from the brain tissue. This is also supported by the fact that iNOS is expressed in the glial cells that have been treated with LPS and LPS plus cytokines.[29,33,34]. However, it is also possible that extracranial tissue, such as endothelium and smooth muscle, produce NO during LPS-induced systemic inflammation. Further study was performed to verify this problem. Rats were decapitated under deep anesthesia with an overdose of pentobarbital after *in vivo* EPR measurement and the brains were removed immediately. When EPR spectra from the heads without brains were measured by using the 700 MHz EPR system, an NO–Fe–DETC signal that was rather weak in intensity was observed (Figure 5(d)). This result suggests that an *in vivo* EPR signal that is detected in the head region of a living rat originates predominantly from the brain but partly from the extracranial tissues.

In this study, NO formation in the brain of septic rats was assessed by employing EPR spectroscopy. It was demonstrated that 700 MHz EPR spectroscopy with an NO-trapping technique is a potent tool for detecting endogenous NO in living animals. Moreover, a low-frequency EPR system would be useful for investigations on *in vivo* spatial distributions of NO in a pathologic brain. The application of this technique is likely to contribute to the assessment of pathophysiological conditions associated with the overproduction of NO.

Acknowledgments

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