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Review Article

Detection and Imaging of Endogenously Produced Nitric Oxide with Electron Paramagnetic Resonance Spectroscopy

SATOSHI FUJII[1] and TETSUHIKO YOSHIMURA

ABSTRACT

Nitric oxide (NO) represents a new paradigm for second messengers in regulation. Despite the numerous physiological and pathophysiological functions of NO, its importance as an endogenous second messenger and a cytostatic and/or cytotoxic agent was unknown until 1987. Recent developments in detection methods for endogenous NO produced directly or indirectly from NO synthases (NOSs) have enabled major advances in our understanding of the role of NO in biological systems. The spin-trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy is a method for analyzing NO production directly both *in vivo* and *in vitro*. Iron complexes with dithiocarbamate derivatives are noteworthy among the spin-trapping reagents for NO because NO has a high affinity for iron complexes. The resultant stable nitrosyl iron complexes exhibit an intense three-line signal at room temperature and an axial signal at low temperature. Besides the facility and wide applicability of this method, its outstanding feature is that noninvasive *in vivo* measurements are available by using a low-frequency EPR spectrometer. In this article, we review on previous and recent developments of *in vitro*, *in vivo*, and *ex vivo* EPR detection and imaging of endogenously produced NO. Antiox. Redox Signal. 2, 879–901.

INTRODUCTION

THE DISCOVERY OF THE PHYSIOLOGICAL ROLE of nitric oxide (NO) in 1987 led to an explosion of research on NO and NO-related compounds in biological systems. The biological role of NO was first established in blood vessel reactivity. It is surprising that NO is biosynthesized from L-arginine by NO synthase (NOS) and that such a simple molecule can convey information concerning the regulation of vascular tone. It is now well recognized that NO is a ubiquitous second messenger and cytotoxic or cytostatic agent in both the nervous and immune systems as well as in the mammalian cardiovascular system (Moncada *et al.*, 1991; Nathan, 1992; Bredt and Snyder, 1994; Kerwin *et al.*, 1995). In addition, L-arginine-derived NO has been found in a wide variety of organisms, including mammals, invertebrates, bacteria, and plants. This recognition of numerous important functions of NO in various biological systems is the fruit of the development of techniques to analyze endogenously produced NO.

NO is a gas under standard temperature and pressure and is a freely diffusible and potentially highly reactive radical. In biological con-

Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, Yamagata 990-2473, Japan.

[1]Present address: Department of Chemistry, Faculty of Science, Konan, University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan

ditions, with the exception of the lung, NO is always found dissolved as a neutral solute but maintains its intrinsic chemical properties as a free radical. Because of its high chemical reactivity in biological systems, a half-life of only 3–5 sec in aqueous solution under physiological conditions (Ignarro, 1990) and the low level of NO produced by NOS (less than μM), it is difficult to detect endogenously produced NO.

Despite the lability and low concentration of NO, interest in its measurements in biological specimens has increased and several methods have been developed (Archer, 1993; Feelish and Stamler, 1996; Packer, 1996). Strategies for detecting NO are classified into two groups. One is direct assays, including: (1) chemiluminescence using ozone; (2) spectrophotometry using hemoglobin (Hb); (3) electron paramagnetic resonance (EPR) using spin traps, such as Hb, organic compounds, iron-dithiocarbamate [(Fe)DTC] complexes; and (4) amperometry using microelectrodes. In direct assays, NO-reactive chemicals or NO-sensitive electrodes are used, and NO itself, NO adduct, or light emission from the reaction of NO with ozone is detected. The other type of assays is indirect, analyzing stable biological metabolites of L-arginine, including nitrite, nitrate, and L-citrulline by spectrophotometry and fluorometry. Although these methods provide a measure of the amount of NO produced, each has both merits and demerits. The most appropriate method will depend upon the aims of the investigation.

EPR spectroscopy is one of the most popular methods currently available by virtue of its facility and wide applicability. NO itself is a radical and an EPR-active molecule. However, NO cannot be detected by simple EPR measurements. EPR detection of NO can be achieved by using the spin trapping technique, which has been used for detection of unstable free radicals such as hydroxyl (·OH) and superoxide ($\cdot O_2^-$). Several spin traps for NO have been developed. Iron complexes with dithiocarbamate derivatives, Fe(DTC), are particularly good spin trapping reagents for NO because of the high affinity of NO for iron complexes. The resultant nitrosyl complexes are fairly stable in air and exhibit characteristic EPR spectra, both at room temperature and low temperature. In addition, the availability of EPR spectra at room temperature, coupled with EPR apparatus operating at low frequencies, makes it possible to carry out *in vivo* noninvasive measurements of endogenous NO in living small animals (Yoshimura *et al.*, 1997).

Although several assays are available for measuring NO in biological systems, we have been employing EPR spectroscopy and Fe(DTC) complexes to study the contribution of NO toward various physiological and pathophysiological processes. In this review, we will summarize studies of EPR detection and imaging of endogenously produced NO, especially focusing on studies using Fe(DTC) complexes.

PROPERTIES OF NO

NO is a small uncharged molecule that can diffuse freely across membranes. However, because of 11 electrons in its second molecular orbital, NO contains an unpaired electron that dominates its chemical properties. As with many other radicals, it has a reactivity toward other substances to stabilize its unpaired electron. NO reacts with radicals such as O_2, $\cdot O_2^-$, ·OOH, and ·OH at an almost diffusion-controlled rate. However, self-reaction (dimerization) and direct nitrosation of organic compounds apparently do not occur. NO is, therefore, one of the less highly reactive radicals.

Under physiological conditions, another important target for NO to stabilize its unpaired electron is a metal ion. NO binds to transition metals, including Fe, Cu, Mn, Co, and Ni (Henry *et al.*, 1991; Butler and Williams, 1993), and shares its unpaired electron with their *d* orbitals. The ligation of NO to metals is crucial to the function of metalloproteins, many of which are important enzymes.

Although reactions of NO in biological systems are very important to understand fully its functions, their discussion is beyond the scope of this review and is available in previous reviews (see, for example, Henry *et al.*, 1991; Nathan, 1992; Archer, 1993; Butler and Williams, 1993; Davies *et al.*, 1995; Kerwin *et al.*, 1995; Lancaster, 1996; Williams, 1996; Yoshimura *et al.*, 1997).

EPR SPECTROSCOPY OF NO

Because NO has an unpaired electron, EPR spectroscopy is the most appropriate tool for analyzing NO in biological systems (for a general explanation of EPR spectroscopy, see, for example, Singel and Lancaster, 1996; Ohinishi, 1998). However, it is difficult to observe the NO radical by simple EPR measurement because of its rapid relaxation time. Direct measurements of NO have been achieved under special conditions, such as a pressure-controlled gas phase (Brown and Radford, 1996; Jinguji *et al.*, 1978), NO adsorbed in inorganic materials (Lunsford, 1967; Kasai and Bishop, 1973), and NO in frozen solution at liquid helium temperature (Arciero *et al.*, 1983; Orville and Lipscomb, 1993; Yoshimura, unpublished result). Accordingly, the direct detection of NO produced in biological samples appears unfeasible. In addition, the short half-life and low concentration ($<\mu M$) of NO in biological systems further render it difficult to detect directly.

Spin trapping combined with EPR spectroscopy has been used for detection and characterization of free radicals, including activated oxygen radical species (·OH, $\cdot O_2^-$, ·OOR) and organic radicals (·CR, ·SR) (Janzen, 1971). This method is also applicable to NO detection (Wilcox and Smith, 1995; Singel and Lancaster, 1996). Spin trapping agents are compounds that react with free radicals to form stable spin adducts (those used for NO detection are listed in Table 1). Typical spin traps, such as nitrones (*e.g.*, 5,5-dimethyl-pyrroline-*N*-oxide; DMPO) and nitroso compounds (*e.g.*, 2-methyl-2-nitrosopropane, MNP; 3,5-dibromo-4-nitrosobenzene, DBNBS) are also available to monitor NO generation, but there are some problems with these (Arroyo and Kohno, 1991; Archer, 1993).

Hb is a particularly good spin trap for NO (Greenberg *et al.*, 1990; Wennmalm *et al.*, 1990; Westenberger *et al.*, 1990; Wang *et al.*, 1991; Kosaka and Shiga, 1996) because it is endogenous (in vertebrates) and so obviates the need to add exogenous traps, thus avoiding perturbation of the samples. Because NO has a high affinity to reduced Hb, NO readily binds to deoxyHb to produce NO-Hb, which exhibits a characteristic EPR spectrum at low temperatures such as 77 K (Henry *et al.*, 1991). The addition of detergents such as inositol hexaphosphate and sodium dodecylsulfate improves the signal intensity by changing 6-coordinated NO-Hb (with a broad EPR signal) to 5-coordinated NO-Hb (with a sharp three-line signal). In the presence of O_2, however, a part of Hb is always in its oxy form. OxyHb reacts with NO to produce metHb and nitrate (Doyle and Hoekstra, 1981), causing inaccuracies in NO measurements. The carbon monoxide (CO) adduct of Hb is a better NO trap than deoxyHb (Kosaka *et al.*, 1992): the affinity of Hb to CO is higher than for O_2 but lower than for NO (Gibson and Roughton, 1957), so NO can displace CO to form NO-Hb and the reaction of NO with deoxyHb is suppressed. However, NO-Hb is an air-sensitive EPR-active species, thus careful treatment is needed during preparation and measurement of samples.

Organic compounds such as nitronyl nitroxides (Akaike *et al.*, 1993; Joseph *et al.*, 1993) and *o*-quinodimethane (Korth *et al.*, 1992, 1994; Korth and Weber, 1996; Paul *et al.*, 1996) have been reported as NO traps. Nitronyl nitroxides (e.g., 2-phenyl-4,4,5,5-tetramethylimidazoline-

TABLE 1. SPIN TRAPPING AGENTS FOR NO DETECTION

Traps	*Lower limit*	*References*
Protein		
deoxyHb	1 n*M*–1 μM	Greengerg *et al.* (1990)
COHb	1 n*M*–1 μM	Kosaka *et al.* (1992)
Organic compounds		
PTIO	1 μM	Akaike *et al.* (1993)
NO cheletropic trap	10 n*M*	Korth *et al.* (1994)
Inorganic compounds		
Fe-DTC	50 n*M*	Mordvintcev *et al.* (1991)

Hb, Hemoglobin; CO, Carbon monoxide; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-yloxy-3-oxide; DTC, dithiocarbamate.

1-yloxy-3-oxide; PTIO) are radicals, and when NO reduces them by abstracting oxygen in a stoichiometric manner, their EPR spectrum at room temperature changes from the five-lined spectrum of nitronyl nitroxide to nine-lined spectrum of imino nitroxide by nature (Akaike *et al.*, 1993). Nitronyl nitroxides are readily reduced by other biological reductants, so special care may be required to obtain an accurate NO level. Az-ma *et al.* (1994) reported the quantitative measurement of NO from cultured endothelial cells using a column to separate the cells from carboxy-PTO. Encapsulation in a liposome vesicle is another good approach to quantitative measurement of NO. Woldman *et al.* (1994) measured the NO production from rat cerebellum by incorporating nitronyl nitroxide trap into large unilamellar phosphatidylcholine liposomes. Akaike and co-workers (1996) further improved the stability and specificity of nitronyl nitroxides by encapsulating them in liposomes prepared by a reverse-phase evaporation method by using L-α-phosphatidylcholine and dimyristoylamido-1,2-deoxyphosphatidylcholine, thus improving reliability. Quantitative measurements of NO produced from cultured RAW 264 cells were carried out with this liposome encapsulated 2-trimethylamino PTIO. Nitronyl nitroxides are also used as a selective NO scavenger. Many physiological applications have been reported (for example, Akaike *et al.*, 1993; Yoshida *et al.*, 1993; Maeda *et al.*, 1994; Yoshida *et al.*, 1994; Konorev *et al.*, 1995a,b).

NO trapping by *o*-quinodimethane is based on the reaction of *cis*-conjugated dienes with free radicals, such as 7,7,8,8-tetramethyl-*o*-quinodimethane which is generated by photolysis of 1,1,3,3-tetramethyl-2-indanone. When it reacts with NO, the cyclic compound 1,1,3,3-tetramethylisoindolin-2-oxyl is formed, which exhibits a three-line hyperfine signal. Thus, this type of compound containing the 7,7,8,8-tetramethyl-*o*-quinodimethane moiety is called NO cheletropic traps (NOCTs). NO formation from cultured liver macrophages, Kupffer cells, was detected by NOCTs (Korth *et al.*, 1992). However, the application of NOCTs to biological specimens is very limited because of the instability and low water solubility of the trap or the NO adducts. Modified NOCTs that are improved in thermal stability and water solubility have been synthesized (Korth *et al.*, 1994; Korth and Weber, 1996). It is an advantage of NOCTs that they can also trap NO_2, and the EPR spectrum of NO_2 adducts is distinguishable from that of NO adducts. Unfortunately, these compounds are not commercially available.

IRON COMPLEXES WITH DITHIOCARBAMATE DERIVATIVES AS NO TRAPPING AGENTS

$Fe(III)(DTC)_3$ complexes and ferrous nitrosyl complexes, $NO\text{-}Fe(II)(DTC)_2$, attracted much attention during the 1960s and 1970s because of their anomalous magnetic and electronic properties (Coucouvanis, 1970, 1979; Fujii and Yoshimura, 2000). Although the EPR spectrum of the $NO\text{-}Fe(II)(DTC)_2$ complex was first reported in 1962 (Gibson, 1962), its first biological application was reported in 1991 by Mordvintcev *et al.* (1991). They used an *N,N*-diethyldithiocarbamate (DETC; Fig. 1) as an exogenous ligand, which combines spontaneously with the ionic Fe in biological tissues, and succeeded in detecting NO in a variety of biological systems. Exogenous iron salts such as iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) are also available as an iron source, but the solubility in water of Fe(DETC) complex is so low that the iron salt and DETC ligand must be administered to samples separately (Ohnishi, 1998). (Hereafter DTCs that form water-insoluble metal complexes are named Group 1.)

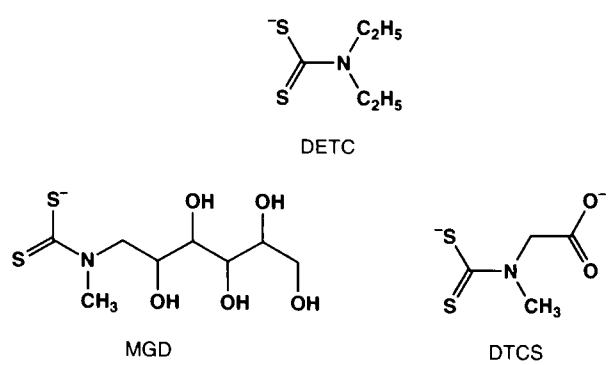

FIG. 1. Dithiocarbamate derivatives used as NO trapping reagents. Abbreviations used: NO, nitric oxide; DETC, *N,N*-diethyldithiocarbamate; MGD, *N*-methyl-D-glucamine dithiocarbamate; DTCS, *N*-(dithiocarboxy)sarcosine.

The application of this detection method was promoted by the development of DTCs that form water-soluble iron complexes (Group 2), including *N*-methyl-D-glucamine dithiocarbamate (MGD; Fig. 1) and *N*-(dithiocarboxy)sarcosine (DTCS; Fig. 1). Because these ligands have a hydrophilic group in addition to the dithiocarboxyl group, their iron complexes dissolve well in water, even after complexation with iron ion. Accordingly, these NO traps can be prepared as a solution of desired concentration or powder before use. Several DTCs that form water soluble iron complexes have been developed (Fujii and Yoshimura, 2000). To date, NO produced from all NOS isoforms can be detected by using this NO trapping technique. Typical experimental procedures are summarized in the literature (for Group 1 DTCs, Mülsch *et al.*, 1992a; Ohnishi, 1998; for Group 2 DTCs, Kotake, 1996, Yoshimura *et al.*, 1996).

In general, the solubility in water of Fe(DTC) complexes and NO-Fe(DTC)$_2$ complexes depends on the DTC substituent, with decreasing solubility in the order: DTC with carboxyl groups (*e.g.*, DTCS) > DTC with hydroxyl groups (*e.g.*, MGD) >> DTC with alkyl groups (*e.g.*, DETC). Water-soluble NO traps can be transported via the circulation system, so they are suitable for *in vivo* real time detection of NO under physiological and pathophysiological conditions. In addition, this mobility enables NO adducts of water-soluble iron complexes to be used as spin labels (Yoshimura *et al.*, 1995, 1997; Yokoyama *et al.*, 1997). Although the facility of the preparation of iron complexes and their mobility are advantages of water-soluble NO traps, they do not permeate into lipophilic organs such as brain (Suzuki *et al.*, 1997; Fujii and Berliner, 1999). Therefore, it is necessary to select the most appropriate ligand for the target organ or tissue required (see section EPR Detection of NO Using Fe-DTC Traps).

Chemical bases of NO trapping by Fe(DTC) complexes

Both Fe(II)(DTC)$_2$ and Fe(III)(DTC)$_3$ complexes react with NO to form NO-Fe(II)(DTC)$_2$ complexes, either in the presence or absence of oxygen (Fujii *et al.*, 1996b). The reaction scheme of this spin trapping is shown in Fig. 2. NO reacts with Fe(II)(DTC)$_2$ complex as a simple addition. This type of reaction was studied by EPR and laser flash photolysis (Paschenko *et al.*, 1996). The reaction of NO with Fe(III)(DTC)$_3$ complex under anaerobic condition has been investigated by EPR, high-performance liquid chromatography (HPLC), LC-electrospray mass spectroscopy, and the technique of pulse-radiolysis. It was found that the reaction occurred in a stoichiometric manner via a new type of reductive nitrosylation (Fujii *et al.*, submitted for publication). The mechanism of the reaction of Fe(II)(DTC)$_2$ and NO from hydroxyurea under aerobic conditions has been proposed recently (Tsuchiya *et al.*, 1999).

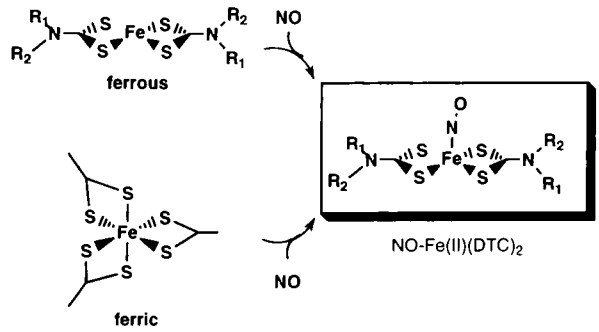

FIG. 2. The reaction scheme of NO trapping. Abbreviations used: NO, nitric oxide; DTC, dithiocarbamate.

The NO-Fe(II)(DTC)$_2$ complex exhibits a characteristic three-line EPR spectrum ($g_{av} \approx 2.04$; $A_N \approx 1.27$ mT) at room temperature (Fig. 3A) and a spectrum with axial symmetry ($g_\perp \approx 2.037$, $g_\parallel \approx 2.015$) at low temperature (Fig. 3B). The EPR spectrum at room temperature is nearly isotropic in aqueous solution. In biological specimens, however, a three-line spectrum with an anisotropic component (tilted three-line) is often observed. This is probably because free rotation of the complex is restricted by the interaction of the NO complex with biological components such as membrane. Three-line splitting originates from the hyperfine interaction of an unpaired electron with the ^{14}N nucleus ($I = 1$) of NO. If an NO source such as L-arginine or nitrate is labeled with ^{15}N ($I = 1/2$), a doublet signal ($g_{av} \approx 2.04$, $A_N \approx 1.8$ mT) is observed (Fig. 3C). This distinctive feature is a great advantage of this method because it enables the NO source to be assigned easily (Lancaster and Hibbs, 1990; Kubrina *et al.*, 1992; Kotake *et al.*, 1995; Zweier *et al.*, 1995b; Kuppusamy *et al.*, 1996a; Xia and Zweier, 1997; Fu-

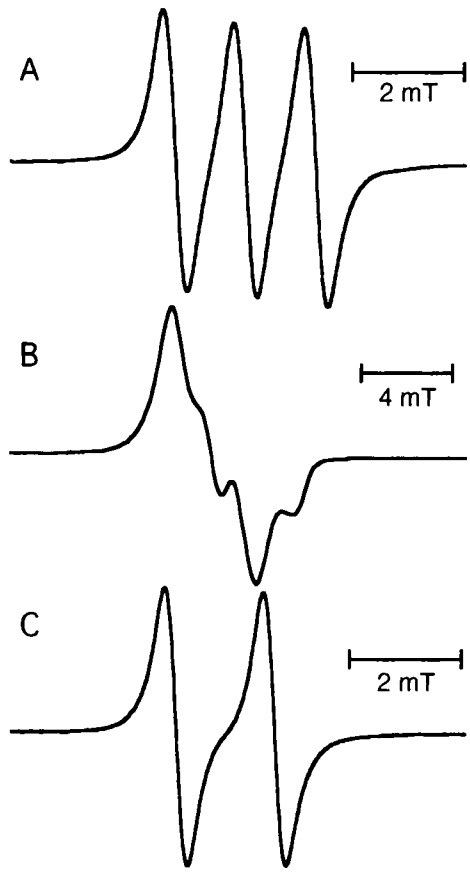

FIG. 3. X-Band EPR spectra of the ^{14}NO-Fe(II)(DTCS)$_2$ complex at room temperature (A), and at 77 K (B), and of the ^{15}NO-Fe(II)(DTCS)$_2$ complex at room temperature (C). The NO complexes were chemically synthesized. DTCS, *N*-(dithiocarboxy)sarcosine.

jii *et al.*, 1997, 1998). The EPR parameters of NO-Fe(II)(DTC)$_2$ complexes are slightly dependent on the DTC substituent. However, it has been reported that the reactivity of some water-soluble Fe(III)(DTC)$_3$ complexes toward NO depends on both the DTC substituent and the solvent used (Fujii *et al.*, 1996b; Fujii and Yoshimura, 2000). Recently, Pou *et al.* (1999b) reported the stability of NO complexes of MGD and DTCS and evaluated the pharmacokinetics of both NO complexes in mice.

The stability of the NO-Fe(II)(DTC)$_2$ complexes is essential for this detection method, but there have been few systematic investigations of the factors contributing to NO complex stability (Fujii and Yoshimura, 2000). The stability in ambient air of the NO-Fe(II)(DTC)$_2$ complexes is an interesting and important property, because it facilitates sample handling and may improve the lower detection limit. In general, transition metal nitrosyl complexes, including Fe complexes with other ligands and Hb, are very sensitive to air. Oxidative degradation occurs immediately after they are exposed to air. However, NO-Fe(II)(DTC)$_2$ complexes degrade gradually and their life-time in ambient air (≈ day) is much longer than that of the spin adducts of most organic spin traps (≈ min). Embedding in liposomes further improves the stability of NO-Fe(II)(DTC)$_2$ complexes (Yoshimura *et al.*, 1997).

Other features of the Fe(DTC) spin trapping method

Whenever DETC is used as an NO trapping agent and the EPR spectra are measured at low temperature (*e.g.*, 77 K), a background signal with four lines is observed (Fig. 4). This four-line signal appears even when only DETC is added (Fig. 4A) (Mordvintcev *et al.*, 1991). It has been assumed that the origin of this background signal is Cu(II)(DETC)$_2$ complex, which is formed adventitiously from exogenous DETC and endogenous Cu(II) ion, although the

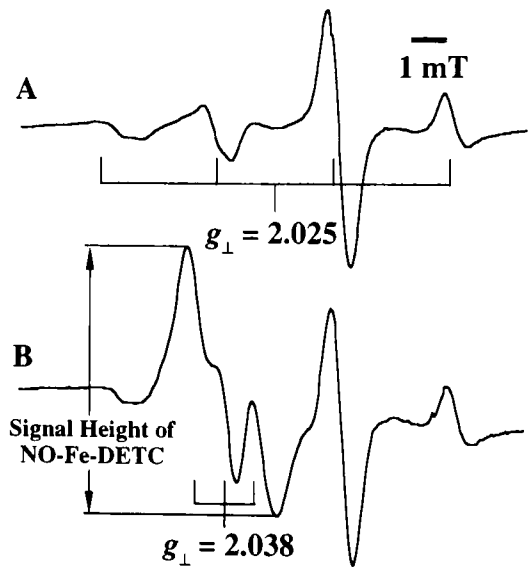

FIG. 4. (A) X-band EPR spectrum at 77 K observed in the brain tissues of sterile saline-treated rat. The rat received a saline vehicle following DETC and Fe citrate. The signal was assigned to a $g_\perp$ signal of a Cu(DETC) complex. **(B)** X-band EPR spectrum of an NO-Fe(DETC)$_2$ complex at 77 K detected in the brain tissue of LPS-treated rat. The NO-Fe(DETC)$_2$ signal was superimposed on the $g_\perp$ signal of Cu(DETC) complex. Reprinted with permission from Suzuki *et al.* (1998), copyright ownership by Overseas Publishers Association, N.V. Abbreviations used: DETC, *N*,*N*-diethyldithiocarbamate.

signal line shape is different from that of typical Cu(II) complexes such as type 1 and type 2 coppers (Vänngård, 1972).

Suzuki *et al.* (1997) clearly showed that the background signal is certainly derived from $Cu(II)(DETC)_2$ complex by comparing EPR spectra in organic solvents, liposomes, albumin suspension, and biological specimens. In some organic solvents, liposomes, and albumin suspension, the $Cu(DETC)_2$ complex exhibits an EPR spectrum similar to the background signal and its $g_\perp$ component corresponds to the four-line signal (^{63}Cu, $I = 3/2$; $g_\perp \approx 2.035$, $A_\perp \approx 4.3$ mT). They also reported that the behavior of DTCs differs with the solubility of their metal complexes in water. DTCs of Group 1, such as *N,N*-dimethyldithiocarbamate (DMTC) and pyrrolidine dithiocarbamate (PDTC), show similar EPR spectra to DETC. However, Group 2 DTCs, such as DTCS and L-proline dithiocarbamate (ProDTC), show different EPR spectra to DETC in organic solvents. This difference is more remarkable in biological samples. When Group 1 DTCs were administered to rats, an EPR spectrum similar to DETC was obtained in the brain, but with Group 2 DTCs, no distinct Cu(II) signal was observed.

It has been suggested that the copper source of $Cu(II)(DETC)_2$ complex is free intracellular Cu(II) (Mordvintcev *et al.*, 1991). Sato *et al.* (1995) reported that the sources of Cu(II) are Cu/Zn superoxide dismutase (SOD) and/or Cu-albumin complex in plasma. Two questions arise concerning the mechanism of introducing DTCs into intracellular space. First, how do Group 1 DTCs penetrate into membranes, including the blood–brain barrier? Second, why do Group 2 DTCs exhibit no Cu(II) signal in brain? Although Nobel *et al.* (1995) have reported that PDTC (Group 1) can transport external copper into the cells, there is not enough information to answer these questions. It is important to clarify the behavior of DTCs to obviate any unexpected biological effects. DETC inhibits the Cu/Zn-SOD by extracting Cu from the protein both *in vivo* and *in vitro* (Heikkila *et al.*, 1976; Misra, 1979; Cocco *et al.*, 1981). This suggests an increase in the concentration of O_2^- *in vivo* and a concomitant decrease in the concentration of NO, because NO and O_2^- reacts at almost diffusion-control rate (Huie and Padmaja, 1993) to form peroxynitrite, a more toxic species than NO. Moreover, it has been reported that, in red blood cells, DETC reacts with oxyHb to produce activated oxygen species, metHb, sulfHb, oxidized DETC, and lipid peroxidation products, besides inhibiting intracellular Cu/Zn-SOD (Kelner and Alexander, 1986; Kelner *et al.*, 1989).

In addition, other biological effects of DTCs have been reported. Although many of these effects are attributed to their metal-chelating properties, some are based on the other properties. For example, it is reported that DTCs inhibit enzymes by making a disulfide bond their sulfhydryl group and free protein thiols (Neims *et al.*, 1966). They also inhibit glutathione *S*-transferases by direct binding to the protein (Dierickx, 1984). Moreover, DTCs can exert either antioxidant or pro-oxidant effects in different situations. As an antioxidant, PDTC has been reported to be a potent inhibitor of oxidative activation of the nuclear transcription factor-κB (NF-κB) (Schreck *et al.*, 1992). NF-κB regulates gene expression in various physiological processes, such as immunity, stress responses, inflammation (Baeuerle and Baltimore, 1996), glial and neuronal function (O'Neill and Kaltschmidt, 1997; Kaltschmidt *et al.*, 1999), and the inhibition of apoptosis (Antwerp *et al.*, 1996; Wang *et al.*, 1996). Therefore, the inhibition of NF-κB activation by PDTC causes various biological phenomena, including inhibition of inducible NOS (iNOS) expression (Eberhardt *et al.*, 1994; Bedoya *et al.*, 1995), inhibition of apoptosis in thymocytes (Wolfe *et al.*, 1994), leukemic cells (Bessho *et al.*, 1994), and, in L929 fibroblasts (Albrecht *et al.*, 1994), induction of heme oxygenase-1 gene expressions (Hartsfield *et al.*, 1998), and so on (Schreck *et al.*, 1992; Hattori *et al.*, 1997; Altura and Gebrewold, 1998; Kaltschmidt *et al.*, 1999). In contrast, as a pro-oxidant, PDTC is an inducer of apoptosis in thymocytes (Nobel *et al.*, 1995). DETC has been reported to inhibit induction of iNOS in the same manner as PDTC (Schreck *et al.*, 1992; Mülsch *et al.*, 1993b).

The biological effects of Group 2 DTCs seem to be different from those of Group 1 DTCs. Representative differences include their membrane permeability and ability to withdraw copper from metalloprotein complexes such as

Cu/Zn-SOD. Group 1 DTCs are usually used as a complex with ionic iron, which may relieve the action of free DTCs on their targets. Recently, however, iron complexes with Group 2 DTCs (DTCS and MGD) have been reported to inhibit NO production from purified neuronal isoform of NOS (nNOS) by interfering with the physiological electron flow from NADPH to nNOS heme iron; although free DTCs did not inhibit the activity (Yoneyama *et al.*, 1999). Because the iron complexes with Group 2 DTCs are considered not to permeate through membranes, it is important to know whether or not similar to those reported *in vitro* can occur *in vivo*.

Although it is essential to clarify the biological effects of the NO traps, there have been few systematic investigations of them. Considering the properties of DTCs, it is expected that those of the same group exert similar effects on biological systems. The effects exerted may also depend on the conditions of use (*in vivo* or *in vitro*). One should, therefore, keep in mind the possibility of such effects when using these NO traps.

IN VIVO REAL-TIME EPR DETECTION AND IMAGING OF NO PRODUCED IN BIOLOGICAL SYSTEMS

Feasibility of continuous NO monitoring

Although the addition of exogenous NO traps causes disturbance to biological specimens to some extent, the feasibility of continuous and quantitative monitoring from the same biological specimen is a great advantage of this method (Kotake, 1996). The reagents used in other NO detection methods are often toxic, and their addition to samples causes serious damage. For example, the reagents used in the Griess method, a commonly used nitrite assay, are highly acidic, so that cells lose their viability.

The LD_{50} of some DTCs alone in mice has been estimated: 1,870 mg/kg (orally, Merck Index 12 ed., 1996) or 1,332 mg/kg (intravenously, Yoshimura *et al.*, unpublished data) for DETC·Na·$3H_2O$; over 2,500 mg/kg (Shinobu *et al.*, 1984) or over 7,000 mg/kg (Yoshimura *et al.*, unpublished results) for MGD·Na, 765 mg/kg for DTCS·$2NH_4$·$2H_2O$; and 1,942 mg/kg for DTCS·2Na·$2H_2O$ (Fujii *et al.*, 1996a). Unfortunately, the toxicity of Fe(DTC) complexes has not been estimated precisely. However, solution of these NO traps are neutral and the doses used are relatively low. Therefore, the addition of these traps does not cause fetal damage to biological systems and continuous observation at ambient temperature is feasible.

In vivo *EPR spectroscopy and imaging*

At conventionally used X-band (9–10 GHz) frequencies, large biological specimens (*e.g.*, tissues, organs, and whole body) cannot be measured because of high dielectric loss of water, which is the major component of biological specimens. Accordingly, large samples should be resected and homogenized or sliced to fit a flat-type EPR cell developed for measuring samples with high dielectric loss.

In vivo EPR spectroscopy has been developed with the aim of measuring paramagnetic species produced *in vivo* noninvasively (Eaton *et al.*, 1991; Colacicchi *et al.*, 1993). EPR spectroscopy at lower frequencies satisfies the requirements of sample volume and diminishing the dielectric loss of water from water-rich samples (Pou *et al.*, 1999a). L-band (0.3–2 GHz) spectrometers are now available, and the whole body of mice or rats can be measured noninvasively. Furthermore, EPR imaging similar to nuclear magnetic resonance imaging (MRI) can be obtained by applying additional magnetic field gradients (Alecci *et al.*, 1990; Kuppusamy *et al.*, 1995a; Yoshimura *et al.*, 1997 and references cited therein). By using these kinds of EPR apparatus, one can characterize free radical species in small animals or isolated organs and obtain information on their spatial distribution. However, it is difficult to detect endogenous free radicals produced *in vivo* by physiological and pathophysiological processes because of their short half-lives and low concentrations. Poor sensitivity of the spectrometer, due mainly to the reduction of sensitivity by lowering the microwave frequency, also makes it hard to detect them (Pou *et al.*, 1999a).

Despite such difficulties, *in vivo* real-time de-

tection of NO by low-frequency EPR is highly attractive, because the concentration of NO produced *in vivo* under some conditions is relatively high ($\approx \mu M$) compared with that of other free radicals and continuous monitoring of NO production at ambient temperature is available, as mentioned above. In fact, several groups have succeeded in detecting and/or imaging NO both *in vivo* and *in situ*. The following sections will summarize our researchers concerning the EPR detection and imaging of endogenously produced NO.

EPR DETECTION OF NO USING Fe-DTC TRAPS

In vitro *NO detection from cultured cells*

Detection of NO from cultured cells using Fe(DTC) traps is straightforward. After co-incubation with the NO trap for appropriate times, the sample cell suspension or supernatant is subjected to conventional X-band EPR measurements (Mülsch *et al.*, 1992b; Vanin *et al.*, 1993; Kotake, 1996; Norby *et al.*, 1997). Although DTCs of either group can be used, Group 2 DTCs are preferable regarding preparation of iron complexes. The advantage of the method is that the NO source is easily distinguishable on using L-[^{15}N]arginine. When L-[^{15}N]arginine is added to the medium instead of L-[^{14}N]arginine, a two-line EPR signal (Fig. 3C) is observed if NO is derived from L-arginine (Lancaster and Hibbs, 1990; Kubrina *et al.*, 1992; Komarov and Lai, 1995; Kotake *et al.*, 1995; Kotake, 1996).

In general, NO produced from macrophages, stimulated by cytokines and/or bacterial lipopolysaccharide (LPS), is readily detectable by this NO trapping technique. The iNOS induced by cytokines and/or LPS produces a high concentration of NO ($\approx \mu M$), thus a distinct EPR signal is obtained at room temperature (Kotake *et al.*, 1996). It is noteworthy that NO produced from endothelial cells can be detected directly by this method, even though the low concentration and short half-life of endothelium-derived NO make direct detection difficult. The only other method to succeed in the direct detection of endothelium-derived NO is the NO-sensitive microelectrode (Malinski and Taha, 1992; Taha *et al.*, 1992). Mülsch *et al.* (1993a) first reported the direct EPR detection of NO from porcine aorta endothelial cells, using a water-insoluble Fe-DETC trap. The lipophilic nature of Fe(DETC) may be an advantage for detection of intracellular NO, like endothelial-derived NO, because endothelial NOS (eNOS) is localized in membranes and neutral NO has a tendency to dissolve in the membrane and lipid phases of cells (Kerwin *et al.*, 1995).

To examine whether or not hydrophilic NO traps can detect such a low level of NO synthesized by eNOS, we applied the water-soluble Fe(DTCS) trap to the measurement of NO production from cultured porcine aorta endothelial cells (1×10^7) (Fujii *et al.*, 1996a). The EPR signal of the NO-Fe(DTCS)$_2$ complex was successfully obtained at low temperature. The signal intensity of the NO adduct was sensitive to the stimulants such as adenosine 5′-triphosphate and calcium ionophore A23187, indicating that NO is certainly generated from eNOS. The NO synthesized by endothelial cells exerts its principal biological effect in adjacent vascular smooth muscle cells, where it activates soluble guanylate cyclase, and ultimately leads to vasorelaxation (Moncada *et al.*, 1991; Kerwin *et al.*, 1995). It is, therefore, meaningful to detect and analyze the intercellular NO diffusing out from endothelial cells by employing water-soluble Fe(DTC) traps.

Recently, the water-soluble Fe(MGD) trap has been used as a probe of NO activity released from control and diabetic rat endothelium (Pieper and Lai, 1999). The detection of NO from unstimulated endothelial cells is very difficult indeed, so the usage as a scavenger of NO (Pieper and Lai, 1996) is unique in that sense.

Ex vivo *NO detection from resected organs or tissues*

Ex vivo NO detection by X-band EPR spectrometry is probably the most common usage of this NO trapping method. After the administration of the NO trap into an animal, target organs or tissues are resected and measured by a conventional X-band EPR spectrometer. NO

TABLE 2. *Ex Vivo* DETECTION OF NO FROM RESECTED BRAIN[a]

Experimental model	*DTCs*	*References*
LPS-induced (mouse)	DETC	Kubrina *et al.* (1993)
LPS-induced (rat)	DETC	Suzuki *et al.* (1998)
	DETC	Fujii and Berliner (1999)
Global ischemia (rat)	DETC	Sato *et al.* (1993)
Forebrain ischemia (rat)	DETC	Tominaga *et al.* (1994)
Focal ischemia and reperfusion (rat)	DETC	Sato *et al.* (1994)
Global ischemia and reperfusion (rat)	DETC	Shutenko *et al.* (1999)
Kainate-treated (rat)	DETC	Mülsch *et al.* (1994)
Experimental meningitis (rat)	DETC	Suzuki *et al.* (1999)

LPS, Lipopolysaccharide; DETC, *N,N*-diethyldithiocarbamate.
[a]Only X-band measurements are listed.

synthesized by NOSs in various organs or tissues has been detected (Tables 2–4). In *ex vivo* experiments, NO produced from LPS-induced iNOS is the main target, as in *in vitro* experiments. Both groups of DTCs are useful because the distribution of their iron complexes is dependent on their solubility to water. In particular, permeability into brain through the blood–brain barrier is a useful property of Group 1 DTCs (Table 2) (Suzuki *et al.*, 1997; Fujii and Berliner, 1999).

Concerning the distribution of Fe(DTC) traps, we have investigated the behavior of some NO-Fe(DTC)$_2$ complexes administered to mice, using a low-frequency EPR spectrometer (Yokoyama *et al.*, 1997). Three NO-Fe complexes (of DTCS, MGD and DETC) were chemically synthesized, and 200 m*M* solutions were injected subcutaneously. The 700-MHz EPR spectra of resected abdominal organs and whole blood from the mice subjected to injections of NO-Fe(DTC)$_2$ complexes were measured 40 min after the injections. The signal amplitude was corrected on the basis of the wet weight of the sample. The resected liver, gastrointestinal organs, and blood sampled after injection of NO-Fe(DTCS)$_2$ gave a triplet signal. The signal amplitude was far higher in the liver than in other samples. With NO-Fe(MGD)$_2$ injection, the triplet signal was detectable in the liver but undetectable in the other samples. The signal amplitude of NO-Fe(MGD)$_2$ was much lower than that treated with NO-Fe(DTCS)$_2$. No signals were observed in any samples obtained from mice receiving NO-Fe(DETC)$_2$ injections. These observations seem to reflect the mobility of NO-Fe(DTC)$_2$ complexes and, further, that of Fe(DTC) complexes.

In addition to NO produced from NOSs, NO

TABLE 3. *Ex Vivo* DETECTION OF NO FROM RESECTED LIVER[a]

Experimental model	*DTCs*	*References*
LPS-induced (mouse)	DETC	Kubrina *et al.* (1992)
	DETC	Kubrina *et al.* (1993)
	DETC	Quaresima *et al.* (1996)
	DETC, MGD	Mikoyan *et al.* (1997)
	DETC	Komarov *et al.* (1998)
	MSD	Nakagawa *et al.* (1998)
LPS-induced (rat)	MGD	Reinke *et al.* (1996)
	DETC	Wallis *et al.* (1996)
	DETC, MGD	Fujii and Berliner (1999)
	MGD	Lecour *et al.* (1999)
Regeneration (rat)	DETC	Obolenskaya *et al.* (1994)

LPS, Lipopolysaccharide; DETC, *N,N*-diethyldithiocarbamate; MGD, *N*-methyl-D-glucamine dithiocarbamate; MSD, *N*-methyl-L-serine dithiocarbamate.
[a]Only X-band measurements are listed.

TABLE 4. *Ex Vivo* DETECTION OF NO FROM RESECTED ORGANS[a]

Experimental model	*Organs*	*DTCs*	*References*
LPS-induced (mouse)	Intestine, lung, heart, kidney, spleen	DETC	Kubrina *et al.* (1993)
	Urine	MGD	Komarov and Lai (1995)
	Blood	DETC MGD	Mikoyan *et al.* (1997)
	Blood	MSD	Nakagawa *et al.* (1998)
LPS-induced (rat)	Bile	MGD	Reinke *et al.* (1996)
	Intestine, kidney	DETC	Wallis *et al.* (1996)
	Kidney, aorta, Heart	MGD	Lecour *et al.* (1999)
STZ-induced diabetes (mouse)	Pancreas	DTCS	Tabatabaie *et al.* (1997)
Ischemia (rat)	Heart	MGD	Zweier *et al.* (1995a)
	Heart	MGD DETC	Komarov *et al.* (1997)
Mouse	Stomach	DETC	Mikoyan *et al.* (1994)

LPS, Lipopolysaccharide; DETC, *N,N*-diethyldithiocarbamate; MGD, *N*-methyl-D-glucamine dithiocarbamate; MSD, *N*-methyl-L-serine dithiocarbamate; STZ, streptozotocin; DTCS, *N*-(dithiocarboxy)sarcosine.
[a]Only X-band measurements are listed.

formation from nitrovasodilators is detected by Fe(DTC) traps. Representative nitrovasodilators including glyceryl trinitrate (Mülsch *et al.*, 1995a,b), isosorbide dinitrate (ISDN) (Mülsch *et al.*, 1995b; Fujii *et al.*, 1998), and sodium nitroprusside (Rao and Cederbaum, 1995) have been investigated by this NO trapping technique and found to release NO under biological conditions.

Ex vivo EPR measurements are also important as preliminary experiments for *in vivo* EPR measurement. Low-frequency EPR spectrometry is not sensitive enough for quantitative analysis of endogenous NO using the EPR spectra of NO adducts, so optimum conditions are first determined by *ex vivo* X-band EPR measurements. Under the conditions determined by these preliminary *ex vivo* EPR measurements, we succeeded in detecting endogenous NO production in rat brain *in vivo* in two experimental models (Suzuki *et al.*, 1998, 1999). Details are summarized in the following section.

In vivo *NO detection from living small animals*

The first *in vivo* EPR spectrum of endogenously produced NO was reported by Komarov *et al.* (1993). They used an S-band (3.5-GHz) EPR spectrometer and the Fe(MGD) trap, and monitored (from the tail) the production of NO from sodium nitroprusside in the blood circulation of a conscious mouse. They also reported the detection of NO produced from a LPS-treated mouse using similar procedures (Lai and Komarov, 1994; Komarov and Lai, 1995). The restricted diameter (4 mm) of the loop-gap resonator of their S-band spectrometer limited the *in vivo* NO detection by their EPR NO trapping method to measurements of the blood circulation in the tail.

To detect the NO production in organs and/or tissues *in vivo*, we used a 700-MHz EPR spectrometer (Yoshimura *et al.*, 1997) and a highly water-soluble Fe(DTCS) trap. The 41-mm diameter of the 700-MHz loop-gap resonator enables us to measure the whole body of mice (~30 grams). Using the 700-MHz EPR spectrometer and the Fe(DTCS) trap, we succeeded in detecting and imaging the NO produced from the upper abdominal region of LPS-treated mice (Yoshimura *et al.*, 1996). Details are described in the next section.

Similar *in vivo* NO detection in the abdominal region of LPS-treated mice have been reported. Quaresima *et al.* (1996) used an L-band (1.14-GHz) EPR spectrometer and Fe(DETC) trap and obtained *in vivo* EPR spectra of NO adduct 6 hr after intravenous (i.v.) injection of LPS. By employing an L-band (1.2-GHz) EPR spectrometer, the Fe(MGD) trap and L-[^{15}N]arginine, Fujii and co-workers (Fujii *et al.*, 1997; Fujii and Berliner, 1998) observed the *in vivo* EPR spectra of both ^{14}NO and ^{15}NO adducts 8 hr after intraperitoneal (i.p.) injection of LPS. Nakagawa *et al.* (1998) reported the *in*

vivo EPR spectrum from the upper abdomen of mice that had received i.v. injection of LPS 7 hr before L-band (1.1-GHz) EPR measurement with iron complex with *N*-methyl-L-serine dithiocarbamate (MSD).

Unfortunately, the diameter of our 700-MHz loop-gap resonator (41 mm) does not allow us to measure the whole body of rat (~200–250 grams), but it is suitable for the head. Therefore, we tried to detect *in vivo* NO production from NOS in the rat brain in two experimental models.

It is well known that the administration of LPS produces a septic shock-like syndrome with significant induction of iNOS in both human and experimental animals (Parratt, 1973; Suffredini *et al.*, 1989). However, no direct detection of NO in the brain of living animals has been reported. We, therefore, prepared a septic model rat induced by i.p. injection of LPS, with or without rat recombinant interferon-γ (IFN-γ), and attempted the direct *in vivo* detection of NO from the head of intact living rats. IFN-γ was used because it has been reported that IFN-γ stimulates the pathways of iNOS induction different from those of LPS (Förstermann *et al.*, 1995).

The optimum doses of LPS and IFN-γ and the optimal interval between stimulation and measurements were determined by *ex vivo* experiments, as described in the previous section. Seven hours after the injection of LPS, the *in vivo* EPR spectra of NO-Fe(DETC)$_2$ were obtained from the living rat head (Fig. 5A) (Suzuki *et al.*, 1998). Combined injection of LPS and IFN-γ enhanced the signal intensity (Fig. 5B). The EPR signal of NO adducts disappeared upon injection of N^G-monomethyl-L-arginine (NMMA), NOS inhibitor, 30 min before NO trap injection (Fig. 5C). When the brain was removed from the head of the rat and given a combined injection of LPS and IFN-γ, the EPR signal intensity was greatly reduced (Fig. 5D). These results clearly demonstrate that the NO-Fe(DETC)$_2$ signal observed in the head region originated from the brain tissue and from iNOS induced by both LPS and IFN-γ.

In vivo NO detection by the NO trapping method was successful also in bacterial meningitis model. In various phases during bacterial meningitis, the involvement of excessive NO

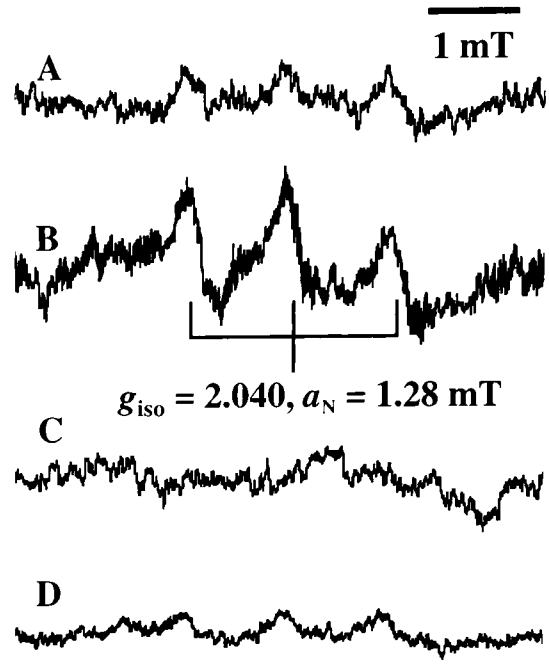

FIG. 5. 700-MHz EPR spectra of the NO-Fe(DETC)$_2$ complex detected in the head regions of septic rats. *In vivo* EPR spectra were recorded at 7 hr after the injection of 40 mg/kg of LPS (**A**) and 40 mg/kg of LPS plus 100 × 10^4 U/kg of IFN-γ (**B**). NMMA (100 mg/kg, i.p., 30 min prior to the NO trap) eliminated the NO-Fe(DETC)$_2$ signal (**C**). When the EPR spectrum from the head without brain (rat was treated in a manner similar to **B**) was measured by using the 700-MHz EPR system, an NO-Fe(DETC)$_2$ signal that was rather weak in intensity was observed (**D**). Reprinted with permission from Suzuki *et al.* (1998), copyright ownership by Overseas Publishers Association, N.V.

synthesis has been reported (Pfister *et al.*, 1992; Tunkel and Scheld, 1993; Koedel *et al.*, 1995; Boje, 1996; Kim and Täuber, 1996; Kornelisse *et al.*, 1996; Leib *et al.*, 1998). However, NO in living animals during meningitis has never been measured directly. Therefore, we examined NO formation in the rat brain under experimental bacterial meningitis using 700-MHz EPR spectrometry (Suzuki *et al.*, 1999). A mixture of LPS and IFN-γ in sterile saline was used as an inducer of experimental bacterial meningitis (Tunkel and Scheld, 1993; Koedel *et al.*, 1995; Boje, 1996).

After *ex vivo* experiments to determine the optimum conditions, *in vivo* EPR measurements were performed on the heads of living rats. Eight hours after the intraventricular inoculation of the LPS/IFN-γ mixture, a weak triplet signal was initially observed. The height of the signal increased, peaked 60–90 min after

the injection of Fe(DETCO) (Fig. 6A-1), and then decreased. The line shape of this signal was identical to that observed in the rat's brain under sepsis (Fig. 5A,B). The EPR signal of endogenous NO adducts was eliminated by treatment of NMMA 30 min before Fe(DETC) injection (Fig. 6A-2). No signal was observed in the control (sterile saline) group (data not shown). These results showed that the *in vivo* EPR signal of NO adducts detected in the heads of living rats originated from NOS induced by experimental meningitis.

Expression of iNOS gene during bacterial meningitis was also verified with reverse transcriptase-polymerase chain reaction (RT-PCR) (Fig. 6B). Before RT-PCR experiments, we failed to detect the expression of iNOS by Western blot analysis under our experimental condition, although this was achieved using brain homogenates of infant rat with meningitis induced by group B. streptococci (Leib *et al.*, 1998). Considering our results, *in vivo* EPR spectroscopy is more sensitive than Western blot analysis in a sense. This is probably because NO produced by iNOS is accumulated by the Fe(DETC) trap.

As with *ex vivo* experiments, NO from NOS-independent pathways has been detected by *in vivo* EPR spectroscopy. NO production from the nitrovasodilator ISDN in mice has been investigated (Fujii *et al.*, 1998), as described in the following section.

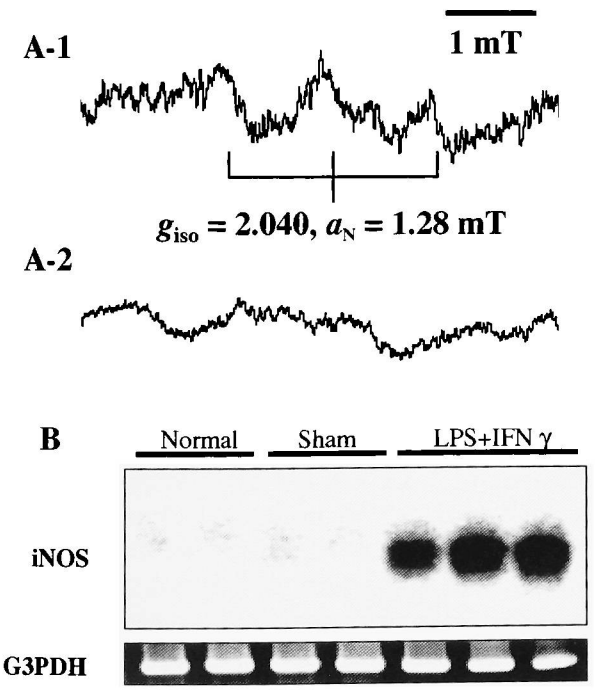

FIG. 6. ***In vivo*** **700-MHz EPR spectra of the NO-Fe(DETC)$_2$ complex detected in the head region of a living rat during experimental meningitis, which was induced by the intraventricular injection of LPS plus IFN-γ.** (**A-1**) EPR spectrum recorded at 8 hr after the injection of LPS plus IFN-γ. (**A-2**) EPR spectrum after the treatment of LPS plus IFN-γ and then of NMMA. (**B**) RT-PCR analysis of the induction of the inducible isoform of NOS mRNA in the brain of the rats during experimental meningitis. Reprinted with permission from Suzuki *et al.* (1999). Abbreviations used: LPS, lipopolysaccharide; IFN-γ, interferon-γ; RT-PCR, reverse transcriptase-polymerase chain reaction; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

IN VIVO EPR IMAGING OF ENDOGENOUSLY PRODUCED NO

Fe(DTC) complexes added to various biological samples can stabilize and accumulate endogenously produced NO to produce an intense EPR signal. By using the Fe(DTC) complexes, therefore, one would be able not only to detect *in vivo* EPR spectrum of the endogenous NO in organs or tissues but also to obtain its *in vivo* EPR image.

Kuppusamy *et al.* (1995b, 1996b) performed pioneer work on EPR imaging of endogenous NO. NO produced in the rat brain during ischemia-hypoxia was trapped by the systemic administration of DETC and iron. Three-dimensional EPR imaging was then performed in the resected brain frozen with liquid nitrogen by employing an EPR imaging system with a microwave frequency of 1.2 GHz. The EPR images of the brain thus obtained exhibited several areas with high NOS activity.

We have been attempting the *in vivo* EPR imaging of NO produced in tissues of living animals by the application of Fe(DTC) complexes as an NO trapper. Here, we report on our results to date.

To obtain *in vivo* EPR spectra and images, we used a home-built EPR imaging system with a 700-MHz microwave unit and a two-gap loop-gap resonator (Yoshimura *et al.*, 1997). The dimension of resonator was 41 mm in diameter and 10 mm in axial length, which is adapted to the head of a rat or the whole body of a mouse. Before we attempted to image endogenous NO

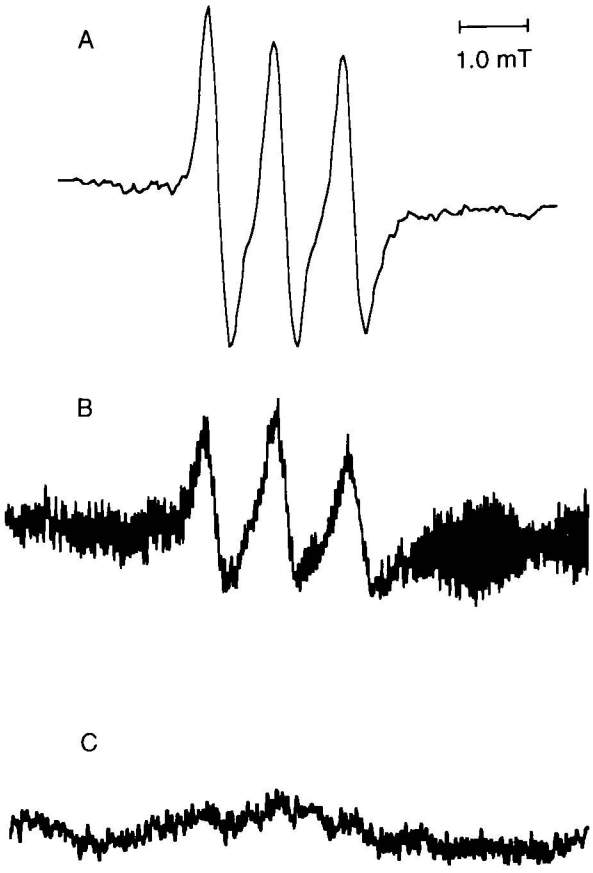

FIG. 7. EPR spectra of NO adduct in an aqueous solution (A) and in the abdominal region of mice treated with LPS only (B) and both LPS and NMMA (NOS inhibitor) (C), measured by a 700-MHz EPR system. Reprinted with permission from Yoshimura *et al.* (1996).

in living animals, we performed an imaging experiment employing NO-Fe(DTC)$_2$ complexes as a spin probe or an imaging agent to assess the resolution quality of spectral data. A phantom consisted of four glass tubes (i.d., 3.4 mm) containing NO-Fe(DTCS)$_2$ solution in an agar base (Yoshimura *et al.*, 1997), and the head of a living rat receiving NO-Fe(DTCS)$_2$ solution (Yoshimura *et al.*, 1995) gave good three-dimensional EPR images (spatial resolution, 3.59 mm) and a two-dimensional EPR image (spatial resolution, 6.0 mm), respectively. *In vivo* EPR spectra in the upper abdomen of a living mouse were measured 40 min after the subcutaneous (s.c.) administration of NO-Fe(DTCS)$_2$, NO-Fe(MGD)$_2$, or NO-Fe(DETC)$_2$ (Yokoyama *et al.*, 1997). EPR computed tomography (CT) images were reconstructed from the spectral data obtained from rats injected with NO-Fe(DTCS)$_2$, but reconstruction was not possible for experiments with NO-Fe(MGD)$_2$ or NO-Fe(DETC)$_2$ because the spectral data had much lower S/N ratios. The high-intensity area in the EPR-CT image obtained (spatial resolution, 3.64 mm) corresponded to the liver. These results demonstrate that the NO-Fe(DTCS)$_2$ complex is the most suitable spin probe or imaging agent among the NO-Fe(DTC)$_2$ complexes.

We next tried to obtain images of endogenously produced NO in living animals. In what follows, two cases of *in vivo* imaging are introduced.

EPR imaging of the endogenous NO produced in the abdominal region of septic-shock model mouse was attempted (Yoshimura *et al.*, 1996). The septic-shock model mice were prepared by i.p. administration of LPS, and Fe(DTCS) complex was used as an NO trap. Distinct three-line EPR spectra characteristics of NO-Fe(DTCS)$_2$ were obtained from the upper abdominal region of living mice 8.5 hr after LPS injection (Fig. 7B). It is reasonable that the *in vivo* EPR signal can be observed about 8 hr after LPS stimulation, because it has been reported that the NO_2^-/NO_3^- (Stuehr and Marletta, 1985) and NO-Hb (Westenberger *et al.*, 1990) levels in the blood reach their maxima at 6–8 hr after stimulation. No EPR signal was detectable from control mice treated with saline instead of an LPS, followed by an Fe(DTCS) solution. Furthermore, administration of NMMA, an NOS inhibitor, after the LPS injection also resulted in no EPR signal (Fig. 7C). These ob-

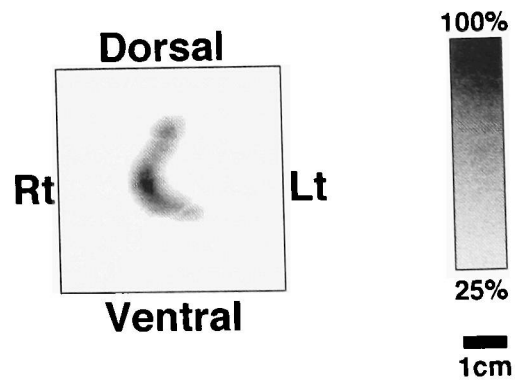

FIG. 8. *In vivo* two-dimensional EPR projection of a cross section of the abdominal region of an LPS-treated mouse. Signals lower than 25% of the maximum signal level in intensity were regarded as noise. The image was reproduced in gray scale. (We have presented the color version of this image in Yoshimura *et al.*, 1996.)

TABLE 5. DISTRIBUTION OF NO-Fe-DTCS COMPLEX IN VARIOUS TISSUES OF LPS-TREATED MICE

Tissue	*NO-Fe-DTCS complex*[a]
Liver	96.0 ± 10.8 nmol/g (wet tissue)
Kidney	19.8 ± 1.3
Whole blood	9.4 ± 1.5 nmol/ml
Urine	13.8 ± 2.6

DTCS, *N*-(dithiocarboxy)sarcosine.

[a]Data are presented as a mean ± SE of those for samples from 6 mice.

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servations indicate that NO observed was indeed produced via an L-arginine-dependent pathway. The image thus obtained showed a signal distribution crescent-wise across the right abdomen of the mouse (Fig. 8). The outline of the image corresponds to that of a liver. This image is the first to show endogenously synthesized free radicals *in vivo*.

To investigate the distribution of NO adduct in various tissues, we determined NO levels from the EPR signal intensities for different tissue homogenates from LPS-treated mice (Table 5). The signal was detected in liver, kidney, whole blood, and urine, but not in brain and spleen. NO levels were found to be markedly high in the liver, which is in agreement with those reported in studies using the iron complexes with DETC (Kubrina *et al.*, 1993) and MGD (Lai and Komarov, 1994) as an NO-trapping reagent. This result gives strong support to the interpretation of the EPR images described above. Detection of the highest concentration of NO in the liver suggests that the NO adduct, formed by injected Fe(DTCS) complex reacting with NO produced by the administration of LPS, accumulates in the liver from the blood circulation.

Next, we attempted *in vivo* EPR imaging of NO produced from ISDN in upper abdomen of the living mice (Fujii *et al.*, 1998). It is generally accepted that the vasodilatory effect of nitrovasodilators is exerted by the release of NO, and ISDN is well known as a long-acting nitrovasodilator commonly used in clinical practice. Both [^{14}N]- and [^{15}N]ISDN were used in this study. *In vivo* EPR spectra in the upper abdomen of mice treated with [^{14}N]- or [^{15}N]ISDN were characterized by triplet or doublet structure, respectively, produced by the formation of ^{14}NO- or ^{15}NO-Fe(DTCS)$_2$ complexes (Fig. 9). The change in line shape from triplet to doublet resulted in improvement in the S/N ratio and in the quality of the image. The three-dimensional images obtained with [^{14}N]ISDN treatment had a spatial resolution of 5.67 mm and they depicted the liver only, whereas [^{15}N]ISDN treatment gave a resolution of 3.95 mm and the images obtained distinctly depicted the liver and kidneys with a more detailed outline of organs. Therefore, ^{15}N substitution clearly provides superior EPR imaging of NO. These results indicate that ISDN is metabolized primarily in the liver.

At present, *in vivo* EPR imaging is not applicable to living systems for physiological levels of NO and oxygen-derived radicals because of the limitations of EPR instrumentation currently available. However, organs or tissues with a locally high, pathological level of NO are suitable targets, so this method has the potential to contribute to the diagnosis of pathophysiological conditions derived from the overproduction of NO.

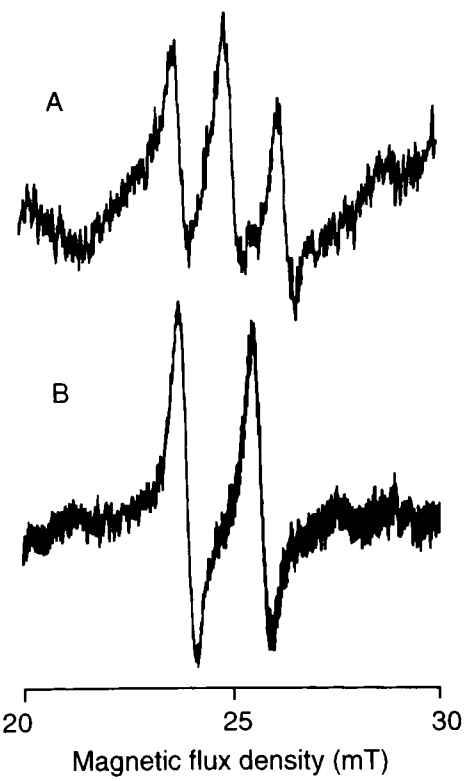

FIG. 9. EPR spectra of NO-Fe(DTCS)$_2$ detected in the upper abdomen of mice treated with (A) [^{14}N]ISDN and (B) [^{15}N]ISDN, measured by a 700-MHz EPR system. Reprinted with permission from Fujii *et al.* (1998).

IRON DITHIOCARBAMATES ACCEPT NO DONATED FROM DINITROSYL DITHIOLATO IRON COMPLEXES *IN VIVO*

The physiological and pathological activities of NO depend not only on NO itself but also on relatively stable endogenous NO carriers or NO donors in which *S*-nitrosothiol (RSNO) and dinitrosyl dithiolato iron complexes (DNIC) are included (Stamler *et al.*, 1992; Vanin and Kleschyov, 1999; Ueno and Yoshimura, 2000). It has been suggested that low-molecular-weight DNIC with cysteine [DNIC-$(Cys)_2$] possesses activities such as an endothelium-derived relaxing factor (Mülsch *et al.*, 1991; Vanin, 1991) and exhibits *S*-nitrosating activity toward cysteine residues of serum albumin *in vitro* (Boese *et al.*, 1995).

DNIC is a stable paramagnetic molecule that exhibits a characteristic EPR spectrum (McDonald *et al.*, 1965). The endogenous production of DNIC has been explained by the binding of NO to iron-sulfur cluster-containing proteins or enzymes in mitochondria and thiol-rich proteins in the presence of free iron (Vanin and Nalbandyan, 1965; Woolum *et al.*, 1968; Reddy *et al.*, 1983; Drapier *et al.*, 1991; Foster and Cowan, 1999). EPR spectral measurements of the abdomen of mice treated with diglutathionyl dinitrosyl iron complex, [DNIC-$(GS)_2$] (Fig. 10), showed that this complex has a relatively high affinity for the liver and kidney (Fig. 11) (Ueno *et al.*, 1999).

Although it is probable that NO donation from DNIC to a variety of *in vivo* targets and their resultant chemical modifications are closely associated with the physiological activities of DNIC, the detailed NO-donation mechanisms remain to be clarified. In transition

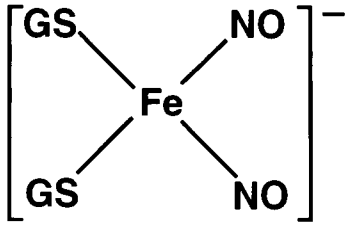

FIG. 10. Chemical structure of diglutathionyl dinitrosyl iron complex. Abbreviations used: GS, glutathionyl group.

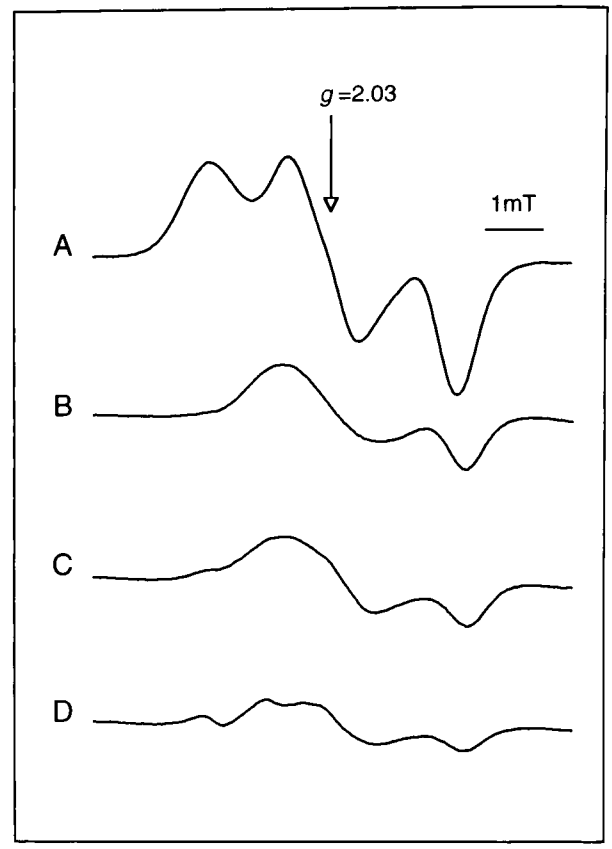

FIG. 11. X-band EPR spectra of resected organs at room temperature: (A) blood; (B) liver; (C) kidney; and (D) spleen. Each organ was isolated 60 min after subcutaneous injection of DNIC-$(GS)_2$ solution. Reprinted with permission from Ueno *et al.* (1999).

metal nitrosyl chemistry, transnitrosylation, in which a coordinated NO-group is transferred to another metal complex, has been observed (Caulton, 1975).

Vedernikov *et al.* (1992) reported that pretreatment of isolated rat aortic ring with DETC attenuated the vasorelaxation induced by DNIC. They assumed that the inhibitory effect of DETC was due to the trapping of NO by a complex of DETC with Fe^{2+} formed in the tissue. This means that the NO-group(s) in DNIC is donated to the Fe(DETC) complex.

Recently, the mechanisms of *in vivo* transnitrosylation of DNIC to Fe(DTC) complex as an NO acceptor have been examined (Ueno *et al.*, submitted). Three kinds of Fe(DTC) complex were administered to mice after the injection of DNIC-$(GS)_2$ and the time course of the EPR spectra of resected organs and *in vivo* spectra from the upper abdomen of living mice were

measured. The spectral changes suggested that the coordinated NO of DNIC-$(GS)_2$ was transferred to the Fe(DTC) complex *in vivo*. The decreasing order of the ability to accept NO from DNIC-$(GS)_2$ was as follows: Fe(DTCS) > Fe(MGD) >> Fe(DETC), which is in accord with that of the water solubility of Fe(DTC) complexes. These results suggest that transnitrosylation from DNIC to metalloproteins or metalloenzymes is a possible *in vivo* NO-donation mechanism and that this reaction can be influenced by the properties of intrinsic NO acceptors.

CONCLUSIONS

We have reviewed current literature ranging from descriptions of chemical properties of DTCs themselves and their iron complexes to their application to *in vitro*, *ex vivo*, and *in vivo* detection of endogenously produced NO, with special focus on our own results. NO produced from three NOS isoforms and NO-releasing compounds can be analyzed by this spin trapping method. Although facility and wide applicability are great advantages of this method, our understanding of the extent of the effects of NO trapping reagents, DTCs and Fe(DTC) complexes, as exogenous substances on biological systems is incomplete. Thus, elucidation of the biological effects of NO trapping reagents is an important area for further research. For the present, attention should be focused upon evaluation of methods to accurately measure levels of NO *in vivo*

The feasibility of continuous monitoring of NO from the same biological specimen is a unique feature of the method. Combined with low-frequency EPR spectroscopy, it enables us to measure the NO production *in vivo*. In addition, the EPR imaging technique provides information about the spatial distribution of NO adducts *in vivo* noninvasively. Unfortunately, current *in vivo* EPR imaging methods are applicable only to living systems with rather high levels of exogenously supplied and endogenously produced NO, because of the limitations of the EPR instrumentation currently available. However, *in vivo* EPR spectroscopy/imaging has great potential as a technique to detect physiological levels of NO and of other radicals in biological systems.

Probably all DTC derivatives can be used as NO trapping reagents. Recently, Fe(DTC) complexes have been also used as NO scavengers or NO acceptors *in vivo*. If novel functions such as accumulation in specific organs is realized by modifying a functional DTC group, DTCs and Fe(DTC) complexes have the potential to become useful and powerful tools for exploring the levels and functions of NO in biological systems.

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ABBREVIATIONS

CO, Carbon monoxide; Cys, L-cysteine; DBNBS, 3,5-dibromo-4-nitrosobenzene; DETC; *N,N*-diethyldithiocarbamate; DMPO, 5,5-dimethyl-pyrroline-*N*-oxide; DMTC, *N,N*-dimethyldithiocarbamate; DNIC, dinitrosyl dithiolato iron complexes; DTC, dithiocarbamate; DTCS, *N*-(dithiocarboxy)sarcosine; eNOS, endothelial isoform of nitric oxide synthase; EPR, electron paramagnetic resonance; EPR CT, electron paramagnetic resonance-computed tomography; GS, glutathionyl group; Hb, hemoglobin; HPLC, high performance liquid chromatography; i.p., intraperitoneal; i.v., intravenous; iNOS, inducible isoform of nitric oxide synthase; ISDN, isosorbide dinitrate; LPS, lipopolysaccharide; MGD, *N*-methyl-D-glucamine dithiocarbamate; MNP, 2-methyl-2-nitrosopropane; MRI, nuclear magnetic resonance imaging; MSD, *N*-methyl-L-serine dithiocarbamate; NF-κB, nuclear transcription factor-κB; NMMA, N^G-monomethyl-L-arginine; nNOS, neuronal isoform of nitric oxide synthase; NO, nitric oxide; NOCTs, nitric oxide cheletropic

traps; NO-Fe(DTC)$_2$, nitrosyl iron complex with dithiocarbamate; NOS, nitric oxide synthase; PDTC, pyrrolidine dithiocarbamate; PTI, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-yloxy; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-yloxy-3-oxide; RSNO, *S*-nitrosothiol; RT-PCR, reverse transcriptase-polymerase chain reaction; s.c., subcutaneous; SOD, superoxide dismutase; STZ, streptozotocin; TMIO, 1,1,3,3-tetramethylisoindoline-2-oxyl.

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Address reprint requests to:
Dr. Tetsuhiko Yoshimura
Institute for Life Support Technology
Yamagata Public Corporation for the Development of Industry
2-2-1 Matsuei
Yamagata 990-2473, Japan

E-mail: yoshi@ymgt-techno.or.jp

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