In Vivo Electron Paramagnetic Resonance Imaging of NO-Bound Iron Complex in a Rat Head

Tetsuhiko Yoshimura,* Satoshi Fujii, Hidekatsu Yokoyama, and Hitoshi Kamada
Institute for Life Support Technology, Yamagata Technopolis Foundation, Kurumanomae-683, Numagi, Yamagata 990

Electron paramagnetic resonance (EPR) imaging of NO-bound iron complex in the head of the living rat has been carried out by L-band EPR imaging system. The NO-bound iron complex used as an imaging reagent was a nitrosyl iron complex with *N*-(dithiocarboxy)sarcosine. Intraperitoneal administration of the imaging reagent to a living rat has provided a clear EPR image derived from the paramagnetic complex in the rat head .

Nitric oxide (NO) has been widely recognized as playing an important role in the biological processes of vascular smooth muscle relaxation, neuronal transmission, and macrophage-induced cytotoxicity.[1-5] To elucidate a variety of biological actions of endogenous NO, information concerning the quantities and distributions of NO in cells, tissues and organs is essential. The concentration of NO formed *in vivo* is very small (0.01- 1 μM) and the half life of NO *in vivo* is very short (3 - 5 s for NO from endothelial cells), making the analysis very difficult.[6] As one of the analytical methods to overcome these difficulties, spin trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy has been used for the determination of unstable free radicals *in vitro* and in vivo.[7-10] Spin trapping reagents react with NO to form relatively stable radical adducts which can be detected easily by EPR spectroscopy.[11-13] Iron complexes with dithiocarbamate derivatives are noted among the spin trapping reagents for NO because NO has a high affinity for the iron complexes and resultant nitrosyl iron complexes exhibit intense three-line signal at room temperature, enabling *in vivo* determination of endogenous NO.[7,10] We have been carrying out studies about the contribution of NO to various physiological and pathophysiological processes by employing an iron complex with *N*-(dithiocarboxy)sarcosine (DTCS) as the spin trapping reagent and the paramagnetic NO-bound (nitrosyl) iron complex, Fe(DTCS)$_2$(NO), as the spin probe. DTCS, a derivative of *N*-methyl glycine, is fairly stable in air and aqueous media compared with other dithiocarbamates[14] and thus the water-soluble iron complex is biologically benign, effective spin trapping reagent. In the course of these studies, we succeeded in EPR imaging of nitrosyl iron complex, Fe(DTCS)$_2$(NO), in the head of a living rat. Here, we demonstrate EPR imaging of the nitrosyl iron complex in the rat head. This is the first report of EPR imaging of small animals that used a nitrosyl iron complex as an imaging reagent within our knowledge.

To obtain EPR spectra and image of a living rat, we used a home-built L-band EPR imaging system, which is composed of an electromagnet (modified RE-3X, JEOL) attached with a pair of field gradient coils (Yonezawa Electric Wire) and field scan coils (Yonezawa Electric Wire), a power supply (Yamagata Stabilizer), an on-line computer (5450 System, Concurrent), a 700-MHz microwave unit (Junkosha), a loop-gap resonator (JEOL), and modulation coils (JEOL). EPR imaging is constructed by the two-dimensional zeumatography as previously described.[15] For zeumatography, linear magnetic field gradients along the X-, Y-, and Z-axis are produced by the gradient coils up to 1 mT/cm in the

range of 20 mm from the center. Samples were held into a loop-gap resonator (i.d., 40 mm) which is situated between a pair of gradient coils attached to the pole faces of an electromagnet.

DTCS diammonium salt together with FeSO$_4$·7H$_2$O was anaerobically dissolved in N$_2$-bubbled Tris buffer (40 mM, pH 7.4) solution (Fe concentration, [Fe] = 50 mM; [DTCS]/[Fe] = 5). NO was bubbled into the solution containing DTCS and Fe(II) for 10 min. Excess NO was removed by bubbling pure N$_2$ gas through the solution. The formula of NO complex in the solution was assumed to be Fe(DTCS)$_2$(NO) by reference to the molecular structure of bis(dithiocarbamato)nitrosyliron(II) complex.[16] (Studies on characterization of the NO complex are under way.) Thus, the solution obtained was used as an Fe(DTCS)$_2$(NO) complex solution.
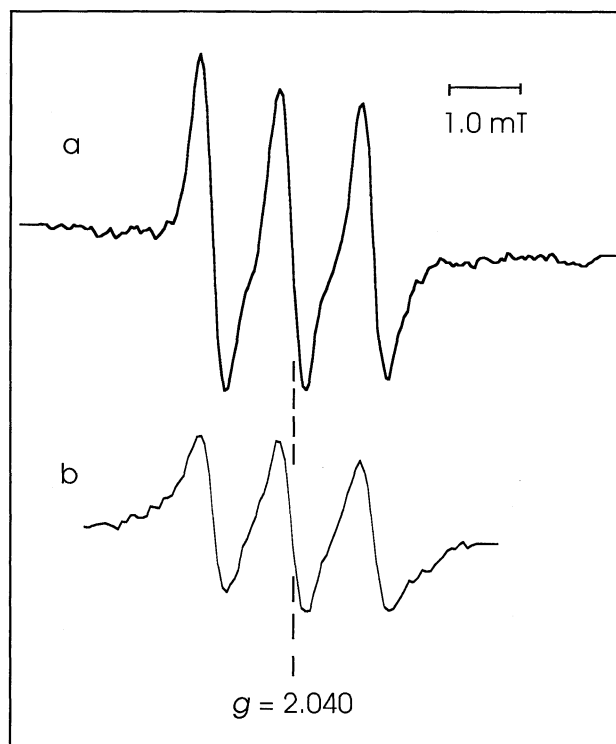

Figure 1. EPR Spectra of Fe(DTCS)$_2$(NO) complex at L-band frequency. (a) is the spectrum of an aliquot (30μl) of the Fe(DTCS)$_2$(NO) solution at ambient temperature. The rat head (male Wistar rat, about 200 g) was held in the loop-gap resonator (thermostated at 37 °C) under pentobarbital anesthesia. The rat received an intraperitoneal administration of 6 ml of the Fe(DTCS)$_2$(NO) solution to obtain a constant concentration in the head for 40 min. (b) is the EPR spectrum of the rat's head. Instrument settings: microwave power, 40 mW and modulation width, 0.2 mT. EPR measurements were started at an operating frequency of about 700 MHz.

The water-soluble $Fe^{II}(DTCS)_2$ complex reacted with NO to give a three-line EPR signal (g = 2.040, a_N = 1.27 mT) at the L-band (Figure 1-a) and X-band frequencies at ambient temperature. This signal was similar to those of nitrosyl iron(II) complexes with diethyl dithiocarbamate[7] and N-methyl-D-glucamine dithiocarbamate[10] in EPR spectral feature and so it is assigned to that of the $Fe^{II}(DTCS)_2(NO)$ complex. Thus since the stable and water-soluble $Fe(DTCS)_2(NO)$ complex has an intense EPR signal at ambient temperature, the $Fe(DTCS)_2$ and $Fe(DTCS)_2(NO)$ complexes are applicable to *in vivo* real time NO assay in biological systems as a spin trapping reagent and a spin probe, respectively.

The rat (Wistar rat; male, 200-250 g) head was held in the loop-gap resonator under pentobarbital anesthesia. The rat received an intraperitoneal administration of the $Fe(DTCS)_2(NO)$ solution to obtain a constant concentration in the head for 40 min and then the EPR spectrum (Figure 1-b) in the blood circulation of the rat head was measured. The EPR spectrum in Figure 1-b is quite similar to those of Figure 1-a in the spectral feature, suggesting that the spin probe does not interact with tissues but behaves as a fluid in the blood.

The resolution of NMR or EPR images is influenced by the line width of the spectrum; it can be improved by narrowing the line width. While a proton NMR spectrum has a line width of about 0.01 mT, nitroxide free radicals of most commonly used EPR imaging or labeling reagents have a line width of the order of 0.14 - 0.16 mT.[13] Therefore, the high resolution in NMR imaging is difficult for EPR imaging at the present time to attain, but EPR imaging is the most effective technique available for noninvasively observing spatial distributions of free radicals. A peak-to-peak widths for the central line in Figure 1-a and -b were 0.38 and 0.46 mT, respectively, of which line broadening unfavorable for EPR imaging originates from the delocalization of an unpaired electron of NO toward the iron d orbital.

Figure 2 exhibits a two-dimensional image of the coronal

section (ZX-plane) in the rat head. As shown in this figure, the high-intensity area (ventral side) is clearly distinguished from the low-intensity area (dorsal side) and corresponds to the region with considerable blood circulation. The low-intensity area corresponds to the space occupied by the rat brain. This result indicates that the nitrosyl iron complex, $Fe(DTCS)_2(NO)$, is scarcely able to pass through the blood-brain barrier.

Thus, intraperitoneal administration of a spin probe, the nitrosyl iron complex, to a living rat has provided a clear EPR image derived from the paramagnetic complex in the rat head, although the spin probe had broad line width that lowers the resolution of an image and so is unfavorable for EPR imaging. One can obtain information on the biological distribution and clearance of a paramagnetic nitrosyl iron complex by analysis of the *in vivo* EPR imaging in small animals, which would be related to the metabolization of endogenous NO.

The results reported here could be the essential first stage for *in vivo* EPR imaging of endogenous NO trapped by the exogenous iron complex.
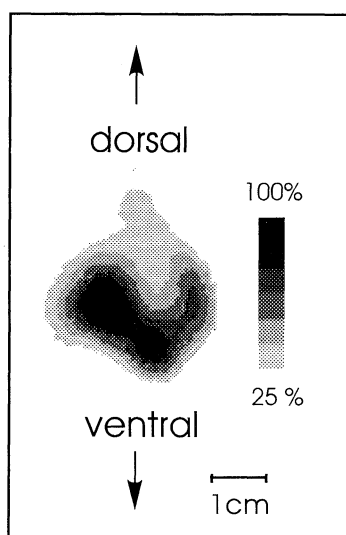

Figure 2. Two-dimensional EPR image of the coronal section (ZX-plane) in the rat head. The rat received treatment similar to that in Figure 1-b. The spatial resolution was 6.0 mm. Instrument settings are similar to those described in Figure 1. The image was reproduced in six gray levels.

References and Notes
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