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In Vivo Distribution and Behavior of Paramagnetic Dinitrosyl Dithiolato Iron Complex in the Abdomen of Mouse

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It has been shown that a dinitrosyl dithiolato iron complex is formed under physiological conditions and that it functions as an NO transporter. In the present study, a diglutathionyl dinitrosyl iron complex [DNIC-$(GS)_2$] was injected into mice and its abdominal distribution and behavior were examined by using electron paramagnetic resonance (EPR) spectroscopy. The X-band EPR signal intensity of the blood, liver, kidney, and spleen decreased with time but signals from the liver and kidney were readily detectable even 24 h after the injection. The time courses of signal intensity were quite similar when the agent was administered via intravenous and subcutaneous injection routes, suggesting that DNIC-$(GS)_2$ can penetrate readily and rapidly through the membranes. Real-time detection of DNIC-$(GS)_2$ in the upper abdomen of the living mice was performed by employing an *in vivo* EPR spectroscopy. These results suggest that DNIC-$(GS)_2$, an endogenous NO carrier, has an excellent membrane permeability and has a relatively high affinity for the liver and kidney.

Keywords: Diglutathionyl dinitrosyl iron complex, *in vivo* electron paramagnetic resonance, endogenous NO carrier

INTRODUCTION

Nitric oxide (NO) is now widely known as an endogenous molecule with critical physiological roles, such as relaxing the smooth muscles, inhibiting platelet activation, acting as a neurotransmitter, and exhibiting nonspecific cytotoxicity.[1,2] It is believed that the physiological activity of NO is derived from its paracrine action in tissues. However, since NO is extremely labile in the biological milieu including dissolved oxygen, metal ions, and various constituents, the paracrine action of NO has not been elucidated completely. NO has markedly high affinity for the metal centers of metalloproteins, and its reaction and binding to the metal ions result in the inhibition or activation of metalloenzymes or the inhibition of iron metabolism. Endogenously generated and exogenously supplied NO has been shown to

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bind iron–sulfur cluster-containing proteins in mitochondria[3] or to thiol-rich proteins in the presence of free iron.[4]

Chemical features of these complexes have been studied earlier.[5,6] Vanin *et al.* reported the detection of paramagnetic dinitrosyl-iron(II) complexes (DNIC) with protein thiol groups in cells and tissues after exposure to endogenous or exogenous NO and demonstrated that NO might be stabilized in biological systems by binding to iron(II)-dithiolate complexes.[7] Other investigators have reported that these complexes are detectable by electron paramagnetic resonance (EPR) spectroscopy in murine cytotoxic activated macrophages;[8] in rat heart allografts during rejection;[9] and in various other sources.[10,11]

Thiol-containing proteins in DNIC can displace low molecular weight thiols (RSH) to yield low molecular dinitrosyl dithiolate iron complexes [DNIC-$(RS)_2$]. It has been reported that DNIC with proteins is in equilibrium with that with low molecular weight thiols in a physiological milieu.[12] Glutathione (GSH) and cysteine (CySH) are representative low molecular weight intracellular thiols and thus the complexes, DNIC-$(CyS)_2$ and DNIC-$(GS)_2$, seem to exist commonly in physiological conditions.[8] The occurrence of these complexes has been demonstrated in cells and tissues after exposure to endogenous or exogenous NO.[3] They are considered to be involved in transmembranous NO transport.

DNIC-$(RS)_2$ can be synthesized *in vitro* and has been utilized in various studies associated with DNIC.[13,14] It has recently been shown that the properties of DNIC-$(CyS)_2$ are very similar to those of an endothelium-derived relaxing factor (EDRF).[15,16] These properties include induction of hypotension[17] and relaxation of vascular smooth muscles.[18]

DNIC-$(GS)_2$ as well as S-nitorosoglutathione (GSNO) are reported to be relatively stable NO carriers and have a rather higher vasodilatory activity than that of DNIC-$(CyS)_2$.[15] It has also been shown that DNIC-$(GS)_2$ irreversibly inhibits glutathione reductase.[19–21] Thus DNIC-$(GS)_2$ may be related to the metabolism of GSH, a major low molecular weight antioxidant in biological systems. Under physiological conditions, DNIC-$(GS)_2$, a significant endogenous NO–iron complex, may be formed in only a small amount; therefore its physiological roles and its detailed pharmacokinetics have not yet been extensively elucidated. Because DNIC are paramagnetic molecules, EPR spectroscopy has been utilized extensively to detect them.

Here we report on the *in vivo* distribution and behavior of DNIC-$(GS)_2$ in the abdomen of mice, which were examined by using both X-band and *in vivo* 7001 MHz EPR spectrometry. For the EPR measurement of paramagnetic molecules, it is common to use X-band microwaves (about 9.5 GHz) which provide high sensitivity. However, X-band microwaves only allow one to measure a small volume (less than 0.1 ml) of aqueous specimens because of the dielectric loss of water in the liquid phase.

Thus X-band EPR detection of endogenous NO has been used for *in vitro* or *ex vivo* studies. By contrast, in an EPR system operating at low microwave frequencies (S-band, ~3 GHz; L-band, ~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.[22] In the present study, a 700 MHz microwave EPR system that had been constructed at our laboratory was utilized for *in vivo* detection of DNIC-$(GS)_2$-injected mice. A combination of these two EPR spectrometry systems enabled us to clarify the novel physiologic mode of DNIC.

MATERIALS AND METHODS

Animals

Female Institute for Cancer Research (ICR) mice, each weighing about 30 g, were used throughout the experiments. Before EPR measurements, mice

were anesthetized with sodium pentobarbital and were sacrificed by dislocation of the neck.

Chemicals and DNIC-$(GS)_2$ Preparation

Dinitrosyl iron complex with GSH $[(GS)_2\text{-}Fe\text{-}(NO)_2]^-$ was used. This complex, the concentration of which was 20 mM, was anaerobically synthesized in a Thunberg vessel by treating $FeSO_4 \cdot 7H_2O$ (Wako, Japan) and GSH (Wako, Japan) solutions with gaseous NO in a 15 mM HEPES buffer (pH 7.4) with a molar ratio of 1 : 2, according to the method described elsewhere.[16] The DNIC-$(GS)_2$ solution (20 mM) thus obtained was stored in a freezer. The DNIC-$(GS)_2$ stock solution was thawed immediately before use. All other chemicals were of the highest grade commercially available and were used without further purification.

X-band EPR Spectroscopy

X-band EPR spectra were measured at room temperature with a JEOL (Tokyo, Japan) TE-200 type ($\sim$9.5 GHz) EPR spectrometer. Experimental procedures were as follows: the blood, liver, kidney and spleen were isolated from the sacrificed mice. To remove blood from the organs, perfusion with saline (approximately 2 ml/min) was performed for several mice before the sacrifices.

These tissue homogenates were drawn into capillary tubes (75 mm in length, 4 µl in inner volume). The capillary tube was inserted first into an EPR quartz tube (o.d., 5 mm) and then introduced into the EPR resonator cavity. Typical instrument settings were: microwave frequency, 9.43 GHz; center field, 331 mT; modulation frequency, 100 kHz; modulation amplitude, 0.32 mT; microwave power, 60 mW; time constant, 0.3 s; sweep width, 15 mT; sweep time, 4 min.

In Vivo 700 MHz EPR Spectroscopy

In vivo EPR spectral measurements were carried out on the abdomen of living mice by using a 700 MHz EPR system that had been constructed at our laboratory.[22] The 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 consists of a two-gap loop-gap resonator (41 mm in diameter; 10 mm in axial length) and modulation coils.

Following the subcutaneous administration of a DNIC-$(GS)_2$ solution (10 ml/kg), the mice were anesthetized (sodium pentobarbital, 0.1 ml/kg, intraperitoneally). Under deep anesthesia, the whole body of the mouse was held in the resonator, which was adjusted to 37°C by a thermostat that was located between a pair of gradient coils that were attached to the pole faces of an electromagnet. The instrument settings were: frequency, 720 MHz; 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 64 scans.

RESULTS

It has been suggested that both diamagnetic $[(NO)_4Fe_2(SR)_2]^{2-}$ and paramagnetic species $[(NO)_2Fe(SR)_2]^-$, which are dimeric and monomeric, respectively, coexist at equilibrium in an aqueous solution containing Fe(II), NO, and thiolate ligands (RS^-); and the dilution of this solution leads to the dominant formation of a monomeric, paramagnetic species.[6] X-ray structural analysis had been performed for a dimeric, diamagnetic complex with bis ethanethiolate ligands $[(NO)_4Fe_2(SC_2H_5)_2]^{2-}$;[5] but the structure of a monomeric, paramagnetic complex has not been reported unequivocally, either in a crystal or an aqueous solution. It has been assumed that iron in the paramagnetic complex has a formal oxidation state +1 ($3d^7$) and that the two unpaired electrons in the nitrosyl groups would be paired.

We prepared a DNIC-$(RS)_2$ by introducing NO gas into a solution containing $FeSO_4 \cdot 7H_2O$ and GSH under anaerobic conditions. [The DNIC-$(GS)_2$ prepared by this method was occurred prevalently in diamagnetic dimeric form.[14] Only small part of the complexes was in monomeric paramagnetic form which can be characterized by the EPR signal with mean *g*-factor 2.03.] Once the DNIC-$(GS)_2$ solution is administered into an animal, it is diluted to yield a paramagnetic species $[(NO)_2Fe(SG)_2]^-$.

X-band EPR Spectroscopy

First, we examined the dependence of the EPR line shape of the DNIC-$(GS)_2$, solution on the magnitude of the modulation amplitude. Figure 1 shows typical X-band EPR spectra of a DNIC-$(GS)_2$ solution at room temperature [modulation amplitude: (a) 0.032 mT, (b) 0.32 mT], which have line shapes of isotropic symmetry with an isotropic *g*-value = 2.03. As shown in Figure 1(a), 13 hyperfine lines that arise from two NO nitrogens and the CH_2 protons adjacent to the coordinated sulfur were detected with the smaller modulation amplitude. The DNIC-$(GS)_2$ solution was considerably stable at room temperature even when exposed to air. In subsequent experiments, we adopted 0.32 mT as a modulation amplitude because of the intensified signal and the simplification of data analysis.

In animal experiments, we measured the EPR spectra of various resected organs: blood, liver, kidney, spleen, pancreas, small intestine (jejunum and ileum), colon, and others. Figure 2 shows representative EPR spectra of the blood, liver, kidney and spleen of a mouse injected with DNIC-$(GS)_2$ (20 mM, 10 ml/kg, s.c.). The spectral line shape of the blood was clearly different from that of other parenchymal organs. The blood exhibited a rhombic-symmetric spectrum with a *g*-value = 2.03 at the zero-crossing, while the liver and kidney exhibited axial-symmetric spectra that were similar. Furthermore, the spectrum of

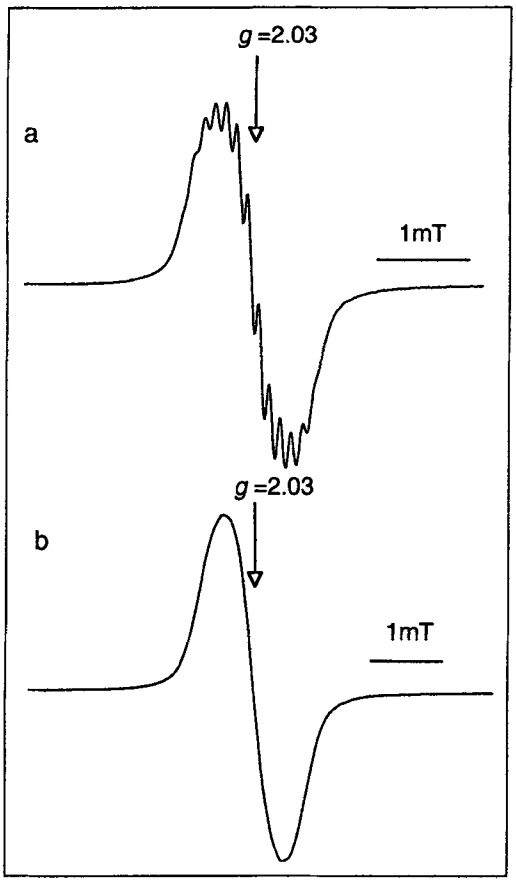

FIGURE 1 X-band EPR spectra of a DNIC-$(GS)_2$ solution at room temperature. The ordinate of (a) was expanded 10-fold compared with that of (b). Instrument settings were: microwave power, 60 mW; microwave frequency, 9.43 GHz; modulation width, (a) 0.032 mT (b) 0.32 mT at 100 kHz.

the spleen had the lowest intensity and appeared to be a combination of spectra of the blood and liver. The pancreas, jejunum, and lung exhibited spectra that were similar to those of the liver and kidney. The ileum and colon did not exhibit any EPR signals. Only a very weak signal was detected emanating from the brain (data not shown).

Figure 3 shows a relationship between the subcutaneous injection dose (up to 25 ml/kg) and the EPR signal intensity for the blood, liver, kidney, and spleen. The data in Figure 3 represent means ± SE ($n = 3$). The signal intensities were obtained by double integration of each signal. As shown in Figure 3, the signal intensity of each

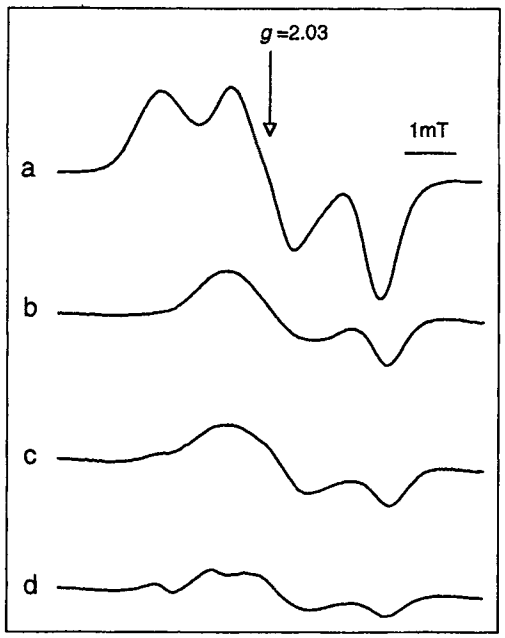

FIGURE 2 Representative X-band EPR spectra of resected organs at room temperature [(a) blood, (b) liver, (c) kidney, (d) spleen]. Each organ was isolated 60 min after subcutaneous (s.c.) injection of a DNIC-$(GS)_2$ solution (20 mM; 10 ml/kg). The tissue homogenates were drawn into capillary tubes (75 mm in length, 46 μl in inner volume), which were inserted first into an EPR quartz tube (o.d., 5 mm), then introduced into the EPR resonator cavity. Instrument settings were: microwave frequency, 9.43 GHz; microwave power, 60 mW; modulation width, 0.32 mT at 100 kHz.

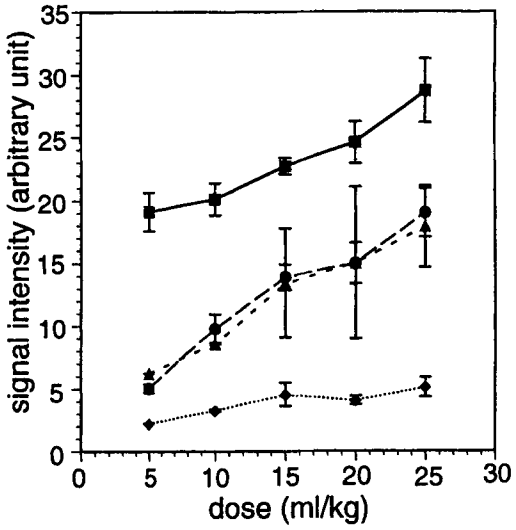

FIGURE 3 Relation between the injection dose and X-band EPR signal intensity: EPR signal intensities of the blood (■), liver (●), kidney (▲), and spleen (◆) were obtained by double integration of each signal. Each plot represents means ± SE ($n = 3$). Thirty minutes before EPR measurement, the mice were injected with DNIC-$(GS)_2$ solutions (20 mM, s.c.). Instrument settings were similar to those listed for Figure 2.

organ increased dose-dependently. The highest signal intensity was detected in the blood. In decreasing order, the signal intensity was: blood > liver, kidney > spleen. After perfusion with saline, the signal intensity barely changed in the liver, kidney, and spleen, while it decreased strikingly in the lung and brain (data not shown).

The X-band EPR spectra of the blood, liver, kidney, and spleen were observed between 0.5 and 24 h after intravenous and subcutaneous injections of the DNIC-$(GS)_2$ solution (20 mM; 10 ml/kg). The time courses of EPR signal intensity through the two routes were quite similar (Figure 4). In all four organs, the signal intensity decreased with time. We roughly estimated the half-lives of signal intensity in the s.c. route. The half-lives thus obtained are 2.0, 2.5, 2.5, and 2.5 h (blood, liver, kidney, and spleen, respectively). During the 3 h following the injections of DNIC-$(GS)_2$, the blood exhibited the highest signal intensity of all; but it decreased in the first 3 h more rapidly than those of the liver, kidney and spleen. From then to 24 h, the signal intensity from the blood was lower than those of the liver and kidney, but higher than that of the spleen. The spleen exhibited the lowest signal intensity of all throughout the experiment.

At 24 h after the injection of DNIC-$(GS)_2$, no signals were detected emanating from the blood and spleen, while signals from the liver and kidney were readily detectable. Based on the data of Figure 4, the relative concentrations of DNIC-$(GS)_2$ in the liver, kidney and spleen vs. blood were evaluated (Figure 5). In the liver and kidney, the relative concentration markedly increased 3 h after the s.c. injection (Figure 5b), and that of the spleen measured less throughout the measurement period. This result was generally duplicated when the animals were injected with DNIC intravenously (Figure 5a).

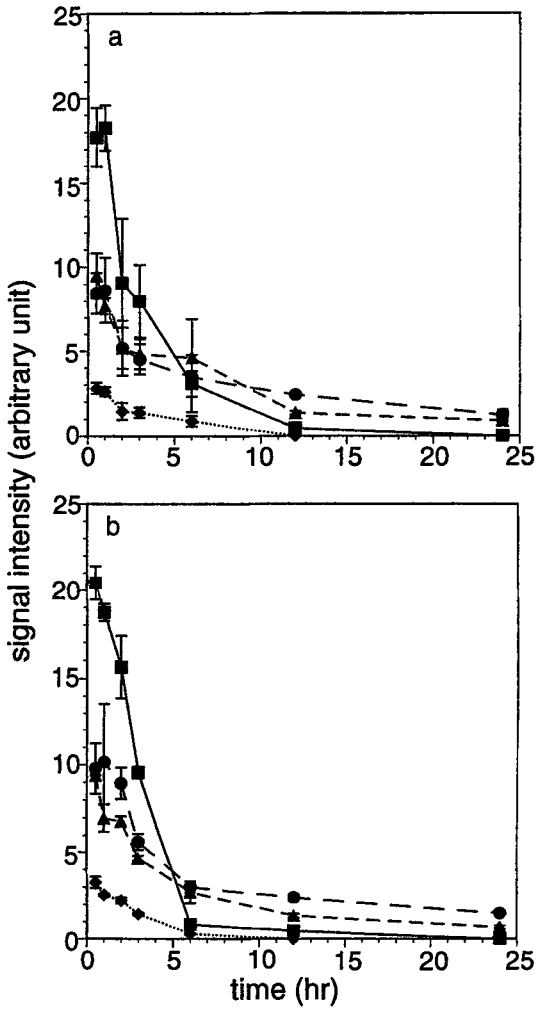

FIGURE 4 Time course of X-band EPR signal intensity of the resected organs [blood (■), liver (●), kidney (▲), and spleen (◆)] at room temperature. Mice were injected with a DNIC-$(GS)_2$ solution (20 mM; 10 ml/kg), intravenously (a), or subcutaneously (b). X-band EPR spectra were recorded between 0.5 and 24 h after the injection. Each plot represents means ± SE ($n = 3$). Instrument settings were similar to those listed for Figure 2.

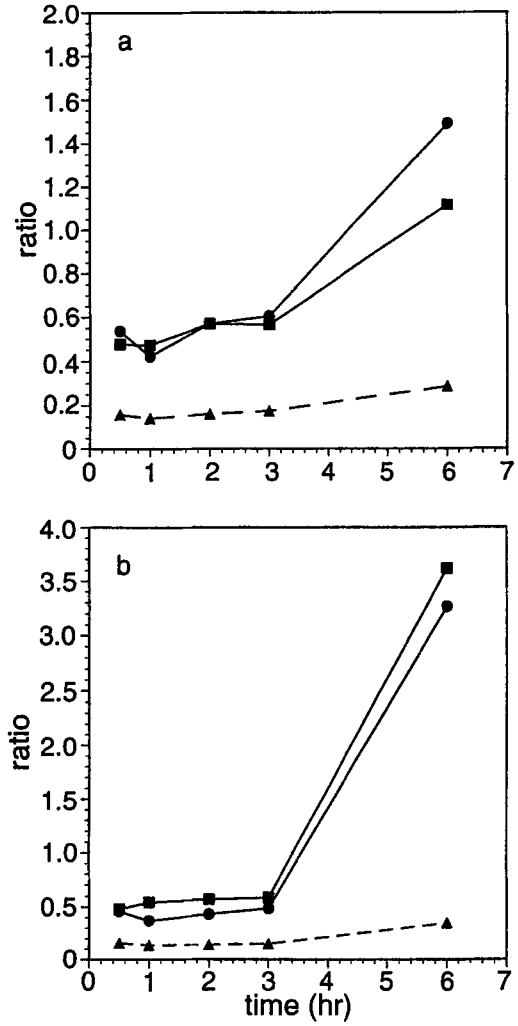

FIGURE 5 Time course of the relative concentration of DNIC in the liver (■), kidney (●), and spleen (▲) vs. that in the blood. The DNIC-$(GS)_2$ solution (20 mM, 10 ml/kg) was injected intravenously (a), or subcutaneously (b). The ordinate represents the ratio of EPR signal intensities, which was evaluated by using the data in Figure 4(a) or (b).

In Vivo 700 MHz EPR Spectroscopy

In line shape, the 700 MHz EPR spectrum of the DNIC solution at room temperature was slightly asymmetric (g-value, 2.03; peak-to-peak width, 0.78 mT, Figure 6). The *in vivo* EPR spectra in the upper abdomen of living mice were measured between 0 and 3 h after the subcutaneous injection of a DNIC-$(GS)_2$ solution (20 mM; 10 ml/kg). The spectral measurements were recorded at 5-min intervals until 60 min after the DNIC-$(GS)_2$ injection; then at 1.5, 2.0, 2.5, and 3 h. (Considering the fatal effect of pentobarbital, anesthesia was interrupted 3 h after the DNIC injection.)

Figure 7 shows the spectra 0, 5, 10, 30, 60, and 90 min after the injection. The EPR spectral line

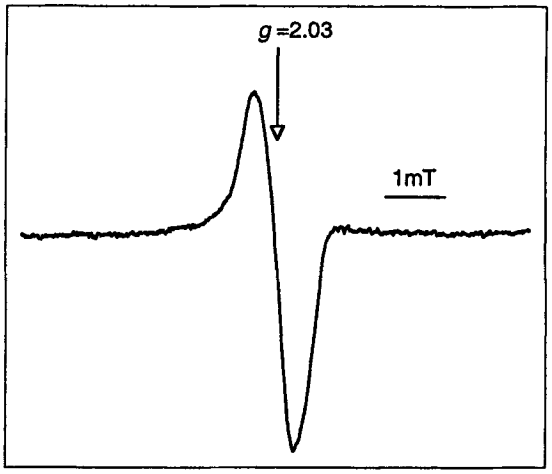

FIGURE 6 Seven hundred MHz EPR spectrum of a DNIC-$(GS)_2$ solution at room temperature (g-value, 2.03; peak-to-peak width, 0.78 mT). Instrument settings were: microwave power, 40 mW; microwave frequency, 738 MHz; modulation amplitude, 0.2 mT; accumulation number, 64.

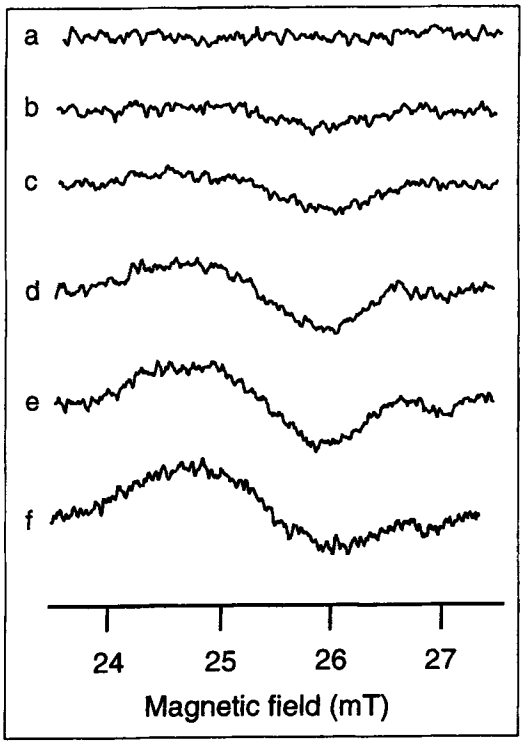

FIGURE 7 Seven hundred MHz EPR spectra of the upper abdomen of a treated living mouse. The spectra were recorded at 0, 5, 10, 30, 60, and 90 min after the injection [(a), (b), (c), (d), (e), and (f), respectively]. Following the administration of DNIC-$(GS)_2$ (20 mM; 10 ml/kg, s.c.), the mice were anesthetized (sodium pentobarbital, 0.1 ml/kg, i.p.). Under deep anesthesia, the whole body of the mouse was held in the resonator. Instrument settings were: microwave frequency, 720 MHz; microwave power, 40 mW; modulation amplitude, 0.2 mT; accumulation number, 64.

shape in the upper abdomen was quite similar to that of the DNIC-$(GS)_2$ solution (Figure 6). Figure 8 shows the time course of signal height of the DNIC detected in the upper abdomen of living mice. Data in Figure 8 are means ± SE ($n = 3$).

In this series of experiments, signal intensity was estimated from the peak-to-peak signal height. The signal intensity increased steeply during the first 30 min, reached a maximum at about 50 min, and then decreased slowly.

Contrary to expectation, most mice treated with DNIC-$(GS)_2$ were well after *in vivo* experiments and remained alive for an extended period despite the exposure to the large dose (20 mM, 10 ml/kg) or even with doubling of the dosage (data not shown).

DISCUSSION

It has been demonstrated that a DNIC is formed under physiological conditions and is stored in a protein-bound form.[23] Protein-bound DNIC in biological systems can be reversibly transformed into DNIC with low molecular weight thiolate ligands, such as GSH and CySH, to function as an intracellular NO transporter.[24,25] Diglutathionyl dinitrosyl iron complex [DNIC-$(GS)_2$] has been reported to inhibit a glutathione reductase irreversibly[19,20] and to exhibit a significant vasodilatory activity similar to EDRF.[15] However, nothing has been revealed yet about the distribution and behavior of this physiologically active iron complex.

The objective of this study was to evaluate the *in vivo* distribution and behavior of administered DNIC-$(GS)_2$ in the abdomen of mice by using EPR spectroscopy. By employing X-band EPR spectroscopy at room temperature, the spectral

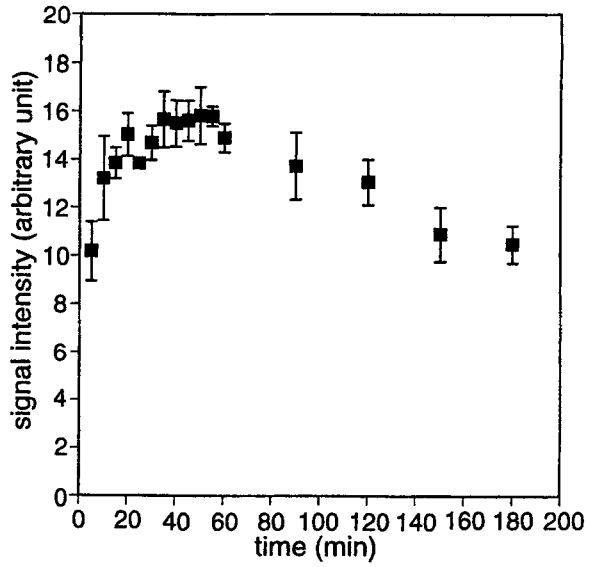

FIGURE 8 The time course of peak-to-peak EPR signal height detected in the upper abdomen of living mice. Each plot represents means ± SE ($n = 3$). The animals were treated in a manner similar to that described in Figure 7. After the administration of DNIC-$(GS)_2$ (20 mM; 10 ml/kg, s.c.), spectral measurements were made every 5 min until 60 min; thereafter at 1.5, 2.0, 2.5, and 3 h by using an *in vivo* 700 MHz EPR spectrometer. Instrument settings were similar to those listed for Figure 7.

characteristics of DNIC-$(GS)_2$ could be detected in the tissues of the abdominal organs of DNIC-$(GS)_2$-treated mice. In addition, real-time detection of DNIC-$(GS)_2$ in the upper abdomen of the living mice was performed by using an *in vivo* 700 MHz EPR system that had been designed and constructed in our laboratory.

X-band EPR Spectroscopy

DNIC-$(GS)_2$-derived signals could be detected, in the X-band EPR spectra at room temperature, in various organ tissues of mice that had been injected with DNIC-$(GS)_2$ subcutaneously or intraperitoneally. These signals from the blood, liver, kidney, and spleen were quite different from those of a DNIC-$(GS)_2$ aqueous solution, which suggest that DNIC-$(GS)_2$ molecules permeate into organ tissues to interact with the constituents and (or) that the mobility of DNIC-$(GS)_2$ molecules is unique in each tissue. Such interactions would lead to the structural transformation of DNIC-$(GS)_2$. Although the chemical structure of DNIC-$(GS)_2$ in an aqueous solution has not been reported yet, the iron can be coordinated by two nitrosyl groups, two thiols (GSH), and one or two solvent molecule(s) in the solution. The coordination site of the solvent can be occupied by tissue constituents in a biological milieu.

In addition, two GSHs of DNIC-$(GS)_2$ can be exchanged for biological thiol-containing compounds, such as serum albumin, coenzyme A, and CySH. DNIC-$(GS)_2$-derived signals from the liver closely resemble those from albumin-bound DNIC prepared *in vitro*.[11] The formation of albumin-bound DNIC in the liver is plausible because serum proteins, including albumin, are synthesized mainly in the liver and occur abundantly. In contrast with the low molecular DNIC, the protein-bound DNIC can occur only in monomeric paramagnetic form.[15,25]

We have reported on the availability of *N*-(dithiocarboxy)sarcosine (DTCS) as a spin trapping agent of endogenously produced NO. The EPR spectra of the spin adduct, $[NO–Fe–(DTCS)_2]^{2-}$, in a biological milieu and in an aqueous solution were essentially identical.[26] Further, the $[NO–Fe–(DTCS)_2]^{2-}$ complex, which is produced endogenously and supplied exogenously, quickly shifts to the liver tissues, while DNIC-$(GS)_2$ can be present in the blood even 6 h after injection. Accordingly, these facts suggest that two water-soluble iron complexes of DNIC-$(GS)_2$ and the $[NO–Fe–(DTCS)_2]^{2-}$ exhibit strikingly different behaviors in a biological milieu because the former can permeate into organ tissues and the latter can hardly do so.

The EPR signal intensities of DNIC-$(GS)_2$ from the blood, liver, kidney, and spleen decreased with time. The time courses of EPR signal intensity were quite similar for both intravenous and subcutaneous injection routes. This suggests that DNIC can permeate readily and rapidly into the membranes. Within 30 min after injection, a relatively large amount of DNIC-$(GS)_2$ shifted to

the blood, liver, and kidney in comparison to the spleen. Furthermore, even 24 h after injection, the EPR spectra of DNIC-$(GS)_2$ were clearly detected in the liver and kidney but not in the blood and spleen. Therefore, in view of the fact that the signal intensity from the liver and kidney did not change after perfusion with saline, it is evident that DNIC-$(GS)_2$ has a particularly high affinity to these organs and that it is metabolized mainly in the liver and kidney. The fact that the EPR spectra of DNIC-$(GS)_2$ were not detected in the feces but in the urine also supports the metabolic pathway that is suggested above.

In Vivo 700 MHz EPR Spectroscopy

By using *in vivo* 700 MHz EPR spectroscopy, a [DNIC-$(GS)_2$]-derived signal could be detected in real time. Especially, this spectroscopy permitted a time-course observation of a DNIC-$(GS)_2$-derived signal even within 30 min after injection, which is essentially impossible with X-band spectroscopy. As can be seen in Figure 8, EPR spectra of DNIC-$(GS)_2$ were detected immediately after injection. The signal intensities of DNIC-$(GS)_2$ rapidly increased during the first 30 min, reached a maximum at about 50 min, and then decreased slowly. These results indicate that DNIC-$(GS)_2$ that has been injected is readily released into the circulating blood and is delivered to the abdominal organs.

In summary, the *in vivo* distribution and behavior of DNIC-$(GS)_2$ in the abdomen of mice have been clarified by using EPR spectroscopy. A combination of X-band and *in vivo* 700 MHz EPR spectrometry made it possible to reveal the novel physiologic mode of DNIC-$(GS)_2$. It has been clearly shown by X-band EPR spectroscopy that DNIC-$(GS)_2$ not only possesses an excellent membrane permeability but also has a relatively high affinity for the liver and kidney. *In vivo* 700 MHz EPR spectroscopy is readily available for the real-time detection of DNIC-$(GS)_2$ in the upper abdomen of a living mouse.

In the present study, the physiological activity of administered DNIC-$(GS)_2$ in the liver and kidney remains to be clarified. Further studies to apply DNIC-$(GS)_2$ as an effective, mild vasodilator to improve the microcirculation of the liver and kidney are currently underway.

Acknowle dgment s

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