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In vivo nitric oxide transfer of a physiological NO carrier, dinitrosyl dithiolato iron complex, to target complex

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Abstract

Dinitrosyl dithiolato iron complex (DNIC) has been identified as an endogenous NO carrier, yet *in vivo* mechanisms of NO donation remain undefined. Transnitrosylation, in which a coordinated NO group is transferred to another metal complex, has been observed in transition-metal-nitrosyl chemistry. In this study, we used three kinds of iron dithiocarbamate complexes (Fe-DTCs) as NO acceptors to elucidate *in vivo* transnitrosylation of diglutathionyl dinitrosyl iron complex [DNIC-$(GS)_2$]. Fe-DTCs were administered to mice after the injection of DNIC-$(GS)_2$ and electron paramagnetic resonance (EPR) spectra were measured both in the resected organs and in the upper abdomen of living mice. The spectral feature gradually changed from an initial DNIC-$(GS)_2$ signal to mononitrosyl iron dithiocarbamate one, suggesting that NO-Fe-DTC was formed through *in vivo* reaction of DNIC-$(GS)_2$ with Fe-DTC. The spectral results in *in vitro* and *in vivo* systems indicate that NO-Fe-DTCs can be formed not only by the transfer of coordinated NO-group(s) in DNIC-$(GS)_2$ but also by the abstraction of Fe-NO group in DNIC-$(GS)_2$ by free DTC ligands. Transnitrosylation proceeded more rapidly in blood than in liver and kidney; and more efficiently in kidney than in liver. Further, the ability to accept NO from DNIC was dependent on water-solubility of Fe-DTCs. Thus, *in vivo* transnitrosylation from DNIC to exogenous iron complex could be observed and this reaction was influenced by biological constituents and properties of iron complex. These results demonstrate that the transnitrosylation from DNIC to intrinsic NO acceptors like metalloproteins has a probable significance in *in vivo* NO transfer process.

Keywords: Dinitrosyl dithiolato iron complex; DNIC; EPR spectroscopy; Nitric oxide; Transnitrosylation; Iron dithiocarbamate complex

1. Introduction

Nitric oxide (NO) is produced in the cells of bacteria, plants, and animals and utilized for cell communication. Its production is associated with crucial biological events, such as control of blood pressure, modulation of neurotransmission, memory formation, and antimicrobial activity [1–5]. These biological activities can be affected not only by NO itself but also by relatively stable physiological NO carriers or NO donors in which *S*-nitrosothiol (RSNO) and DNICs are included [6–9].

DNIC is a stable paramagnetic molecule that exhibits a characteristic EPR spectrum [10–17]. It is generated in cells and tissues from various sources following exposure to endogenous or exogenous NO and can be detected by EPR spectroscopy [11–16,18–28]. 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 [15,17,19,29,30].

It has been demonstrated that under physiological conditions, DNIC is generated in two forms having low molecular-weight thiols or cysteine residues of protein as ligands [15,31,32]. Although it has been suggested that a low molecular-weight DNIC with cysteine [DNIC-$(Cys)_2$] possesses activities such as an endothelium-derived relaxing factor (EDRF) [31,33] and exhibits *S*-nitrosating activity toward cysteine residues of serum albumin *in vitro* [32,34], its physiologic role has not been

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Abbreviations: DETC, *N*,*N*-diethyldithiocarbamate; DNIC, dinitrosyl dithiolato iron complex; DNIC-$(GS)_2$, diglutathionyl dinitrosyl iron complex; DTC, dithiocarbamate; DTCS, *N*-(dithiocarboxy)sarcosine; EPR, electron paramagnetic resonance; MGD, *N*-methyl-D-glucamine dithiocarbamate; NO, nitric oxide.

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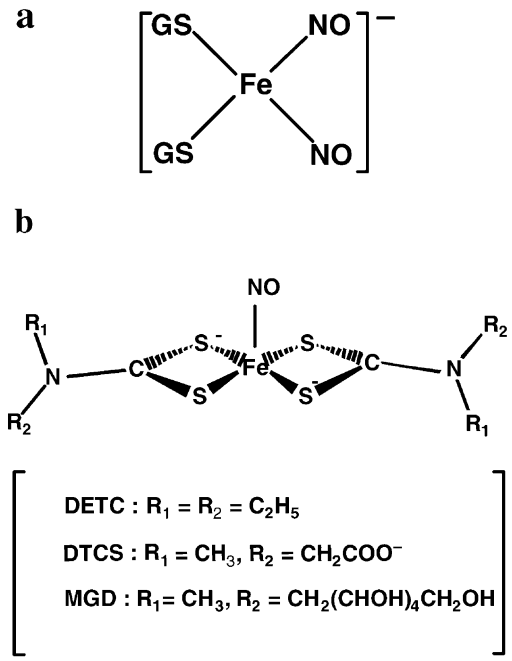

Fig. 1. Chemical structures of DNIC-$(GS)_2$ (a) and NO-Fe-DTC complex (b).

extensively elucidated. One of the reasons may be that *in vivo* detection of DNIC is very difficult because it can be formed in only a limited quantity under physiological conditions. To clarify the *in vivo* distribution and behavior of low molecular-weight DNIC, we recently performed EPR spectral measurements on the abdomen of mice that had been treated by the DNIC-$(GS)_2$ (Fig. 1a) [35]. The EPR spectra attributable to DNIC-$(GS)_2$ were detected in the blood, liver, kidney, and spleen; and it was shown that this complex has a relatively high affinity for the liver and kidney.

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. At present, however, the detailed NO donation mechanisms remain to be clarified. In transition-metal-nitrosyl chemistry, transnitrosylation, in which a coordinated NO group is transferred to another metal complex, has been observed [36]. DNIC would be also able to transfer the coordinated NO groups to metal complexes. In a biological system, therefore, DNIC may transfer NO groups to various metalloproteins or metalloenzymes as targets and modify their biological activities through transnitrosylation.

To elucidate *in vivo* transnitrosylation of low molecular-weight DNIC, an exogenously supplied Fe-DTC complex was selected as an NO-acceptor because the *in vivo* level of metalloproteins and metalloenzymes as intrinsic targets is too low for the detection of the transnitrosylation by employing EPR spectroscopy. The Fe-DTCs react with NO to yield fairly stable mononitrosyl iron complex with intense three-line EPR signals at room temperature and thus, they are commonly utilized as an NO-trapping agent for the EPR analysis of endogenously produced NO [37] (Fig. 1b). In biological systems, it is possible that Fe-DTC will react with DNIC to yield mononitrosyl-iron complex. Therefore, an EPR spectral change from the initial DNIC signal to one indicating the presence of NO-Fe-DTC gives direct evidence of transnitrosylation. In the present study, *in vivo* evidence of NO transfer and Fe-NO transfer from DNIC-$(GS)_2$ and a resultant formation of mononitrosyl-iron complex in the abdomen of mice, by using both X-band and *in vivo* 700 MHz EPR spectroscopy, is reported.

2. Materials and methods

2.1. Animals

Female ICR (Institute for Cancer Research) mice, each weighing about 30 g, were used throughout the experiments. Before EPR measurements, the animals were anesthetized with sodium pentobarbital and sacrificed by dislocation of the neck. All animal-use procedures described herein were performed according to the criteria outlined in the guideline for animal experimentation by Japanese Association for Laboratory Animal Science, 1987.

2.2. Chemicals

Dinitrosyl-iron complex with glutathione $[Fe(I)(GS)_2(NO)_2]^-$, (DNIC-$(GS)_2$) was used. A solution of DNIC-$(GS)_2$ (20 mM) was anaerobically prepared 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 [38]. The DNIC-$(GS)_2$ solution (20 mM) thus obtained was stored in a freezer (-80°). The DNIC-$(GS)_2$ stock solution was thawed immediately before use. The three iron complexes with dithiocarbamate (DTC) derivatives, *N*-(dithiocarboxy)sarcosine (DTCS), *N*-methyl-D-glucamine dithiocarbamate (MGD), and *N*,*N*-diethyldithiocarbamate (DETC) were used as NO acceptors. Disodium salt of DTCS was purchased from Dojindo Laboratory (Kumamoto, Japan) and monosodium salt of MGD was synthesized according to the method of Shinobu, *et al.* [39]. DETC sodium salt was purchased from Wako (Osaka, Japan). Fe(III)$(DTCS)_3$ and Fe(III)$(MGD)_3$ complexes (hereinafter, referred to as "Fe-DTCS" and "Fe-MGD", respectively) were prepared as previously reported [40]. These water-soluble iron complexes were dissolved in a sterile saline prior to use. Because DETC reacts with iron to yield a water-insoluble Fe-DETC complex, DETC and Fe salt were administered separately to the animals, as described below. All other chemicals were of the highest grade commercially available and were used without further purification.

2.3. X-band EPR spectroscopy

In vitro samples were prepared as given below. Saline solutions (0.1 mL) with various concentrations of a water-soluble Fe-DTCS or Fe-MGD complex were added to a 15 mM HEPES buffer solution (20 mM, 0.1 mL) of DNIC-$(GS)_2$. Five minutes later, the mixture thus obtained was subjected to EPR measurement. In addition, a saline solution (0.1 mL) with various concentrations of DTCS or MGD was added to 15 mM HEPES buffer solution (20 mM, 0.1 mL) of DNIC-$(GS)_2$. The mixtures were also subjected to EPR measurement. (*In vitro* experiments for the Fe-DETC complex could not be performed because the complex is insoluble in aqueous media.)

Ex vivo samples were prepared as follows. Fe-DTC complexes (0.3 M, 3.3 mL/kg) were injected into mice 60 min after subcutaneous (s.c.) injection of DNIC-$(GS)_2$ (20 mM, 10 mL/kg). Water-soluble Fe-DTCS and Fe-MGD complexes were injected s.c. The DETC solution and Fe-citrate mixture ($FeSO_4 \cdot 7H_2O$ plus sodium citrate) were injected intraperitoneally (i.p.) and s.c., respectively. At 10, 30, or 60 min after the injection of Fe-DTC complexes, the blood, liver, and kidney were isolated from the sacrificed mice. In addition, saline solutions of the ligand alone, such as DTCS, MGD, and DETC (0.9 M, 3.3 mL/kg), were injected 60 min after the injection of DNIC-$(GS)_2$. DTCS, MGD, and DETC were injected via the same route, as described above. Thirty minutes after the injection of DTCs, the blood, liver, and kidney were isolated and subjected to EPR measurement.

X-band (~9.5 GHz) EPR spectra were measured at room temperature with a JEOL TE-200 EPR spectrometer (Tokyo, Japan). The mixture or tissue homogenates were drawn into capillary tubes (75 mm in length, 46 μL in internal volume), which had been inserted first into an EPR quartz tube (o.d., 5 mm), then introduced into the cavity.

Typical instrument settings were: microwave frequency, 9.43 GHz; center field, 331 mTorr; modulation frequency, 100 kHz; modulation amplitude, 0.32 mT; microwave power, 60 mW; time constant, 0.3 s; sweep width, 15 mT; and sweep time, 4 min.

2.4. In vivo 700 MHz EPR spectroscopy

Fe-DTC complexes (0.3 M, 3.3 mL/kg) were injected into the mice 60 min after the s.c. injection of DNIC-$(GS)_2$ (20 mM, 10 mL/kg). *In vivo* EPR spectral measurements were conducted on the abdomen of live mice by using a 700 MHz EPR system that had been constructed at our laboratory [37]. The system was composed of the following: power supplies; a personal computer; a main electromagnet (air-core, water cooled, two-coil Helmholtz design) 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 administration of an Fe-DTC complex, 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, located between a pair of gradient coils that were attached to the pole faces of an electromagnet. The resonator temperature was adjusted to 37° by a thermostat. 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; and microwave power, 40 mW. An average spectrum was calculated from 64 scans.

3. Results

3.1. X-band EPR spectroscopy

When EPR measurements at room temperature and an X-band frequency were carried out under an appropriate small modulation amplitude, DNIC-$(GS)_2$ exhibited an isotropic EPR signal at $g_{iso} = 2.03$, with 13 hyperfine lines as has been described previously [24,37]. As the modulation amplitude increased, the spectrum was intensified and transformed into an isotropic signal without hyperfine lines (Fig. 2a). In subsequent EPR measurements, a large

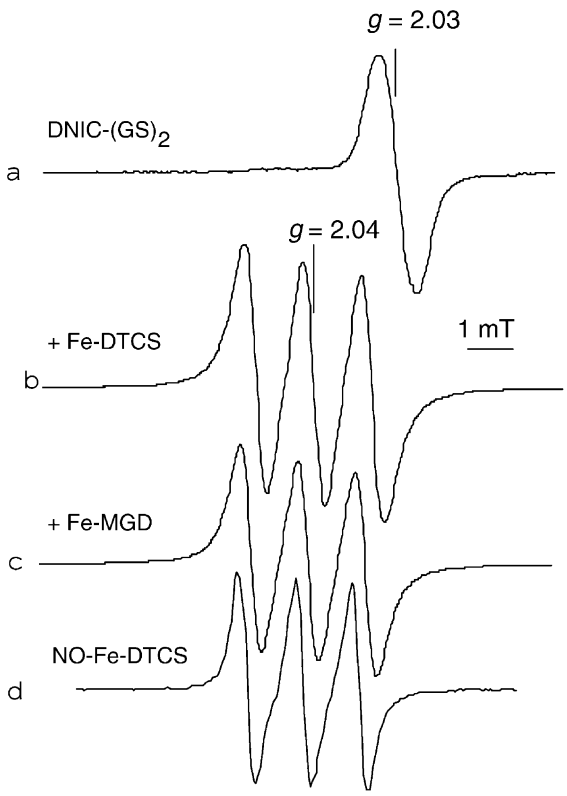

Fig. 2. X-band EPR spectra of DNIC-$(GS)_2$ aqueous solution (20 mM) (a); a mixture of DNIC-$(GS)_2$ (20 mM) and Fe-DTC solution (20 mM) [Fe-DTCS (b) and Fe-MGD (c)] (molar ratio of DNIC-$(GS)_2$ with Fe-DTC complex was unity); and NO-Fe-DTCS solution (1 mM); (d) at room temperature. Instrument settings were: microwave frequency, 9.43 GHz; microwave power, 60 mW; and modulation width, 0.32 mT at 100 kHz.

modulation amplitude (0.32 mT) was selected because of the intensified signal and simplified data analysis.

It has been demonstrated that Fe(III)$(DTC)_3$ complexes readily react with endogenously produced NO to form NO-Fe(II)$(DTC)_2$ and free DTC^- in the presence of chemical or biological reductants (X) such as thiols and ascorbic acid [40–42]. Eq. (1) indicates that the reaction occurs via reductive nitrosylation.

$$\mathrm{Fe(III)(DTC)_3 + NO + X} \rightarrow \mathrm{NO\text{-}Fe(II)(DTC)_2 + DTC^- + X^+} \quad (1)$$

At room temperature and at 0.32 mT for modulation amplitude, NO-Fe(II)$(DTC)_2$ (hereinafter, NO-Fe-DTC) complexes exhibited an isotropic signal (Fig. 2d) at $g_{iso} = 2.04$, with a triplet line shape of $A_N = 1.27$ mT, which originates from the hyperfine interaction of an unpaired electron with an NO-nitrogen nucleus [37].

Both DNIC-$(GS)_2$ and NO-Fe-DTC complexes, when in solution, are fairly stable in air [8,37].

3.2. *In vitro systems*

When the concentration of a water-soluble Fe-DTC complex (20 mM) was comparable to that of DNIC-$(GS)_2$, the EPR spectrum immediately changed from that of DNIC-$(GS)_2$ (Fig. 2a) to those shown in Fig. 2(b,c) upon the addition of the former to the latter complex. These spectra (Fig. 2b and c), having a triplet signal with a *g*-value = 2.04, suggested the formation of a NO-Fe-DTC complex. However, when the Fe-DTC complex with a concentration much lower (20 or 200 μM) than that of DNIC-$(GS)_2$ was added, the EPR spectra consisted of two spectral components: DNIC-$(GS)_2$ and NO-Fe-DTC (data not shown). Thus, the reaction of DNIC-$(GS)_2$ with Fe-DTC complex occurs *in vitro* and is rapidly completed to yield an NO-Fe-DTC complex. It is likely that NO-Fe-DTC formation apparently involves the transfer of an NO group in DNIC-$(GS)_2$ to Fe-DTC.

The EPR spectra obtained by mixing DNIC-$(GS)_2$ with DTCS or MGD were similar to those of DNIC-$(GS)_2$ mixed with Fe-DTCS or Fe-MGD (data not shown). This suggests that free DTC ligands abstract an Fe-NO group from DNIC-$(GS)_2$ to yield an NO-Fe-DTC complex.

The NO-Fe-DTC formation process via the abstraction of Fe-NO group can be involved in the reaction of DNIC-$(GS)_2$ with Fe-DTC complex, because free DTC is generated in the course of reaction of Eq. (1) in which X may be glutathione dissociated from DNIC-$(GS)_2$. Therefore, the formation of NO-Fe-DTC through the reaction of DNIC-$(GS)_2$ with Fe-DTC can be associated with following two processes: (1) a transnitrosylation from DNIC-$(GS)_2$ to Fe-DTC and (2) an abstraction of Fe-NO group from DNIC-$(GS)_2$ by a free DTC.

After dissociation of coordinated NO-group(s), DNIC-$(GS)_2$ seems to be unable to maintain its chemical form because of its electronic and steric structures [8,29,34,43,44]. Therefore, it is probable that this transnitrosylation is an irreversible reaction.

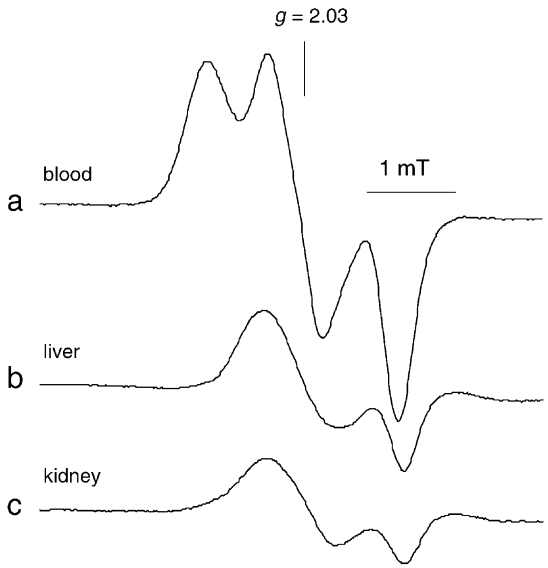

Fig. 3. Representative X-band EPR spectra of resected organs of DNIC-$(GS)_2$-treated mice, at room temperature (top, blood; middle, liver; and bottom, kidney). Each organ was isolated 60 min after s.c. injection of a DNIC-$(GS)_2$ solution (20 mM; 10 mL/kg). Instrument settings were similar to those described in Fig. 2.

3.3. *Ex vivo detection of NO-Fe-DTC formation*

Fig. 3 shows the representative X-band EPR spectra of the blood, liver, and kidney which were isolated 60 min after s.c. injection of DNIC-$(GS)_2$ into mice. In the previous study, the EPR signals from the blood, liver, and kidney showed a markedly high intensity about 60 min after an injection, indicating that the DNIC-$(GS)_2$ that has been administered is conspicuously localized in these organs [35].

In the present study, Fe-DTCs (0.3 M, 3.3 mL/kg) were injected into mice 60 min after injecting DNIC-$(GS)_2$ (20 mM, 10 mL/kg). EPR spectra were measured within the subsequent 60 min (this procedure for injection is hereinafter noted as "(DNIC + Fe-DTC)-type"). If the formation of NO-Fe-DTC through the reaction of DNIC-$(GS)_2$ with Fe-DTC occurs in the blood, liver, and kidney, typical three-line EPR spectra attributable to NO-Fe-DTC can be observed emanating from each organ.

A high intensity signal from DNIC-$(GS)_2$ in the blood could be observed up to 5 hr after injecting DNIC-$(GS)_2$ alone [35]. For the (DNIC + Fe-DTC)-type injection, however, the signal coming from the blood disappeared immediately after the injection of Fe-DTC and an alternate signal with a triplet appeared (Fig. 4a, top). This triplet signal could be observed only on the injection of Fe-DTC and the *g*- and *A*-values agreed with those characteristic of NO-Fe-DTC. Accordingly, this new signal was definitely assigned to that of NO-Fe-DTC. The signals from

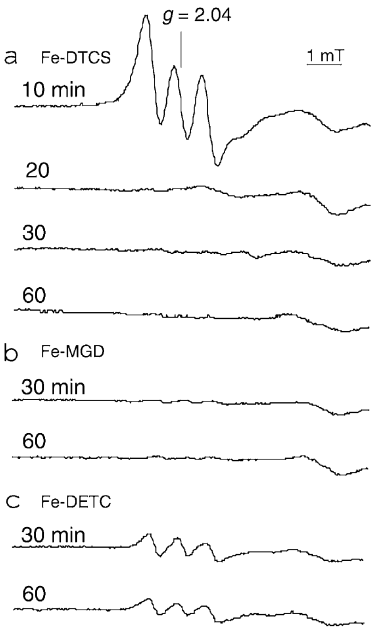

Fig. 4. X-band EPR spectra of the blood of mice treated with DNIC-$(GS)_2$ and Fe-DTCs, at room temperature. Mice were injected with DNIC-$(GS)_2$ solution (20 mM; 10 mL/kg) prior to the injection of Fe-DTC complexes. EPR spectra were measured at 10, 20, 30, and 60 min after the injection of Fe-DTCS complex (a), at 30 and 60 min after the injection of Fe-MGD complex (b), at 30 and 60 min after the injection of Fe-DETC complex (c). Instrument settings were similar to those described in Fig. 2.

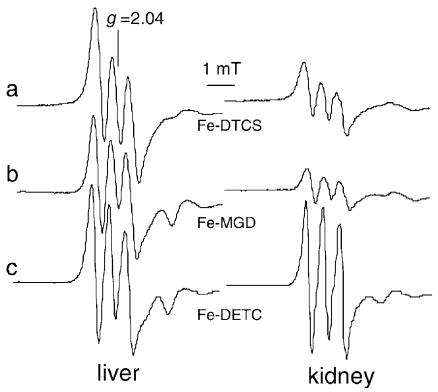

Fig. 5. Representative X-band EPR spectra of the liver and kidney of mice treated with DNIC-$(GS)_2$ and Fe-DTCs, at room temperature. EPR spectra were measured at 60 min after the injection of Fe-DTC complexes (0.3 M; 3.3 mL/kg). Fe-DTC complexes [Fe-DTCS (a), Fe-MGD (b), and Fe-DETC (c)] were injected at 60 min after the injection of DNIC-$(GS)_2$ solution (20 mM; 10 mL/kg). Instrument settings were similar to those described in Fig. 2.

NO-Fe-DTC in the blood were detectable at least up to 10 min after Fe-DTCS injection (Fig. 4a), 30 min after Fe-MGD (Fig. 4b), and 60 min after Fe-DETC (Fig. 4c), after which no signals were detected coming from the blood.

It has been shown that the signal from DNIC-$(GS)_2$ in the liver and kidney can be clearly recognized even 24 hr after injection of DNIC-$(GS)_2$ alone [35]. As for (DNIC + Fe-DTC)-type injection, the EPR spectra of the liver and kidney exhibited an overlapping signal of DNIC-$(GS)_2$ and NO-Fe-DTC at 30 min after Fe-DTCs injection. Fig. 5 shows representative spectra from the liver (left) and from the kidney (right) 60 min after the injection. Signals from DNIC-$(GS)_2$ and NO-Fe-DTC were observed as a small minimum on the high field side and as an intense triplet on the low field side, respectively. The DNIC-$(GS)_2$ signal intensity was much weaker than that of NO-Fe-DTC; and it was found to decrease gradually after Fe-DTCs injection.

For (DNIC + Fe-DTC)-type injection, the EPR signal from DNIC in the blood disappeared more rapidly than it did in the liver and kidney, as shown in Figs. 4a and 5a. Because the signal emanating from the blood was present for a much longer time with the injection of DNIC alone [35], this rapid disappearance may have resulted from the additional injection of Fe-DTC. These suggest that the additional injection of Fe-DTC causes the rapid migration of pre-administered DNIC and formed NO-Fe-DTC from blood to liver and kidney.

The efficiency of the formation of NO-Fe-DTC through the reaction of DNIC-$(GS)_2$ with Fe-DTC in the liver and kidney was examined. The peaks of NO-Fe-DTC on the lowest field side (peak height of positive lobe, A) and DNIC-$(GS)_2$ on the highest field side (peak height of negative lobe, B) are found to have a little overlap with the spectrum of DNIC-$(GS)_2$ and NO-Fe-DTC, respectively. Therefore, the A/B ratio may signify the efficiency of NO-Fe-DTC formation. As shown in Table 1, the A/B ratio generally increased dose-dependently for the kidney and liver, and the order of kidney > liver was apparent. These tendencies were found to be independent of the type of Fe-DTC complexes. The ability to take NO from DNIC-$(GS)_2$ was in decreasing order as follows: Fe-DTCS > Fe-MGD > Fe-DETC.

In the case of administration of free DTC ligands after injecting DNIC-$(GS)_2$ to mice, the spectral line shapes observed for the blood, liver, and kidney (Fig. 6) were quite similar to the case of administration of Fe-DTCs after

Table 1
The change of the ratios in EPR signal intensity, NO-Fe-DTC/DNIC-$(GS)_2$, in the liver and kidney of mice treated with DNIC-$(GS)_2$ (20 mM) and Fe-DTCs (30, 100, 300 mM)[a]

	Concentrations of Fe-DTC (mM)		
	30	100	300
Liver			
Fe-DTCS	0 ± 0	0 ± 0	5.8 ± 2.3
Fe-MGD	0 ± 0	0 ± 0	1.3 ± 0.9
Fe-DETC	0 ± 0	0.08 ± 0.02	2.9 ± 1.0
Kidney			
Fe-DTCS	0 ± 0	0.6 ± 0.1	9.6 ± 1.9
Fe-MGD	0.7 ± 0.4	1.8 ± 0.7	7.7 ± 4.3
Fe-DETC	0.7 ± 0.4	1.5 ± 0.5	6.3 ± 0.4

[a] The animals were treated in a manner similar to that described in legend of Fig. 5. All values are means ± SEM; $n = 3$.

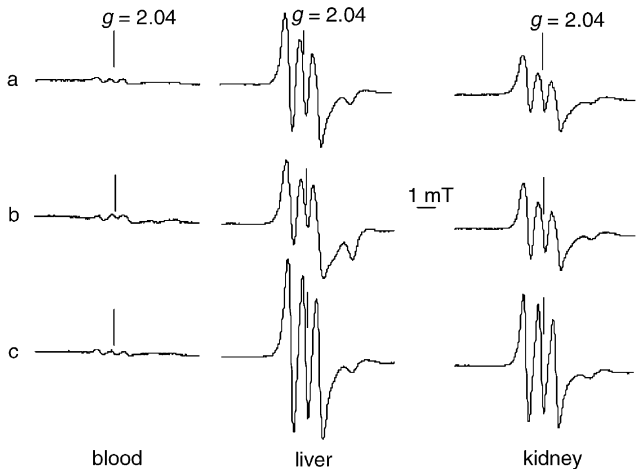

Fig. 6. Representative X-band EPR spectra of the blood, liver, and kidney of mice treated with DNIC-$(GS)_2$ and free DTCs, at room temperature. EPR spectra were measured at 60 min after the injection of free DTCs (0.9 M; 3.3 mL/kg). Free DTCs [DTCS (a), MGD (b), and DETC (c)] were injected at 60 min after the injection of DNIC-$(GS)_2$ solution (20 mM; 10 mL/kg). Instrument settings were similar to those described in Fig. 2.

DNIC-$(GS)_2$, suggesting the formation of NO-Fe-DTC. The *in vivo* processes of NO-Fe-DTC formation in the free DTC administration are assumed as follows: (1) in analogy with *in vitro* system, free DTCs may abstract an Fe-NO group from DNIC-$(GS)_2$ to yield an NO-Fe-DTC complex; (2) free DTCs could be coordinated to endogenous iron in tissues to form an Fe-DTC complex and then Fe-DTC may react with DNIC-$(GS)_2$ to yield an NO-Fe-DTC complex.

Since free DTC is generated in the reaction of DNIC-$(GS)_2$ with Fe-DTC complexes (Eq. (1)), it could participate in *in vivo* NO-Fe-DTC formation in the (DNIC + Fe-DTC)-type injection, through the processes just described above. But, the contribution of free DTC route to the *in vivo* NO-Fe-DTC formation may be limited, because the quantity of free DTC can formally be equal to that of Fe-DTC given according to Eq. (1). Accordingly, both a transnitrosylation from DNIC-$(GS)_2$ to Fe-DTC and an abstraction of Fe-NO group from DNIC-$(GS)_2$ by a free DTC can be responsible for *in vivo* formation of NO-Fe-DTC in the (DNIC + Fe-DTC)-type injection.

3.4. *In vivo* 700 MHz EPR spectroscopy

The 700 MHz EPR spectrum of DNIC-$(GS)_2$ at room temperature was a slightly asymmetric singlet ($g = 2.03$) in line shape [35]. The *in vivo* 700 MHz EPR spectra were measured in the upper abdomen of living mice injected with DNIC-$(GS)_2$ (20 mM, 10 mL/kg) prior to the administration of the Fe-DTC complexes (0.3 M, 3.3 mL/kg). The spectra were measured at 5-min intervals up to 60 min after injecting Fe-DTC (Fig. 7). Only the signal from DNIC-$(GS)_2$ ($g = 2.03$) was detected before the injection of Fe-DTC complexes. Typical three-line signals derived from NO-Fe-DTC complexes ($g = 2.04$) were detected 5 min after injecting the Fe-DTC complexes. Thus, the NO-Fe-DTC signal could be observed immediately after the additional injection of Fe-DTC, suggesting the rapid *in vivo* formation of the NO complexes. Time courses of peak-to-peak signal height of the NO-Fe-DTC complexes are shown in Fig. 8. The signal height, which increased and reached a maximum in about 20–30 min, came down rapidly for DNIC + Fe-DTCS and DNIC + Fe-MGD injection or gradually for DNIC + Fe-DETC one.

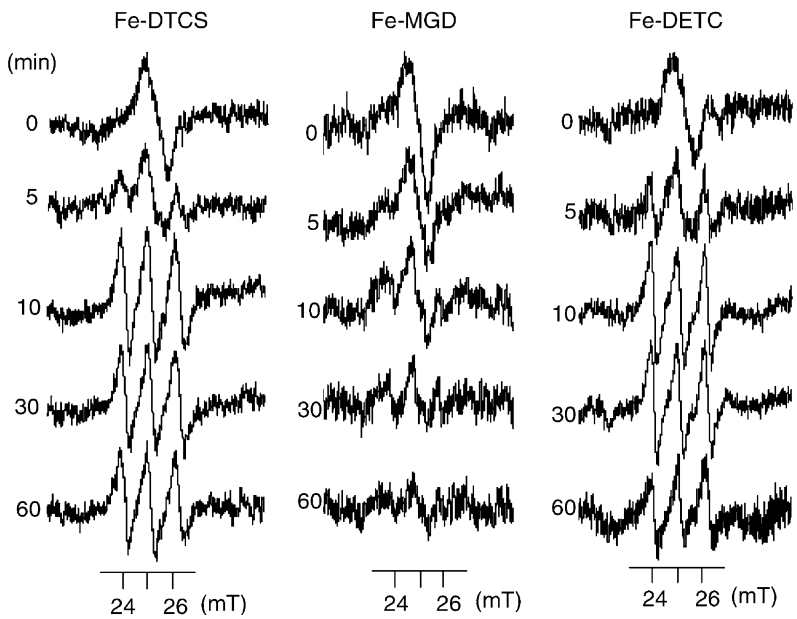

Fig. 7. *In vivo* 700 MHz EPR spectra in the upper abdomen of living mice injected with DNIC-$(GS)_2$ prior to the injection of Fe-DTC complexes (left, Fe-DTCS; center, Fe-MGD; and right, Fe-DETC). Instrument settings were: microwave frequency, 720 MHz; microwave power, 40 mW; modulation amplitude, 0.2 mT; and accumulation number, 64.

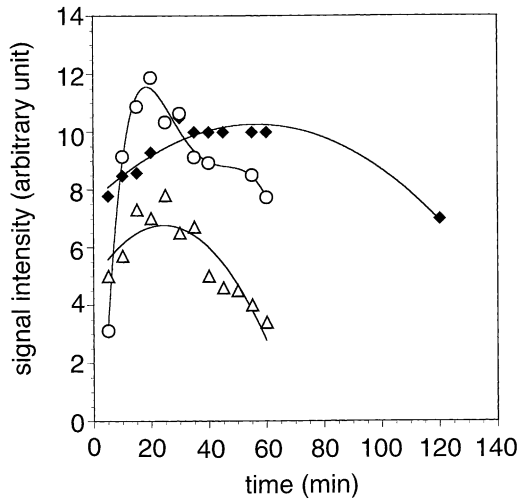

Fig. 8. Time course of peak-to-peak 700 MHz EPR signal height measured in the upper abdomen of living mice injected with DNIC-$(GS)_2$ prior to the injection of Fe-DTC complexes [Fe-DTCS (○), Fe-MGD (Δ), and Fe-DETC (◆)]. Instrument settings were similar to those listed in Fig. 6. Each plot represents means of two experiments.

4. Discussion

It has been shown that low molecular-weight DNIC, an endogenous NO carrier or *S*-nitrosating agent, possesses EDRF-like activity [31,33]. In addition, the low molecular-weight DNIC has been shown to modulate antioxidant activity through the inhibition of glutathione-dependent enzymes, such as reductase, transferase, and peroxidase of glutathione [45–48]. Despite experimental evidence on the NO or NO^+-donor of DNIC [6–9], the amount of DNIC formed in biological systems is so limited that the detailed NO-donation mechanism *in vivo* has not yet been elucidated.

Because transnitrosylation has been found in transition metal nitrosyl chemistry [36], it is most likely that the process is a mechanism of NO donation in biological systems, where coordinated NO group(s) in DNIC are transferred to metalloproteins or metalloenzymes. DETC was reported to inhibit irreversibly the vasodilatory activity of DNIC-$(Cys)_2$ in isolated rat vessels [49], suggesting that transnitrosylation of DNIC to an Fe-DETC complex formed in tissue took place *in vitro*.

To obtain evidence of *in vivo* transnitrosylation of DNIC to iron proteins, we used DNIC-$(GS)_2$ as a low molecular-weight DNIC and three Fe-DTCs as a model complex for iron proteins. Three Fe-DTCs (Fe-DTCS, Fe-MGD, and Fe-DETC) have different water-solubilities [37]. DNIC-$(GS)_2$ and subsequently Fe-DTCs were administered to mice, which is termed (DNIC + Fe-DTC)-type injection and the progress of the reaction of the two complexes in their abdomens was assessed by employing both X-band and *in vivo* 700 MHz EPR spectroscopy. Direct evidence of the formation of mononitrosyl-iron complex (NO-Fe-DTC) through the reaction of DNIC-$(GS)_2$ with Fe-DTC was provided by EPR spectral change from the initial DNIC signal (Fig. 2a) to that of mononitrosyl iron dithiocarbamate (NO-Fe-DTC) (Fig. 2b–d) because paramagnetic DNIC-$(GS)_2$ and NO-Fe-DTC complexes have different EPR spectral features.

In the previous study, we demonstrated that the EPR signal derived from DNIC-$(GS)_2$ could be distinctly observed up to 5 hr in the blood (half-life, about 2 hr) and up to 24 hr in the liver and kidney (half-life, about 2.5 hr) when DNIC-$(GS)_2$ alone was injected subcutaneously into mice [35]. On the other hand, on the (DNIC + Fe-DTC)-type injection in this study, the signal in the blood disappeared immediately after the injection of Fe-DTC (Fig. 4) and that in the liver and kidney (Fig. 5) or upper abdomen (Fig. 7) rapidly weakened after the injection. The decay of DNIC-$(GS)_2$ signal in these tissues was followed by the appearance of NO-Fe-DTC one. The NO-Fe-DTC signal in the blood was reduced and disappeared in 20–60 min after the Fe-DTC injection while in the kidney and liver it was still maintained at a high intensity level 60 min after the injection. These results indicate that the injection of Fe-DTCs causes the rapid migration of pre-administered DNIC and formed NO-Fe-DTC from blood to liver and kidney and in other words, it promotes the clearance of DNIC and NO-Fe-DTC in the blood.

It has recently been reported that an exogenously supplied NO-Fe-DTCS complex reached the liver of the mice within 40 min after the s.c. injection [50]; and that endogenously produced NO was detected in the liver and kidney by using X-band and *in vivo* 700 MHz EPR spectroscopy after the injection of Fe-DTCS [51,52]. These reports support the finding of this study, in which the intense signal detected in the liver and kidney was derived not only from the NO-Fe-DTC complex formed in the organs but also from that being transported via the blood circulation. The NO-Fe-DTC complex and its metabolite in the liver and kidney appear to be excreted into the bile and urine [53,54].

The EPR spectral results in *in vitro* and *in vivo* systems indicate that NO-Fe-DTCs in these systems can be formed not only by the transfer of a coordinated NO group (transnitrosylation) in DNIC-$(GS)_2$ but also, to some extent, the abstraction of an Fe-NO group in DNIC-$(GS)_2$ by free DTC ligands which is generated by the dissociation of Fe-DTC complex. In this study, we used Fe-DTC complexes as a model for iron proteins in order to obtain the clear evidence of *in vivo* transnitrosylation. It is probable that such strong chelating ligands to iron as dithiocarbamates do not exist in the biological systems. Therefore, the latter process including the abstraction of an Fe-NO group in DNIC-$(GS)_2$ by biological strong ligands may be negligible in *in vivo* NO donation from DNIC. On one hand, the *in vitro* transfer of Fe-$(NO)_2$ group from low molecular-weight DNIC to the thiol groups in reduced adrenodoxin has recently been reported [55]. In this case, resultant products were not a mononitrosyl iron complex

like NO-Fe-DTC but a protein-thiol containing (or high molecular-weight) DNIC.

We demonstrated that when a low molecular-weight DNIC-$(GS)_2$ was injected into living mice, different EPR spectral line-shapes were observed emanating from their isolated organs [35]. The appearance of such spectral conversions has been explained mainly as the result of chemical modifications by the reaction of low molecular-weight DNIC with thiol-containing compounds like serum albumin [32]. Accordingly, it was postulated that transnitrosylation can also take place between chemically modified DNIC and iron proteins in biological systems.

DNIC has been reported to react with low molecular thiols or protein thiols to yield *S*-nitrosothiols [32,56]. Further, DNIC may nitrosate endogenous secondary amines with formation of nitrosamines [32]. In biological systems, therefore, transnitrosylation by DNIC would be in competition with nitrosation, which may be associated with the result that the efficiency of transnitrosylation is lower in the liver, a thiol-rich organ, than in the kidney (Table 1).

In this study, we adopted three Fe-DTC complexes as NO-acceptors, in which Fe-DTCS and Fe-MGD are water-soluble and Fe-DETC is water-insoluble but lipid-soluble. The differences in solubility cause differences in membrane permeability, affinity for tissues, and metabolic mode. As a result, apparent differences were found in the rate and efficiency of transnitrosylation in three NO acceptors. This shows that transnitrosylation by DNIC *in vivo* can be influenced by the properties of intrinsic NO acceptors.

In conclusion, we succeeded in obtaining *in vivo* evidence of NO transfer and Fe-NO transfer (Fig. 9) from low molecular-weight DNIC and a resultant formation of mononitrosyl-iron complex in the abdomen of mice by employing both X-band and *in vivo* 700 MHz EPR spectroscopy. These results suggest that transnitrosylation, in which a coordinated NO group in the low molecular-weight DNIC is transferred to metalloproteins or metalloenzymes, is a possible *in vivo* NO donation mechanism.

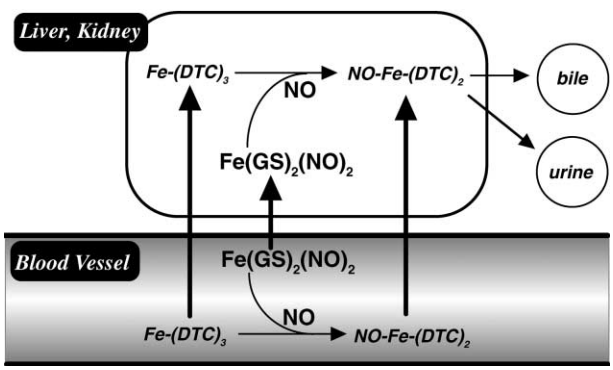

Fig. 9. Possible and simplified pathways of the formation of NO-Fe-DTC through the reaction of DNIC-$(GS)_2$ with Fe-DTC in biological systems. As described in the text, free DTC as a product of reaction of Fe-DTC with NO (Eq. (1)) can contribute to *in vivo* NO-Fe-DTC formation after the administration of DNIC-$(GS)_2$ and Fe-DTC. Here, DNIC-$(GS)_2$ was expressed as a $Fe(GS)_2(NO)_2$ to make the transfer of NO group prominent.

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