In vivo three-dimensional EPR imaging of nitric oxide production from isosorbide dinitrate in mice

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Fujii, Satoshi, Yasuhiro Suzuki, Tetsuhiko Yoshimura, and Hitoshi Kamada. In vivo three-dimensional EPR imaging of nitric oxide production from isosorbide dinitrate in mice. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G857–G862, 1998.—Recently, in vivo electron paramagnetic resonance (EPR) spectroscopy and imaging have been widely used to investigate free radical distribution and metabolism in tissues, organs, and whole body of small animals. Endogenous nitric oxide (NO) is an attractive target of this method. In the present study, NO production from a nitrovasodilator, isosorbide dinitrate (ISDN), in live mice was investigated by in vivo EPR spectroscopy and imaging combined with the spin-trapping technique. A highly water-soluble Fe complex with N-(dithiocarbamoyl)sarcosine (DTCS) was used as an NO-trapping agent. Mice received [14N]ISDN, and the Fe-DTCS complex subcutaneously exhibited the characteristic triplet EPR signal of the NO adduct [14NO-Fe(DTCS)2]2−. Using [15N]ISDN instead of [14N]ISDN, we were able to observe that the doublet EPR signal stemmed from the 15NO adduct, which directly demonstrated that NO was produced from ISDN. The three-dimensional EPR images of the upper abdomen of living mice showed that the NO adducts were distributed in the liver and the kidneys. This EPR image combined with the ex vivo EPR measurements of the blood suggested that NO production from ISDN occurred in the liver in this experimental condition.

nitrovasodilators; in vivo electron paramagnetic resonance; spin-trapping; N-(dithiocarbamoyl)sarcosine; iron-dithiocarbamate complex

IT HAS BEEN KNOWN for over 130 years that organic nitrates and nitrites have vasodilating effects. Indeed, they have been used as therapeutic agents for the treatment of angina pectoris and congestive heart failure for a long time, but the mechanism of their actions was not fully understood. During the past decade, much has been learned about the mechanism of action of these nitrovasodilators, including inorganic nitroso compounds such as sodium nitroprusside. The most significant aspect of the mechanism is that the vasodilator effect of these compounds is exerted by releasing nitric oxide (NO) (3, 4, 7). In addition to the vasodilator effect, nitrovasodilators mimic endothelium-derived NO in many respects.

Among the nitrovasodilators commonly used in clinical practice, isosorbide dinitrate (ISDN), is known to be a long-acting agent. After administration, ISDN is converted to isosorbide-5-mononitrate (IS-5-MN) or, to a lesser extent, to isosorbide-2-mononitrate (IS-2-MN) and NO (2). These metabolites, IS-5-MN and IS-2-MN, exert a further vasodilating effect by their conversion to NO and isosorbide (20). This two-step NO release from ISDN seems to be a reason for its long-acting property. However, the metabolic pathway of ISDN, as well as that of other nitrovasodilators, has not been elucidated completely.

When we study endogenous NO-related phenomena, information concerning the quantity and distribution of NO in cells, tissues, and organs is essential. However, it is rather difficult to determine the quantity and distribution of NO because of its scarcity and its short half-life in living systems.

To overcome these problems, a spin-trapping technique combined with electron paramagnetic resonance (EPR) spectroscopy has been used to study unstable free radicals such as superoxide anion, hydroxyl radical, and hydroperoxy radical in vitro and in vivo (10). This spin-trapping technique can also be applied to NO. There are a number of reports on the use of conventional X-band (9.5 GHz) EPR spectroscopy and Fe complexes with dithiocarbamate (DTC) derivatives as NO-trapping agents for the determination of NO production in biological systems (5, 13, 18). Among the spin-trapping agents for NO, Fe-DTCS complexes are noted because of the high affinity of NO for Fe complexes. The resultant NO adducts are fairly stable in air and exhibit an intense triplet signal even at ambient temperatures. The availability of ambient temperature EPR spectra coupled with EPR instruments operating at low frequencies makes it possible to detect NO production in living small animals (16, 28). Recently we developed an Fe complex with a kind of DTC, N-(dithiocarbamoyl)sarcosine (DTCS; Fig. 1), as a highly water-soluble NO trap (6) and succeeded in detecting the in vivo EPR spectra and obtaining an EPR image of the nitrosyl complex derived from the endogenous NO and Fe-DTCS complex in the abdominal region of lipopolysaccharide (LPS)-treated mice (28).

In the present study, we report on the application of this in vivo EPR technique to NO production from [14N]ISDN and [15N]ISDN in mice. To investigate the spatial distribution of the NO adducts formed in vivo and, if possible, to specify sites of ISDN metabolism, we carried out in vivo EPR measurements in mice that had been treated with ISDN and an Fe-DTCS complex via a subcutaneous route with a 700-MHz EPR apparatus that had been constructed in our laboratory (8, 26). In addition to the in vivo experiments, we conducted an ex vivo analysis of the blood obtained from the ISDN-treated mice to confirm the kinetics of the Fe complex.

MATERIALS AND METHODS

EPR-CT apparatus. We used a 700-MHz EPR-computer tomography (CT) system that had been constructed at our laboratory (8, 26). The system was composed of the following: power supplies, a personal computer, a main electromagnet...
Fig. 1. Structural formula of N-(dithiocarboxy)sarcosine dianion.

(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 consisted of a two-gap loop-gap resonator (41 mm diam; 10 mm axial length) and modulation coils. The magnetic field can be scanned by regulating the current through the field scan coils at a maximum rate of 15 mT/s. Linear magnetic field gradients along the x-, y-, and z-axes can be produced by the gradient coils (up to 1 mT/cm in a range 20 mm from the center).

In vivo EPR experiments. Female Institute for Cancer Research (ICR) mice, each weighing 30 g, were used in this experiment. They were treated with a 300 mM saline solution alone or simultaneously with Fe-DTCS complex. The blood samples were collected from the hearts under deep anesthesia with pentobarbital at 5, 15, or 30 min after the injections and were quickly placed in capillary tubes (75 mm in length and 46 µl in volume). The capillary tube was inserted into a standard quartz EPR cell (5 mm OD) and measured by Jeol TE-200 X-band (~9.5 GHz) EPR spectrometer at ambient temperature. The amount of NO was estimated from the signal intensity of the NO-Fe-DTCS complex, as previously described (6). Instrument settings were as follows: modulation amplitude, 0.32 mT; time constant, 0.3 s; sweep time, 4 min; and microwave power, 60 mW.

RESULTS

[15N]ISDN. Each mouse was treated with an Fe-DTCS saline solution via a subcutaneous route. Thirty minutes after the NO trap injection, ISDN was injected, also subcutaneously, into the contralateral side of the lower back. Under deep pentobarbital anesthesia, the animal was placed at the center of the resonator for in vivo EPR measurements. About 15 min later, a three-line EPR signal from the upper abdomen of the mouse was detectable, and the signal intensity increased gradually. A typical EPR spectrum (64 accumulations of 1-s scans) is shown in Fig. 2A. This signal of isotropic g (giso) = 2.040 and hyperfine coupling constant (aH) = 1.27 mT, which originates from the hyperfine interaction of an unpaired electron with a nitrogen nucleus (l = 1) of 15NO, is a characteristic signal of [15NO-Fe(DTCS)2]2+ (6), suggesting that the injected Fe-DTCS complex reacts with NO to form a nitrosyl complex. The control mice that received the Fe-DTCS solution alone exhibited no EPR signal (data not shown). Therefore, it is most likely that the NO of the EPR-active nitrosyl complex is produced from ISDN. The validity of this postulate can be ascertained by using [15N]ISDN instead of [14N]ISDN (see below).

Thirty minutes after ISDN injection, a positive signal-to-noise ratio (S/N) (~10) EPR signal was obtained (Fig. 2A). We then started to measure the EPR spectra under the field gradient to obtain EPR images. EPR-CT images were successfully reconstructed from 81 spectra with four accumulations each. Figure 3A shows a slice of the EPR-CT images in the z-x plane (transverse slice). The spatial resolution was 5.67 mm FWHM. The image shows that the signals are distributed predominantly in the right ventral part of the abdomen of the mouse. The outline of the image corresponds anatomically to that of the liver (Fig. 3, A and B). This observation agrees with that of the LPS-treated mouse reported previously, although the latter was a two-dimensional projection (28).

[14N]ISDN. The procedures for the [14N]ISDN experiments were repeated. About 15 min after [14N]ISDN injection, an in vivo EPR spectrum could be observed as in the [15N]ISDN experiments. In this case, however, a doublet signal (giso = 2.040 and aH = 1.78 mT) was...
observed (Fig. 2B), because the nuclear spin of $^{15}$N is $\frac{1}{2}$. The EPR parameters of the $^{15}$NO-Fe-DTCS complex were similar to those of the $^{14}$NO complex with other DTCs (15). This observation clearly demonstrates that the NO trapped by the Fe-DTCS complex originated from ISDN.

A good S/N ($\sim 15$) EPR signal was obtained (Fig. 2B) about 30 min after the injection of $[^{15}$N]$\text{ISDN}$. The improvement in S/N ($\sim 1.5$) was mainly due to the change of the line shape from triplet to doublet, which makes the signal intensity 1.5 times higher if the signals have the same line width; thus higher spatial resolution images were expected. A typical set of EPR-CT images in the z-x plane of the upper abdomen of a mouse is shown in Fig. 4. The number at the bottom of each slice shows the distance from the center of the loop-gap resonator in the caudal direction. The thickness was 0.5 mm for each slice. The spatial resolution was 3.95 mm FWHM.

The images of $[^{15}$N]ISDN (Fig. 4) were similar to those of $[^{14}$N]ISDN (Fig. 3A). However, two interesting features were deduced from the series of transverse slices. First, a low-signal-intensity (< 25% of maximum signal intensity) rift at the ventral side of the largest high-intensity area (HIA; > 25% of maximum signal intensity) became even larger (from -3.0 mm slice to the caudal side and finally dividing HIA into small parts). These changes seem to reflect the shape of the liver on the caudal side. Probably the rift with a low signal intensity corresponds to the stomach, where the nitrosyl complex either did not exist or could not be detected by this EPR apparatus. Second, an additional two parts of HIA in a symmetrical position can be seen in slices from -3.0 to +6.0 mm in Fig. 4. One is a small round section of the left dorsal side of the area, which corresponds to the liver that is shown separately, in

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**Fig. 2.** In vivo electron paramagnetic resonance (EPR) spectra (700 MHz) of nitrosyl-iron complex with N-(dithiocarboxyl)sarcosine (DTCS) ([NO-Fe(DTCS)$_2$]$^2_2^2$) formed in mice treated with $^{14}$N-labeled isosorbide dinitrate (ISDN) (A) or $[^{15}$N]$\text{ISDN}$ (B). Mice were injected subcutaneously with Fe-DTCS complex solution (300 mM, 10 ml/kg), followed by ISDN injection (acetone solution, 3.3 g/kg) 30 min later. Mice were then placed at the center of the resonator for in vivo EPR measurements. Instrument parameters: scan time, 1 s; receiver time constant, 1 ms; microwave power, 40 mW; modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; accumulation number, 64. Spectral parameters: $[^{14}$NO-Fe(DTCS)$_2$]$^2_2^2$: isotropic g value, $g_{iso} = 2.040$; hyperfine coupling constant, $a^N = 1.27$ mT; peak-to-peak width $\Delta B_{pp}$, 0.42 mT (A); $[^{15}$NO-Fe(DTCS)$_2$]$^2_2^2$: $g_{iso} = 2.040$; $a^N = 1.78$ mT; $\Delta B_{pp}$, 0.39 mT (B).

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**Fig. 3.** Typical transverse slices (z-x plane; thickness, 0.5 mm) of three-dimensional (3D) EPR computer tomography (CT) images of ISDN-treated mice (A, C) and illustration (B) of upper abdominal region of the mouse. A: slice from 3D EPR-CT images of $[^{14}$N]ISDN-treated mouse. B: schematic drawing of the cross-section of the mouse upper abdomen. C: a slice from the 3D EPR-CT images of $[^{15}$N]$\text{ISDN}$-treated mouse. Number at bottom of A and C is distance from center of the loop-gap resonator in the direction of the caudal side (note that the origin of A is not the origin of B). Three pictures do not necessarily show identical cross-sections.
slices from −0.5 to +6.0 mm. The other is an elliptical part of the right dorsal side, which borders on the largest HIA (liver), in slices from +0.5 to +6.0 mm. These two small parts of HIA, located nearly symmetrically on the dorsal side, resemble the kidneys. Compared with a cross-section of the upper abdomen of a mouse (Fig. 3B), these two parts of HIA and the low-intensity rift mentioned above correspond exactly to the kidneys and the stomach, respectively. To our knowledge, this is the first example of three-dimensional EPR images of free radicals produced in living animals, although the three-dimensional images of spin labels have previously been reported (1, 26).

Fig. 5 shows EPR-CT reconstructed images of the signal distribution in the upper abdomen of a [15N]ISDN-injected mouse. These images were reproduced from the same data as in Fig. 4, using a Macintosh computer and NIH Image program. Figure 5 depicts views from the nasocaudal, ventrodorsal, and right lateral sides. It is obvious from Fig. 5 (nasocaudal and right lateral
views) that the three-dimensional forms of the area, in which the NO adduct formed in vivo was distributed, resemble the kidneys as well as the liver.

Ex vivo EPR measurements of blood. To investigate the amount of the NO-Fe-DTCS complex formed in vivo from ISDN and Fe-DTCS complex in blood, we also measured the EPR spectra of the blood of the ISDN-treated mice with conventional X-band EPR instruments. After administration of the NO trap and ISDN, each of the blood samples was collected from the heart and immediately placed in a capillary tube. Interestingly, no EPR signal could be observed in the blood when the samples were processed through a procedure identical to that of the in vivo EPR experiment (i.e., Fe-DTCS injection followed by ISDN injection, from 5 to 30 min after ISDN injection). On the other hand, the blood samples prepared by ISDN injection first, followed by Fe-DTCS injection or simultaneous injections of the two, exhibited the EPR spectra of an NO-Fe-DTCS complex (data not shown). The concentrations of the NO adduct in blood were 1–5 μM, 5–30 min after these reagents were injected (n = 3 in each experiment).

DISCUSSION

In the present study, ISDN and the Fe-DTCS complexes were injected subcutaneously into the lower back of mice, and in vivo EPR measurements of the NO adduct were attempted. We detected NO production from ISDN and succeeded in obtaining three-dimensional EPR images of the upper abdominal region of the mice. The images clearly demonstrated that the nitrosyl-Fe complex formed in vivo was distributed mainly in the liver. By using [15N]ISDN, we could also obtain images of the kidneys with a more detailed outline of the organs. These features, which could be obtained only with [15N]ISDN, were probably due to the improvement of S/N and better resolution of the hyperfine doublet structure (15). In fact, the spatial resolution of the images improved from 5.67 mm FWHM ([14N]ISDN) to 3.95 mm FWHM ([15N]ISDN). Therefore, [15N] substitution is a great advantage in EPR imaging of NO.

The distribution of the NO adduct is similar to that of the experiment in which LPS-treated mice were used (28). The adduct was primarily distributed in the liver, followed by the kidney, whereas in the two-dimensional image only the liver could be portrayed (28). In this instance, LPS was administered via an intraperitoneal route and the NO thus trapped was certainly produced from inducible NO synthase (iNOS). Although iNOS was induced in the peritoneal macrophages, the possibility of the induction of iNOS in the liver could not be excluded. Accordingly, the site where the NO-Fe-DTCS complex was formed could not be specified.

Is it possible that NO was produced from ISDN in the internal organs depicted in the images described above? Although the metabolic pathways of ISDN have not been elucidated (4, 11, 12, 14), it is presumed that ISDN undergoes a presystemic biotransformation primarily in the liver (11), where glutathione S-transferase (GST) is thought to metabolize it (12). Accordingly, there is a strong possibility that most ISDN releases NO in the liver. Then NO reacts with the Fe-DTCS complex in the vicinity to form an EPR-active NO-Fe-DTCS complex, and the NO adduct remains in the liver.

The results of the ex vivo measurements of blood support this assumption. No EPR signal could be observed when the Fe-DTCS complex was injected 30 min before ISDN injection, although the EPR signal of the NO-Fe-DTCS complex could be observed when the reagents were injected in reverse order or injected simultaneously. These facts suggest that the amount of the Fe-DTCS complex in blood circulation was very small 30 min after the injection and that most of the Fe-DTCS complex was attached to organs (particularly to the liver and the kidneys) during metabolism and subsequent excretion. Therefore, we think that most of the NO-Fe-DTCS complexes are formed in the liver, as shown in the EPR images (Figs. 3 and 4).

Of course, we cannot exclude the possibility that the NO-Fe-DTCS complex was formed in other organs or tissues and then accumulated in the liver. Because the Fe-DTCS and NO-Fe-DTCS complexes are highly water-soluble, they readily move to the abdominal organs from subcutaneous tissue via circulating blood and tend to accumulate in the liver (25), although they hardly permeate through the membranes, including the blood-brain barrier (23, 27). Therefore, the images of the liver obtained from these ISDN experiments may also be produced by the NO-Fe-DTCS complex that was delivered to the liver via the blood circulation.

In fact, the [14C]ISDN studies showed that the concentration of ISDN is significantly higher in vascular tissue than in other tissues or in the blood (19, 21). Tam et al. (24) reported that the lung and intestine homogenates metabolize ISDN with a potency about two-thirds the potency of the liver homogenates, although the liver homogenates of rabbits are more effective than several other types of tissue homogenates of the same animal species in this metabolic process. Moreover, GST-independent pathways of ISDN metabolism, such as thiol-dependent mechanisms (4) and unknown mechanisms in rat erythrocytes (14), have been pointed out. Thus NO presumably is generated in all tissues in which ISDN is distributed. However, this NO may not be trapped by the injected Fe-DTCS complex because of its low concentration at the site of generation.

These possibilities may be applied to the kidney. In the experiment using rabbit homogenates, the kidney shows about 40% of the metabolic activity of the liver (24). Because the kidney is the central organ of excretion, it is no wonder that the water-soluble NO-Fe-DTCS complex is delivered to the kidneys. In fact, feces and urine of ISDN-injected mice exhibited the EPR spectra of the NO adduct (data not shown). Therefore, the NO adduct is excreted from the liver and kidneys and may accumulate in the biliary tract and urinary system.

Both NO adducts generated on-site and those delivered to the site must contribute to the EPR images that were obtained here. For the reasons mentioned above, we believe that the NO adduct generated on-site is predominant, although it is difficult to distinguish
which adduct is predominant at this stage of the development in the sensitivity and time resolution of the in vivo EPR instruments available.

To summarize, we measured NO production from ISDN in mice by in vivo EPR spectroscopy and obtained three-dimensional EPR images of the upper abdominal region. The images corresponding to the liver were obtained with $^{14}\text{N}$ ISDN, whereas the ones corresponding to the liver and the kidneys were obtained with $^{15}\text{N}$ ISDN. This EPR imaging method will be useful for noninvasive investigations of the in vivo spatial distribution of NO in pathological organs or tissues with locally high levels of NO. Further improvements in instrumentation will bring out details of the pathological and physiological pathways of NO-related compounds.

We thank Dr. Hidekatsu Yokoyama of the Institute for Life Support Technology for comments and technical assistance with in vivo EPR instrumentation. We also thank EsiCo, Ltd., for generously providing $^{14}\text{N}$ ISDN and $^{15}\text{N}$ ISDN.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (Molecular Biometallics) (08249107) to T. Yoshimura, and by a Grant-in-Aid for Scientific Research (08271518) to S. Fujii from the Ministry of Education, Science, Sports and Culture, Japan.

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Received 8 September 1997; accepted in final form 12 January 1998.

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