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Importance of volume limitation for tissue redox status measurements using nitroxyl contrast agents: A comparison of X-band EPR bile flow monitoring (BFM) method and 300 MHz in vivo EPR measurement

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Abstract

Methods proposed for in vivo redox status estimation, X-band (9.4 GHz) electron paramagnetic resonance (EPR) bile flow monitoring (BFM) and 300 MHz in vivo EPR measurement, were compared. The spin probe 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) was utilized for both methods, due to its suitable lipophilicity. EPR signal decay of a nitroxyl spin probe in the bile flow and in the liver region (upper abdomen) of several rat groups with different selenium status were measured by both the BFM and the in vivo EPR method, respectively. The nitroxyl radical clearance measured with in vivo EPR method may be affected not only by the redox status in the liver but also by information from other tissues in the measured region of the rat. On the other hand, the time course of nitroxyl radical level in the bile flow of rats was found to be a reliable index of redox status. Measurement site and/or volume limitation, which was achieved by the BFM method in this paper, is quite important in estimating reasonable EPR signal decay information as an index of tissue/organ redox status.

Keywords: Nitroxyl radical; Tissue redox status; Contrast agent; Electron paramagnetic resonance imaging; Magnetic resonance imaging

1. Introduction

In vivo redox status in specific organs and/or tissues of the living body is important in understanding pathogenic mechanisms of free radical related diseases. Nitroxyl free radicals are used as spin probes to monitor changes in biological redox status using in vivo electron paramagnetic resonance (EPR) spectroscopy [1–3] and imaging [4,5]. When a nitroxyl spin probe is administered intravenously to a living animal, in vivo EPR signal intensities of the spin probe decrease as a function of time at a rate governed by pharmacokinetics and redox state [6,7]. After the development of L-band in vivo EPR spectrometer/imager, nitroxyl radicals, with piperidine (TEMPO) and/or pyrrolidine (PROXYL) skeleton structures, were used to monitor tissue redox status of living mice and rats [8–12]. The in vivo decay constant of the EPR signal intensity of the spin probe depends on the tissue being examined and is variable with physiological and/or pathological conditions [1,4].

Liver is an important organ in the metabolism of drugs administered. Liver is known to have an environment that reduces nitroxyl radicals [13]. The first trial of an in vivo EPR experiment, which employed TEMPOL as a spin probe, was carried out using X-band frequencies with a helical coil type resonator surgically implanted into a rat liver [14]. In vitro EPR studies suggest that enzymatic one electron reduction of nitroxyl free radicals in liver

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microsome, mitochondria, and cytosol are responsible for the loss of EPR signal [15–18]. The reduction of nitroxyl radical in liver has been extensively investigated using in vivo and in vitro models [9,11,13–20]. In addition, several oxidative stress modalities were found to modify the in vivo decay constant of nitroxyl probes [21–28]. However, EPR signal loss as an indicator of in vivo redox status in intact liver is more difficult to determine due to the simultaneous pharmacokinetic clearance of the spin probe. To extract tissue redox status information, it is necessary to correct for the pharmacokinetic decay from the EPR signal loss. To obtain biologically and/or clinically useful redox information, a measurement of a specific organ/tissue of interest is desired. Localized regions of interest in an intact animal can be studied using resonators such as surface coil [29] and/or narrow length loop-gap resonators [5,28].

Since the initial part of the bile duct is put together with hepatic cells, the hepatic cell plasma and bile were partitioned only with hepatic cell membrane. Therefore, concentration of materials in the bile reliably reflects the dynamics of the materials in the hepatic cells. When the bile duct of an experimental animal is cannulated, and nitroxyl radical in bile flow is directly measured by X-band EPR [30,31], EPR signal decay in the bile flow may reflect redox status of liver cells. Such a focused measurement of a targeted organ/tissue (bile-duct) is capable of providing a measurement of the global redox status of the organ involved (liver, in this case).

In this paper, time course of nitroxyl radicals in the bile flow of selenium-deficient (SeD) rat was measured by X-band (9.4 GHz) EPR. The data were compared with the spin clearance in the liver of the SeD rat measured using in vivo 300 MHz EPR with a bridged loop-gap resonator. Estimation of redox status in the SeD rat liver compared to selenium-adequate control (SeC) and normal rats with sensitivity and selectivity is discussed. Importance of site and volume limitation of EPR redox measurements was described.

2. Results

The EPR signal intensity of carbamoyl-PROXYL, TEMPOL and CAT-1 as a function of time in the bile after injection were compared in the normal rat. The spin probes were administered with i.v. injections of 0.3 mmol/kg b.w. This was sufficiently lower than maximum tolerated dose of CAT-1 for mouse (<0.50 mmol/kg), which is the most toxic of the three probes [7]. The X-band EPR signals of carbamoyl-PROXYL, TEMPOL, and CAT-1 in normal rat bile at several time points after injection were shown in Fig. 1. The EPR signal intensity of CAT-1 in the bile was markedly weaker compared with that of either carbamoyl-PROXYL or TEMPOL. The maximum concentration in the bile were 1.46 ± 0.08 ($n = 4$), 1.70 ± 0.22 ($n = 5$), and 0.10 ± 0.05 ($n = 4$) mM (values are means $\pm$ SD, n indicates number of rats) for the carbamoyl-PROXYL, TEMPOL and the CAT-1, respectively. The total excreted amount of intact radical detected in the bile during the measurements were 0.22 ± 0.02, 0.15 ± 0.01, and 0.022 ± 0.006% of the total dose for carbamoyl-PROXYL, TEMPOL and CAT-1, respectively. The maximum concentration of carbamoyl-PROXYL and TEMPOL in the bile was almost same as that in the blood, while the maximum concentration of CAT-1 in the bile was around 1/20 of the maximum blood concentration (data not shown).

The X-band EPR signal of carbamoyl-PROXYL in bile showed a triplet line with similar spectrum to that of in vivo EPR except that signal to noise ratio was very high compared with the in vivo experiment. In the bile, the signal height of the peak obtained at highest magnetic field was always slightly lower than other two (Fig. 1A). This EPR signal behavior suggests a weak hyperfine anisotropy of spin probe molecules. The EPR signal intensity in the bile decreased with biphasic pattern (Fig. 2A) similar to observations in previous reports [32]. The 300 MHz in vivo EPR signal of the carbamoyl-PROXYL in the liver section of the rats showed similar triplet lines as obtained by X-band.

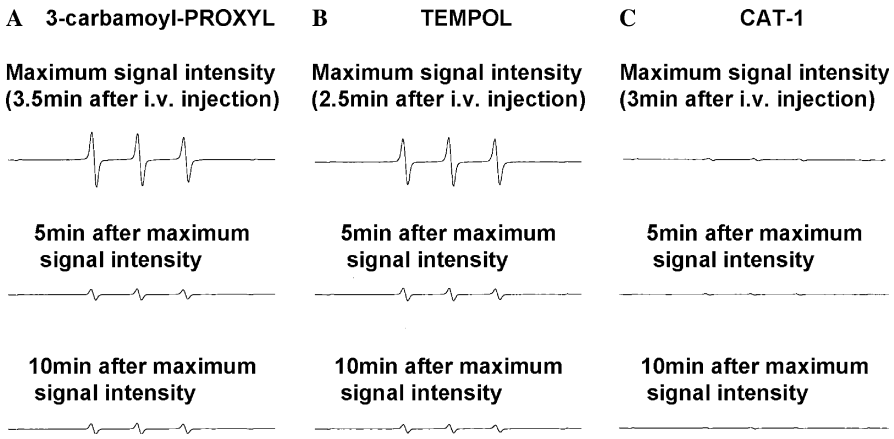

Fig. 1. X-band ESR spectrum of the carbamoyl-PROXYL, TEMPOL and CAT-1 measured in bile. Maximum signal intensity was obtained at 2.5–3.5 min after i.v. injection of (A) carbamoyl-PROXYL, (B) TEMPOL, and (C) CAT-1. Microwave frequency was 9.4 GHz; microwave power was 10 mW; field modulation width was 0.1 mT; field modulation frequency was 100 kHz.

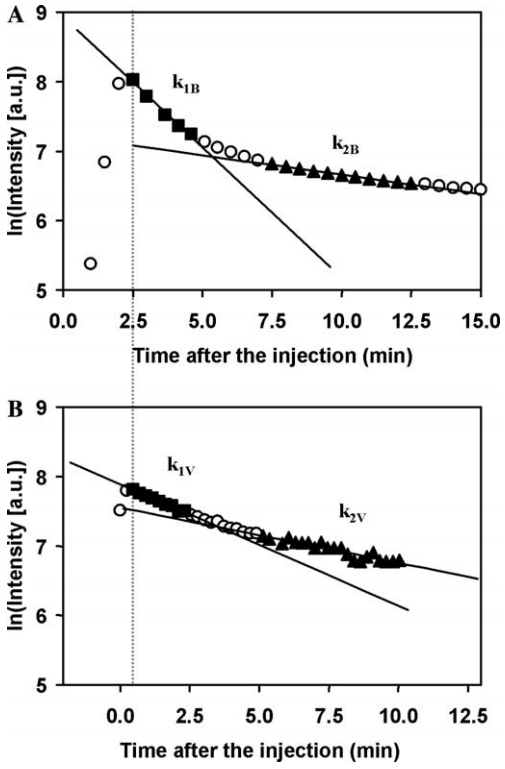

Fig. 2. Typical decay profiles of the EPR signal intensity of carbamoyl-PROXYL in the normal rat obtained by two featured methods in this paper. (A) A typical decay curve in bile flow (BFM method). (B) A typical decay curve in upper abdomen (in vivo measurement). Both x- and y-axes of the figures were indicated with the same scales. The horizontal axis indicates the time after i.v. administration of carbamoyl-PROXYL. Expected peak points of both decay profiles were arranged on a dotted line. Decay curves show biphasic-like decay profiles in ether BFM and in vivo methods. Decay constant k_{1B} was estimated with the gradient of a period from 0.5 to 2.5 min after maximum signal intensity (the data points were indicated as closed square) was obtained. The decay constant k_{2B} was estimated with the gradient of a period from 5 to 10 min after maximum signal intensity (closed triangle). Decay constant k_{1V} was estimated with slope of the period from 0.5 to 2.5 min after the administration of the probe (closed square). The slope of the latter period from 5 to 10 min after the administration of the probe (closed triangle) was estimated as decay constant k_{2V}. Open circles were data points not used for any calculations.

However, the signal to noise ratio of 300 MHz in vivo EPR spectrum was markedly lower than the X-band EPR BFM method. The in vivo EPR signal intensity decreased as biphasic (Fig. 2B), which was similar to observations in previous reports [1,28].

Table 1 shows decay constants k_{1B} and k_{2B} measured in bile flow of several groups of rats. No significant difference was obtained between the k_{1B} values of the normal and the SeC groups. The k_{1B} values of SeD group were significantly lower compared with both the normal and the SeC groups. No significant differences were obtained for k_{2B} values among the three groups of rats. Also no significant differences were obtained for the maximum signal intensity among the three groups of rats.

Table 2 shows the in vivo decay constants of carbamoyl-PROXYL in the liver of several rats. The decay constant, k_{1V}, of SeD group shows significantly lower values

Table 1
Decay constants of carbamoyl-PROXYL in the bile flow measured by BFM method

Rat group	n	k_{1B} (min^{-1})	k_{2B} (min^{-1})	Maximum intensity
SeD	4	$0.235 \pm 0.039^{*,\#}$	0.063 ± 0.015	3800 ± 346
SeC	4	0.308 ± 0.008	0.040 ± 0.007	3866 ± 326
Normal	5	0.327 ± 0.046	0.042 ± 0.013	3245 ± 936

Values are indicated by means ± SD. n is number of rat.
* Significant difference at $p < 0.05$ compared with normal group.
$^{\#}$ Significant difference at $p < 0.05$ compared with SeC group.

Table 2
Decay constants of carbamoyl-PROXYL in the liver part of rats measured by in vivo method

Rat group	n	k_{1V} (min^{-1})	k_{2V} (min^{-1})	Maximum intensity
SeD	17	$0.129 \pm 0.027^{**}$	$0.064 \pm 0.011^{\#}$	2105 ± 249
SeC	16	$0.145 \pm 0.027^{**}$	0.075 ± 0.016	2030 ± 323
Normal	6	0.197 ± 0.014	0.060 ± 0.011	1873 ± 170

Values are indicated by means ± SD. n is number of rat.
** Significant difference at $p < 0.001$ compared with normal group.
$^{\#}$ Significant difference at $p < 0.05$ compared with SeC group.

compared with the normal group. Although this behavior is similar to that observed in a previous report [28] which examined 6-week-old rats, the k_{1V} values of 8-week-old rats appears to be higher than 6-week-old rats (0.101 ± 0.026 min^{-1}, $n = 5$ and 0.181 ± 0.017 min^{-1}, $n = 5$ for SeD and normal groups, respectively). SeD and SeC groups showed significantly lower levels of k_{1V} values compared with the normal group. The SeD group showed lower k_{1V} values than the SeC group, although no statistically significance difference was obtained between them. No marked difference was obtained between the k_{2V} values of SeD and normal groups. Although the SeC group showed slightly higher k_{2V} value compared with other two groups, only the SeD group showed significant difference.

3. Discussion

Since the EPR signal decay of the CAT-1 in circulating mouse blood was markedly slower than TEMPOL [7] and the CAT-1 is a membrane impermeable molecule, it is possible that blood and bile were partitioned by hepatic cells. The nitroxyl spin probes must pass through hepatic cells when it is transported from blood to bile. These results suggest that the transport of carbamoyl-PROXYL and TEMPOL from blood to bile is rapid and the EPR intensity of carbamoyl-PROXYL and TEMPOL in the bile may represent its levels in liver cells. It is also suggested that the membrane permeable nitroxyl contrast agents, such as carbamoyl-PROXYL and TEMPOL, can be tissue redox probes.

Most of our previous reports employed carbamoyl-PROXYL as the in vivo redox probe for EPR measurements [1,22,24,27,28], because of its suitable reduction rate for in vivo EPR measurement. In addition, no marked toxicity was reported for carbamoyl-PROXYL in the

generally used dose (~3.0 μmol/g b.w.). Therefore, carbamoyl-PROXYL was again employed for this paper for a comparison between the BFM method and the in vivo measurement.

Selenium-adequate control rats showed lower k_{1V} values and higher k_{2V} values compared with the normal rats. This may be due to the differences in the measured volume as well as the contribution of several organs/tissues. The in vivo decay rate of spin probe in the normal rat may reflect its kinetics in the liver, while in the SeC rat the decay rate may reflect a greater contribution from other organs/tissues in the measured volume. The same observation might occur when 6- and 8-week-old rats are compared. Therefore, appropriate in vivo experiments should be carried out using suitably sized resonators for each animal or carried out with animal models with minimal differences in sizes. Differences between SeD and SeC groups can only be compared in the present in vivo EPR measurement, because these two groups have similar body weight and size.

No significant difference was obtained for the maximum signal intensity in bile among the three rat groups. This fact suggests that animal body weights (distribution volumes) did not effectively affect the maximum EPR signal intensities in the bile, which was quantitated using a standard curve, when the dose ratio (mmol/kg b.w.) of carbamoyl-PROXYL was the same. This suggests that the assessment of liver redox status by the X-band EPR BFM method was less affected by the differences in body weight of the animals.

The reduction rate constant, k_{1B}, measured in the bile flow, gives a more reliable assessment of in vivo redox status in the liver compared to that obtained from in vivo studies. No significant difference was obtained for the maximum in vivo EPR signal intensity among the three rat groups. However, the normal and treated groups can not be compared accurately because sample volume in the resonator affects the detection sensitivity. Generally, in vivo EPR signals are not quantitative because of variations in sample volumes and detection sensitivity. An identical sample volume is required in order to compare several EPR data quantitatively. Therefore, the sample volume restriction (i.e., bile sampling) is critical when comparing in vivo EPR data.

The time course of nitroxyl radicals in bile flow of SeD rat helps define the reasonable redox status, which is reflected by reduction and re-oxidation of the spin probe in the SeD rat liver with high sensitivity and selectivity. Results from a previous paper [1] suggested that re-oxidation of hydroxylamine to the nitroxyl radical is faster in the SeD rat liver compared to other groups, since the hydrogen peroxide (H_2O_2) level in the SeD liver is higher. Consequently, the SeD group showed significantly lower k_{1B} than the other groups. However, the volume effect masks difference among redox status in the liver region, i.e., the k_{1V} values, of the three rat groups.

To obtain biologically and/or clinically useful information, the information from specific organs/tissues of interest is desired. It should be noted that the in vivo EPR spectroscopic data reflects information not only from the organ/tissue of interest but also from other surrounding tissues. When animals with different sizes are measured using the same resonator, the sampled regions in the animals may include different contributions of organs/tissues. Therefore, reasonable detection of in vivo tissue/organ redox status requires suitable limitation of measurement sites and volumes, which will be available with topical detection technique [7,29,33] and/or imaging techniques [4,5]. These results relate directly to FOV, ROI, and slice selection in future modality approach to quantitative pathophysiology using MR imaging techniques.

4. Conclusions

Although the BFM method is an invasive measurement, its high sensitivity and selectivity suppresses the contribution of the pharmacokinetic effect. Therefore, the BFM method will give useful information about the pharmacokinetics and redox status of nitroxyl spin probes in the liver. The results in this paper emphasized the importance of choosing the correct location volume and physiologically relevant site in order to obtain highly localized and resolved redox information.

5. Experimental

5.1. Materials

The spin probe 3-Carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (carbamoyl-PROXYL) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The cell impermeable probe 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT-1 which is also referred as TEMPO-choline) was purchased from Molecular Probes Inc. (Eugene, OR). Selenium-deficient diet was purchased from Oriental Yeast Co., Ltd (Tokyo, Japan) [34,35]. Other materials were in analytical grade.

5.2. Animals

Wistar rats (15th day after pregnancy) were fed with Se-deficient diet and ultra pure water. Newly born rats were kept with their own mothers for 4 weeks. After 4 weeks the young rats were weaned, and fed with selenium-deficient diet and ultra pure water until experiments (SeD group) were done. The SeC group was prepared in the same manner as the SeD group except for that 0.1 ppm as selenium was added in the drinking water. The normal group was healthy male Wistar rats purchased from Japan Laboratory Animals, Inc. (Tokyo, Japan). Eight-week-old rats were used for all experiments. Body weight of SeD, SeC, and normal group at 8 weeks of their age was 131.8 ± 23.1 g ($n = 24$), 146.5 ± 22.2 g ($n = 21$), and 221.0 ± 9.7 g ($n = 11$) (values are indicated by

means ± SD, *n* indicates number of rats), respectively. The animal experiments were carried out in compliance with the Guidelines for Animal Care and Use at Showa Pharmaceutical University (2001), and approved by the Ethical Committee for Animal Care and Use of Showa Pharmaceutical University.

5.3. X-band EPR measurements in bile flow

Rats were anesthetized with intraperitoneal injection of 0.05 mg/g b.w. pentobarbital (Nembutal, Dinabot, Osaka, Japan). The right upper abdomen just under the ribs was opened with a 2 cm vertical cut. The bile duct was cannulated with a 50 cm segment of polyethylene (PE-10) tubing. The cannula tubing traversed through the X-band EPR TE mode cavity. Bile was collected at end of the cannula tubing. Then, an isotonic aqueous solution of a nitroxyl spin probe was administered as an i.v. injection (0.3 mmol/kg b.w.). EPR signals of the spin probe administered were measured with an X-band EPR spectrometer (FA-100, JEOL, Tokyo, Japan). The center line of the triplet was monitored repeatedly for 20 min with at 30 s intervals. EPR conditions were as follows: microwave frequency was 9.4 GHz; microwave power was 10 mW; field modulation width was 0.1 mT; field modulation frequency was 100 kHz; scan rate was 2.5 G/s, scan range was 50 G; and time constant was 0.01 s, respectively.

Natural logarithmic values of signal height of the center line, which was less affected by anisotropy, were plotted with the time after i.v. injection of the spin probe to the rat. A typical decay profile in the bile was shown in Fig. 2A. The nitroxyl signal was acquired starting 2–3 min after injection and peaked at 3–4 min. Decay constant k_{1B} was estimated with slope of the initial period (2.5 min after peak). The slope of the latter period (5 min to 10 min after peak) was estimated as decay constant k_{2B}.

5.4. In vivo EPR measurements

Rats were anesthetized with intraperitoneal injections of pentobarbital (0.05 mg/g b.w.). Once anesthetized, isotonic aqueous solution of carbamoyl-PROXYL was administered by i.v. injection (0.3 mmol/kg b.w.). The EPR signals were measured at the upper abdomen (liver part) with a low frequency (300 MHz) in vivo EPR spectrometer (JEOL, Tokyo, Japan). The center line of the nitroxyl triplet was repeatedly measured every 14–15 s for 10 min. A bridged loop-gap resonator (70 mm diameter, 5 mm axial length) was used for all measurements. EPR conditions were as follows: microwave frequency was 300 MHz, microwave power was 1.0 mW, field modulation width was 0.1 mT, and field modulation frequency was 100 kHz; scan rate was 1 G/s, scan range was 10 G; and time constant was 0.3 s, respectively.

The natural logarithmic values of signal height of the center line of the triplet EPR signal of the nitroxyl radical were plotted against the time after i.v. injection of the spin probe. A typical in vivo EPR decay profile was shown in Fig. 2B. The decay constant, k_{1V}, was estimated using the slope of the initial period (0.5–2.5 min) while the slope of the latter period (5–10 min) was used to the decay constant, k_{2V}.

5.5. Statistical test

The statistical differences were estimated using an alternative Student's or Welch's *T* test. The suitable test for the data was automatically selected according to the variance of the data. Grades of significance were estimated by $p < 0.05$, $p < 0.01$, and $p < 0.001$.

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