A novel lipophilic spin probe for the measurement of radiation damage in mouse brain using in vivo electron spin resonance (ESR)

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Abstract As a possible lipophilic spin probe of in vivo electron spin resonance (ESR), 3-methoxy carbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (MCPROXYL) was examined. The permeability of the blood-brain barrier to this compound was evaluated with a brain uptake index and autoradiography, with result that this probe is well distributed in the brain. The in vivo ESR spectra were measured in the head and the abdomen of MCPROXYL-injected living mice. The rate of signal decay of MCPROXYL in the head measured at one hour after X-irradiation was about 75% of that of the controls. The decrease in the head seems to be related to the early response of the brain to X-irradiation. This is the first report that the behavior of free radical such as MCPROXYL in the brain is influenced by X-irradiation. MCPROXYL is thus useful as a novel spin probe for in vivo ESR to monitor the radiation damage in the brain.

Key words: In vivo electron spin resonance; Nitroxyl radical; Brain; Radiation; Blood-brain barrier; Free radical

1. Introduction

Oxidative stress such as radiation is known to generate active oxygens in living organisms, to vary the redox state significantly, and to cause various types of tissue damage. The brain may be especially susceptible to oxidative stress for the following reasons [1]: (1) The brain is exposed to a high amount of oxygen, because of its high and constant oxygen requirement, utilizing about one-fifth of the oxygen consumed by the whole body; (2) the brain membrane lipids enrich in oxidizable polyunsaturated fatty acids; (3) the brain has low levels of the antioxidant enzyme, catalase and only moderate amounts of superoxide dismutase and glutathione peroxidase; (4) the presence of non-protein-bound Fe^{3+} in the cerebrospinal fluid increases the formation of highly reactive hydroxyl radicals through the Haber-Weiss reaction. Therefore, oxidative damage in the brain is especially worthy of investigation.

A stable nitroxyl radical such as 3-carbamoyl-2,2,5,5-tetramethyl pyrrolidine-1-yloxy (carbamoyl-PROXYL) or 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-yloxy (hydroxy-TEMPO) is often used for the in vivo ESR as a probe. The redox reaction of the nitroxyl radicals has been reported to correlate to the generation of free radicals and to changes of the physiological redox state caused by aging, ischemia-reperfusion, hyperoxia and hypoxia [2–6].

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In our previous study [7], we reported that X-irradiation increases the signal decay rate, i.e. the reduction rate of carbamoyl-PROXYL in the mouse abdomen at 1 h postirradiation, indicating that early redox response of the mouse to the X-irradiation is detectable by an in vivo ESR system. However, carbamoyl-PROXYL is hydrophilic and does not pass through the blood-brain barrier (BBB) [8]. Similarly, most of the other nitroxyl compounds used conventionally are very soluble in water, and unlikely to pass through the BBB. Therefore, choosing a probe which can pass through the BBB is essential to monitor the redox reaction in the brain by in vivo ESR.

In the present study, we synthesized a lipophilic nitroxyl spin probe (3-methoxy carbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy, MCPROXYL) as a novel spin probe which can monitor the physiological redox state in the brain. The permeability of the BBB to this probe and the effects of X-irradiation on the reduction rates of MCPROXYL in the head of living mice were examined.

2. Materials and methods

2.1. Chemicals

[^{14}C]Methanol (3 mCi/mmol) and [^{3}H]water (1 mCi/g) were purchased from Moravek Biochemicals, Inc. (Mercury Lane • Brea, CA, USA). [^{14}C]Sucrose was purchased from NEN Research Products (Boston, MA, USA). Soluene 350 (a tissue solubilizer) and Hionic-Fluor (scintillation mixture) were purchased from Packard Instrument Co. (Downers Grove, IL, USA). 3-Carboxy-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (carboxy-PROXYL) was purchased from Tokyo Kasei Kogyo, Co. (Tokyo, Japan). Pentobarbital (50 mg/ml) was obtained from Dainabot Co. (Osaka, Japan). Other reagents were of the highest purity commercially available.

2.2. Animals

Male Wistar rats (6 weeks, ca. 150 g body weight) for the brain transport studies, and female ddY mice (3–4 weeks, 15–20 g body weight) for the autoradiography and the ESR studies were purchased from Japan SLC (Hamamatsu, Japan).

2.3. Synthesis

MCPROXYL was synthesized from carboxy-PROXYL and diazomethane by the method of Rozantzev and Krinitzkaya [9]. The synthesized MCPROXYL was purified by column chromatography and distillation in vacuo: bp, 100°C (3 mm Hg). The purified MCPROXYL was characterized by [^{1}H]NMR and FT-IR. Since the NMR spectra of the nitroxyl radicals are quite broad, MCPROXYL was reduced to a diamagnetic *N*-hydroxyl derivative by the addition of a 1.5 equiv. of phenylhydrazine, in an NMR tube containing the nitroxyl dissolved in $CDCl_3$ [10]. [^{1}H]NMR δ 1.31, 1.22, 1.14, 1.02 (each 3H, each s, 4x-CH_3), 1.74 (1H, dd, J = 12.6, 8.4 Hz, H-4), 2.13 (1H, dd, J = 12.6, 11.0 Hz, H-4), 2.75 (1H, dd, J = 10.6, 8.3 Hz, H-3), 3.67 (3H, s, $-OCH_3$); ir ν_{max} (neat) 1740 cm^{-1}. [^{14}C]MCPROXYL was synthesized from carboxy-PROXYL and [^{14}C]methanol via the acid chloride of carboxy-PROXYL. To synthesize acid chloride, thionyl-

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chrolide was added dropwise to a benzene solution of carboxy-PROXYL in the presence of pyridine. The synthesized [^{14}C]MCPROXYL was purified by column chromatography, and the purified nitroxyl showed a single spot in TLC. To verify the validity of this synthetic method, unlabeled MCPROXYL was synthesized by the same method via acid chloride. The MCPROXYL synthesized via acid chloride showed a [^{1}H]NMR spectrum identical to the one described above. The specific activity of [^{14}C]MCPROXYL was 2.92×10^{8} Bq/mol.

2.4. Brain transport studies

The brain transport of MCPROXYL was measured by the tissue-sampling single-injection technique (brain uptake index method) developed by Oldendorf [11]. A Wistar rat was anesthetized with intraperitoneal pentobarbital (80 mg/kg). The left common carotid artery of the rat was isolated and the mixture of [^{14}C]MCPROXYL and the internal reference compound, $^{3}H_2O$, was rapidly injected into the common carotid artery as an approximately 200 μl bolus of injection solution. The rat was decapitated 15 s after the injection. The cerebral hemisphere ipsilateral to the injection was removed from the cranium, solubilized in 1.5 ml of soluene 350 at 50°C for 3 h in an incubator, decolorized with 30% H_2O_2 and mixed with 10 ml Hionic-Fluor before double-isotope liquid scintillation counting. The brain uptake index (BUI) was calculated as follows [12]:

$$\text{BUI}\ (\%) = \frac{^{14}\text{C}/^{3}\text{H dpm (in brain)}}{^{14}\text{C}/^{3}\text{H dpm (in injection solution)}} \times 100.$$

2.5. Autoradiography

Mice were anesthetized with intraperitoneal pentobarbital. The mice were injected intravenously with [^{14}C]MCPROXYL or [^{14}C]sucrose at a dose of 0.3 μCi/mouse. Five min after the injection, the mice were frozen in dry ice/acetone after embedding in the 8% solution of carboxymethyl cellurose (CMC). The frozen mouse was cut into sections 20 μm thick by a microtome in a cryostat maintained at −10 to −15°C. The frozen sections of the mouse were adhered to cover slips at room temperature for a few seconds and then allowed to dry at −20°C for 2–3 days. The cover slips containing the freeze-dried sections were then covered by a thin plastic film (Lumilar Membrane, Nakagawa Co. Ltd., Tokyo, Japan) and placed in contact with an imaging plate (Type BAS III, Fuji Photo Film Co. Ltd., Tokyo, Japan) for 5 days. The radioactive images recorded on the imaging plate were read by a laser beam scanner and analyzed by an attached computer (BAS-2000, Fuji Photo Film Co. Ltd., Tokyo, Japan). Analysis settings were as follows: pixel, 100×100 μm; sensitivity, 10000; latitude, 4. In this image-analyzing system, the radioactivities in the tissue sections were provided as intensities of photostimulated luminescence. The ^{14}C concentrations in the tissue sections were reported to be proportional to the levels of photostimulated luminescence in the wide range [13].

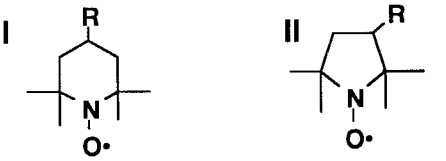
2.6. X-irradiation

The irradiation of ddY mice was performed with X-rays (200 kV, 20 mA), using 0.5 mm copper and 0.5 mm aluminum filters, at a dose rate of 0.62–0.65 Gy/min. Several mice were simultaneously given a single whole body exposure in the irradiation chamber separated for individuals. Sham irradiation for control mice included comparable immobilization in the same irradiation chamber.

2.7. ESR measurement

MCPROXYL was dissolved in sodium phosphate-buffered saline (pH 7.4) at 140 mM, and the solution of MCPROXYL was sterilized before the injection to mice. The in vivo ESR measurements were performed as follows. Mice were anesthetized by an intramuscular injection of pentobarbital (120 mg/kg) and fixed on a Teflon holder. The mouse fixed on the holder was placed in a resonator of the in vivo ESR apparatus. Immediately after the injection of the sterilized solution of MCPROXYL to a tail vein (80 μl), ESR spectra were measured in the head or the abdomen. In vivo ESR spectrometer (JES-PE, JEOL, Tokyo) was equipped with an L-band microwave power unit (ES-LB1A, JEOL) and a loop-gap resonator. The kinetic constants of signal decay of MCPROXYL were calculated from the slope of the spin clearance curves, which were determined from semilogarithmic plots of the peak heights of the ESR signal at the lower magnetic field, as described previously [7].

Table 1
Partition coefficients of various nitroxyl compounds



Nitroxyl compounds	Structure	Partition coefficients
CAT1	I R = $N^{+}(CH_3)_3I^{-}$	0.11
Carbamoyl-PROXYL	II R = $CONH_2$	0.87
Hydroxy-TEMPO	I R = OH	4.83
MCPROXYL	II R = $COOCH_3$	14.4

1-Octanol/water partition coefficients were determined.

3. Results and discussion

Since the permeability to the BBB is dependent on the lipophilicity and molecular weight of the compound, we examined the partition coefficients of various nitroxyl compounds. Table 1 partition coefficients between 1-octanol and water. The partition coefficients of carbamoyl-PROXYL and hydroxy-TEMPO were approximately consistent with those reported by Fuchs et al. [14]. MCPROXYL was more lipophilic than carbamoyl-PROXYL and the other spin probes, predicting the good permeability to the BBB of this compound.

Table 2 summarizes the BUI values for MCPROXYL. The BUI was calculated as described in Section 2. The BUI values for MCPROXYL were considerably higher than those for diazepam [15], suggesting that MCPROXYL can pass through the BBB and distribute well in the brain. This finding is consistent with the results reported by Sano et al. [16]. The result that the BUI decreased with the increase of the concentration of MCPROXYL coincided with the previous report for choline by Oldendorf and Braun [17].

We have confirmed the penetration of MCPROXYL to BBB and its distribution to the brain tissue by autoradiography using [^{14}C]sucrose as a control. Fig. 1 shows typical auto-

Table 2
Brain uptake index for MCPROXYL

Drug	BUI ± S.E. (%)	Reference
Acetylsalicylic acid (0.3 mM)	4.1 ± 1.1	[15]
Chloramphenicol (0.3 mM)	9.7 ± 0.7	[15]
Diazepam (0.3 mM)	94.4 ± 3.7	[15]
MCPROXYL (114.3 mM)	95.2 ± 4.6 (3)	This work
MCPROXYL (31.8 mM)	97.3 ± 1.5 (5)	This work
MCPROXYL (15.9 mM)	132.0 ± 2.8 (6)	This work
MCPROXYL (7.9 mM)	179.0 ± 6.8 (6)	This work

Values in parentheses are numbers of rats.

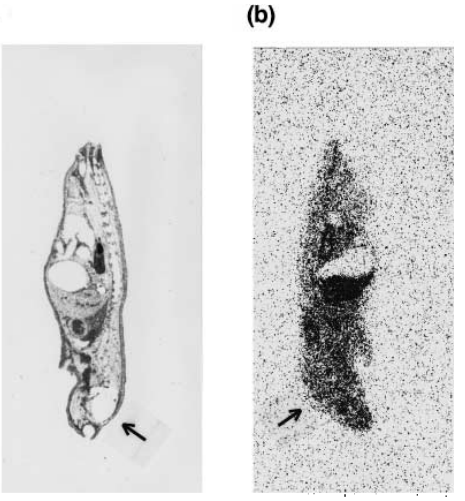


Fig. 1. Autoradiograms of the mice 5 min after injection. a: [^{14}C]Sucrose and b: [^{14}C]MCPROXYL. Details of the preparation are described in Section 2. The arrows show the mouse brain.

radiograms 5 min after injection of [^{14}C]sucrose (Fig. 1a) and [^{14}C]MCPROXYL (Fig. 1b), respectively. Fig. 2 shows a photograph of the corresponding section to Fig. 1. As shown in Figs. 1 and 2, [^{14}C]sucrose, which is known to be impermeable to BBB, was distributed to the blood vessel well but not to the brain cavity. On the other hand, [^{14}C]MCPROXYL was distributed to the liver and the brain cavity as well as to the blood vessel. The difference between the MCPROXYL and sucrose distribution to the brain was quantitatively estimated by analyzing the BAS image obtained through the simultaneous contact of MCPROXYL and sucrose sections (autoradiograms not shown). The areas of the regions of interest were 5.2 mm^2 for both the brain and the background. The intensities of photostimulated luminescence in three regions of interest in the brain were measured, subtracted the level of background, and then averaged. The intensity of the photostimulated luminescence, which is proportional to the ^{14}C concentration, was 1.07 ± 0.38 for the mouse to which sucrose was injected, while it was 5.52 ± 0.31 for the MCPROXYL-injected mouse. The ^{14}C concentration in the brain cavity of MCPROXYL-injected mouse was more than 5 times larger than that of sucrose-injected mouse. The results clearly show that MCPROXYL can penetrate to BBB and be well distributed to the brain tissue.

Fig. 3 shows the in vivo ESR spectra in the head (a) and the abdomen (b) of mice 1 min after the injection of MCPROXYL. In both regions, the spectrum consisting of three lines due to nitroxyl was observed. The hyperfine splitting was 1.65 mT, and the intensity of the three lines was not equivalent. The non-equivalent intensity due to a restriction of the rotational motion may be derived from the binding of MCPROXYL with biological materials such as protein. In both the head and the abdomen, a weak signal of the nitroxyl in the lipid phase was observed as a shoulder at the highest magnetic field peak. Since hyperfine structures and g-tensors of the ESR spectrum depend on the polarity of the environment in which the spin probe exists, the high field line may partially resolve into two lines. This result was consistent with the spectra of 2,2,6,6-tetramethyl piperidine-1-yloxy [18] and 2,2,6,6-tetramethyl-4-oxopiperidine-1-yloxy [19] in phospholipid membranes measured by X-band ESR. Komarov et al. have also

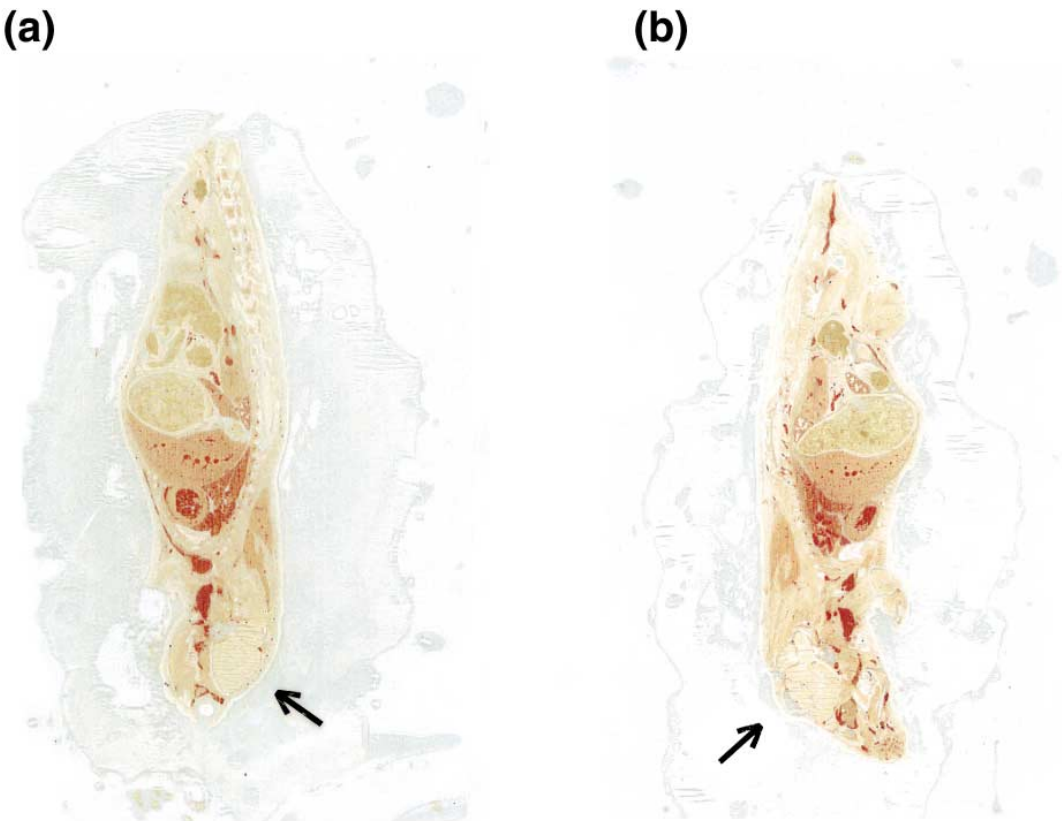


Fig. 2. A photograph of the same section as Fig. 1. a: [^{14}C]Sucrose and b: [^{14}C]MCPROXYL. Details of the preparation are described in Section 2. The arrows show the mouse brain.

Table 3
Effects of X-irradiation on kinetic constants of signal decay in the mouse head and abdomen

Dose of X-irradiation (Gy)	Head		Abdomen	
	1 h after irradiation	4 days after irradiation	1 h after irradiation	4 days after irradiation
0	0.142 ± 0.014 (6)	0.131 ± 0.019 (4)	0.137 ± 0.012 (6)	0.167 ± 0.026 (6)
1	0.135 ± 0.009 (6)	0.132 ± 0.017 (6)	–	–
3	0.105 ± 0.008 (4)*	0.142 ± 0.009 (5)	0.129 ± 0.010 (6)	0.170 ± 0.015 (6)
7.5	0.104 ± 0.010 (5)*	0.131 ± 0.015 (7)	0.128 ± 0.024 (4)	0.152 ± 0.015 (7)
15	O.110 ± 0.007 (5)*	–	0.125 ± 0.025 (3)	

*$P < 0.01$, different from that of 0 Gy.
The kinetic constants (min^{-1}) are the means ± S.D. Values in parentheses are numbers of mice.

demonstrated the two components, derived from an aqueous phase and a lipid phase, of 2,2,6,6-tetramethyl piperidine-1-yloxy injected to a mouse measured by S-band ESR [20]. Since this non-equivalent intensity and the appearance of the component in the lipid phase in the ESR spectrum were not detected using the water-soluble spin probes, these are very characteristic of MCPROXYL with high lipophilicity.

The signals of MCPROXYL in both regions decreased gradually with time after the injection. The kinetic constants of signal decay were calculated as described in Section 2. The effects of X-irradiation on the kinetic constants of signal decay of MCPROXYL were examined (Table 3). In the head, the decay rates of MCPROXYL were decreased (−25%) 1 h after X-irradiation at the doses of 3 and 7.5 Gy. A decrease in the decay rate was not observed 4 days after irradiation. In the abdomen, radiation effects were not observed at 1 h and 4 days after irradiation. These findings demonstrate that the X-irradiation decreased the rates of signal decay of MCPROXYL in the brain immediately after the irradiation. We have reported that the decay rates of carbamoyl-PROXYL were increased by X-irradiation in the mouse abdomen [7]. The opposite effects in the decay rate between MCPROXYL and carbamoyl-PROXYL may be presumably considered both the difference of the measuring region, i.e. the head and the abdomen, and the difference in the pharmacokinetics between MCPROXYL and carbamoyl-PROXYL. The different pharmacokinetics is probably derived from the following factors; (1) tissue and sub-cellular distribution, (2) reactivities with metabolic enzymes, bioreductants and bioradicals, and (3) the rate of excretion.

In conclusion, it was demonstrated that MCPROXYL is fairly lipophilic and can permeate through the BBB to brain. The rate of the signal decay of MCPROXYL was decreased by X-irradiation in the head region. These findings indicate that MCPROXYL is a novel spin probe for in vivo ESR, which can monitor the changes of the physiological redox state in the brain and/or the damage of the brain function caused by X-irradiation.

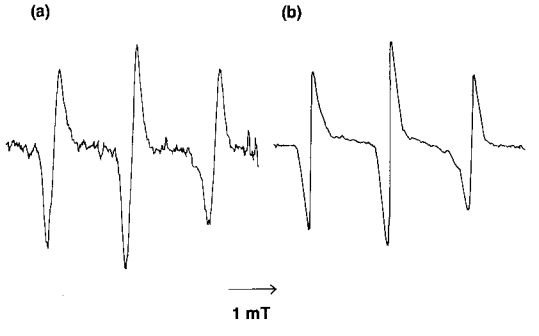


Fig. 3. In vivo ESR spectra of MCPROXYL in the head and abdomen of a living mouse. An anesthetized mouse was placed in a resonator. One min after the injection of MCPROXYL solution (140 mM, 80 μl) to a tail vein, the ESR spectrum was measured in the head (a) and the abdomen (b). The in vivo ESR spectrometer was equipped with an L-band microwave power unit and a loop-gap resonator. Details are described in Section 2.

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