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Separable detection of lipophilic- and hydrophilic-phase free radicals from the ESR spectrum of nitroxyl radical in transient MCAO mice

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Abstract

Free radicals are believed to be key factors that promote ischemia reperfusion injury in the brain. This study used the characteristic spectrum of methoxycarbonyl-PROXYL to detect free radical reactions in hydrophilic and lipophilic compartments in a transient middle cerebral artery occlusion (MCAO) mouse model. Methoxycarbonyl-PROXYL, which has a high water/octanol partition coefficient, allows the detection of nitroxyl radical in both compartments simultaneously. Free radicals generation was analysed from the enhanced ESR signal decay rate of methoxycarbonyl-PROXYL. The signal decay rate in the lipidic compartment was significantly enhanced 1 h after reperfusion following MCAO. The enhanced signal decay rate was significantly suppressed by Trolox. The accumulation of lipid peroxidation products increased by 6 h post-reperfusion and was suppressed by methoxycarbonyl-PROXYL or Trolox. These results demonstrate that information pertaining to different sites of free radical generation *in vivo* can be obtained simultaneously and that lipid-derived radicals are generated in transient MCAO mice.

Keywords: *Nitroxide, EPR, ischemia reperfusion, lipid peroxidation, free radical*

Introduction

Reactive oxygen species (ROS) and free radicals are believed to be important in promoting ischemia reperfusion injury in the brain. ROS are generated by the actions of NADPH oxidase [1] and xanthine oxidase [2]. These species induce lipid peroxidation via lipid-derived free radical generation [3,4], because polyunsaturated fatty acids present in the membrane are particularly susceptible to ROS damage. The lipid peroxidation of cellular membranes is associated with cerebral ischemic damage, such as oedema or infarction [5–8]. In fact, Zini et al. [9] demonstrated that carbon-centred free radical adducts are generated during ischemia and early reperfusion, consistent with the presence of an oxidative attack on membrane lipids. Lipid peroxidation might also be linked with cell death in the developing white matter [10], which contains more total lipid than the gray matter [11]. Furthermore, the plasma level of malondialdehyde is significantly increased in ischemic stroke patients, suggesting that the generation of lipid-derived free radicals might be involved in human disease states [8].

The direct detection of free radical reactions is imperative for clarifying the pathophysiological process of ischemia reperfusion. For this purpose, nitroxyl radicals (i.e. Nitroxide) have been used as spin probes and as antioxidants in a variety of biological experiments, for which electron spin resonance (ESR) spectroscopy is a convenient method of detection. In addition, the macroscopic assessment of tissue damage can be useful for determining the rates

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of ESR signal decay in oxidative stress. Nitroxyl radicals react with $O_2^{\cdot -}$ [12–14] and with •OH [15,16] in the presence of reducing agents. They also react with peroxyl, alkoxyl, and alkyl radicals [17–20], resulting in the suppression of lipid peroxidation in biological systems [19,21–25]. These reactions form the chemical basis for using nitroxyl radicals as spin probes for *in vivo* ESR spectroscopy, to determine ROS generation non-invasively [26–30]. Our results from a previous study using three types of nitroxyl radicals with different membrane permeabilities suggested that ROS are generated *in vivo* at the interface of the cerebrovascular cell membrane in rats with transient middle cerebral artery occlusion (MCAO) [31]. In that case, the enhanced signal decay of 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl (carbamoyl-PROXYL), which was used as the index of ROS generation, was suppressed by superoxide dismutase (SOD). However, direct evidence of *in vivo* free radical generation in the membrane itself has not been obtained, although many reports suggest that lipid peroxidation occurs in the ischemic brain.

Spin probes with a high water/octanol partition coefficient allow the simultaneous detection of nitroxyl radicals in both lipidic and aqueous compartments, depending on the spin probe's specific distribution [31–35]. The simultaneous observation of two compartments in liposomes was reported by Shimshick and McConnell [34]. In liposomes, the spin probe 3-methoxycarbonyl-2,2,5,5-tetramethy lpyrrolidine-l-oxyl (methoxycarbonyl-PROXYL) partitions into both the aqueous and lipidic phases, which can be clearly separated in ESR spectra by using a high magnetic field hyperfine manifold. The ratio of lipidic-to-aqueous components detected increases with an increase in liposomal lipid, indicating that the lipidic signal is due to the probe's localizing to membranes [31]. In our earlier studies of transient MCAO rats with methoxycarbonyl-PROXYL as a spin probe, we first used 300 MHz ESR spectroscopy for detection [31]. We used the centre peak of methoxycarbonyl-PROXYL for analysis, because the large modulation width in the 300 MHz ESR decreased the spectral resolution and distorted the lines at lower and higher magnetic fields. However, the signal decay rate of the centre peak of methoxycarbonyl-PROXYL in the transient MCAO rat did not change compared with that of sham-operated rats. On the other hand, when we used the higher-frequency L-band ESR for detection, we observed that the spectra for methoxycarbonyl-PROXYL showed hydrophilic and lipophilic components at lower and higher magnetic fields in living mice after its intravenous injection, depending on the probe's penetration into the aqueous and lipidic phases [31,32]. Therefore, methoxycarbonyl-PROXYL may enable the *in vivo* analysis of free radical reactions in different locations simultaneously, using L-band ESR for detection.

For effective antioxidant therapy, it is important to clarify where the targeted species are located. Therefore, in this study, we used the characteristic spectrum of methoxycarbonyl-PROXYL to examine the free radical reactions occurring in the hydrophilic and lipophilic compartments in transient MCAO mice. We also evaluated the effect of the water-soluble vitamin E homologue, Trolox, which is a scavenger for lipid-derived radicals, on the *in vivo* free radical generation [36].

Materials and methods

Chemicals

3-Carboxy-2,2,5,5-tetramethylpyrrolidine-l-oxyl (carboxy-PROXYL) was purchased from Aldrich Chemical Co. (Milwaukee, WI). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,6-di-*t*-butyl-4-methyphenol (BHT), 1,1,3,3-tetraethoxypropane (TEP) and thiobarbituric acid (TBA) were purchased from Wako Pure Chemical Industries, Inc. (Osaka, Japan). All other reagents were of the highest purity commercially available.

3-Methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl (methoxycarbonyl-PROXYL) was synthesized from carboxy-PROXYL as described previously [37]. Isotonic solutions containing methoxycarbonyl-PROXYL at a final concentration of 100 mM were prepared by mixing the probe with saline, sterilized by filtration (0.2 μm) and stored at -20°C before use.

Animals

Male ddY mice (9–10 weeks) were purchased from Kyudou Co., Ltd. (Saga, Japan). The mice were housed in a temperature- and humidity-controlled room and fed a commercial diet (MF; Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*. Reversible focal cerebral ischemia was induced with an intraluminal suture to produce a model of MCAO. In brief, anaesthesia was induced with 2% isoflurane (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) in the air and maintained with 1% isoflurane using a facemask. A 1 cm midline incision was made on the anterior neck and the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed. The CCA and ECA were ligated and a suture was placed around the ICA for ligation. An embolus was made by inserting a 6-0 nylon surgical thread into the ICA via a small incision. The MCA was occluded by advancing the embolus into the internal carotid artery to block the origin of the MCA. After 1 h of MCAO, the MCA was reperfused by withdrawing the embolus. The ICA, CCA and ECA of the sham-operated mice were

ligated with a suture, but the nylon thread was not inserted. The rectal temperature was maintained by placing the mouse on a heating pad under the anaesthesia.

All the procedures and animal care were approved by the Animal Care and Use Committee, Kyushu University, and carried out in accordance with the Guideline for Animal Experiments, Kyushu University.

ESR measurements

For the *in vivo* ESR measurements, methoxycarb onyl-PROXYL solution was administered intravenously (0.4 mmol/kg body weight). ESR spectra were taken at regular intervals at the head domain using an L-band ESR spectrometer (JEOL Co. Ltd., Akishima, Japan) with a loop-gap resonator (33 mm i.d. and 30 mm in length), as reported previously [27,38]. The power of the 1.1 GHz microwave was 10 mW. The amplitude of the 100-kHz field modulation was 0.063 mT. The ratio of lipidic-to-aqueous components of methoxycarbonyl-PROXYL in the head region was calculated using each signal height according to the method of Shimshick and McCo nnell [34]. The signal decay rate, which was used as an index of ROS generation, was determined from a semi-logarithmic plot of signal height vs time after probe injection. Trolox (0–0.4 mmol/kg body weight, dissolved in saline) was administered simultaneously with the probe to confirm the relationship between the signal decay of the nitroxyl probe and free radical generation.

In vitro *and* in vivo *lipid peroxidation*

The concentration of thiobarbituric acid reactive substances (TBARS) was measured by fluorometric analysis. *In vitro* effect of antioxidants on copper (5 μmol/L)-induced lipid peroxidation was tested in brain tissue, prepared from normal mice. For *in vivo* experiment, the animals were sacrificed by transcardiac perfusion with heparinized saline 1 h after reperfusion. The brain was homogenized (3:7, W/V) in 1.15% KCl and 5 mM BHT. A 0.1-ml sample of the homogenate was mixed with reagents to give a final concentration of 2 mM EDTA, 7.5% acetic acid and 0.4% SDS and then reacted with 0.3% TBA in a boiling water bath for 45 min. After cooling, the chromogen was extracted in n-butanol:pyridine (15:1, V/V). The concentration of TBARS was calculated from the n-butanol-extracted supernatant at excitation wavelength 510 nm/emission wavelength 550 nm. TEP was used as the standard. Metho xycarbonyl-PROXYL or Trolox (0.4 mmol/kg body weight) was administered intravenously twice: immediately and 3 h after reperfusion.

Quantification of methoxycarbonyl-PROXYL and Trolox in brain

Methoxycarbonyl-PROXYL was injected in the tail vein of mice (0.4 mmol/kg body weight). Brain samples were homogenized with 3-fold volume saline at 5 min after injection. The homogenate solution was mixed with ferricyanide solution (1 mmol/L). Ferricyanide quantitatively oxidizes the hydroxylamine produced as a result of *in vivo* reduction back to the oxidized form [14]. X-band ESR spectra were recorded at room temperature using a JEOL JES-1X ESR spectrometer (microwave power: 5 mW; amplitude of 100 kHz field modulation: 0.063 mT; time constant: 0.03 s; sweep rate: 5 mT/min) and were analysed with an ESR Data Analyser (JEOL Co. Ltd., Akishima, Japan). The spin concentration of sample was estimated from the signal intensity using a calibration curve obtained with aqueous solutions of methoxycarbonyl-PROXYL.

Trolox was also injected in the tail vein of mice (0.4 mmol/kg body weight). Brain tissue was homoge nized in 1:4 (W/V) methanol containing 0.33 mg/mL of BHT at 5 min after injection. This homogenate was centrifuged for 15 min at 20 000 g at 4°C. Trolox was assayed by HPLC coupled to UV detection according to previous reports [39,40]. Separation was achieved with a C18 reverse-phase column (MCM column 150*4.6 mm, MC Medical Inc., Tokyo Japan). The mobile phase contained 10 mM lithium acetate with 30% (v/v) methanol. The HPLC flow rate was 0.5 mL/min. UV detection was carried out with a UV detector (UV-2070 Plus; Jasco Co., Tokyo, Japan) at 290 nm. Trolox concentration of sample was calculated using the standard compound.

Statistical analysis

All of the results are shown as the mean ± SD. Statistical significance was analysed using the two-tailed Student's *t*-test or Dunnett's test. A probability value of 0.05 was set as the minimum level of statistical significance.

Results

In vivo *ESR measurement in transient MCAO mice*

Figure 1A shows the experimental set-up for the L-band ESR spectroscopy. An aqueous solution of methoxycarbonyl-PROXYL was given intravenously to sham-operated or transient MCAO mice and then the *in vivo* ESR spectra of methoxycarbonyl-PROXYL were recorded from the head of living mice. Figure 1B shows typical *in vivo* ESR spectra of methoxycarbonyl-PROXYL obtained from a sham-operated and a transient MCAO mouse. The *in vivo* spectra of methoxycarbonyl-PROXYL had two components: aqueous (open circles) and lipidic (closed circles). The signal from the aqueous component had

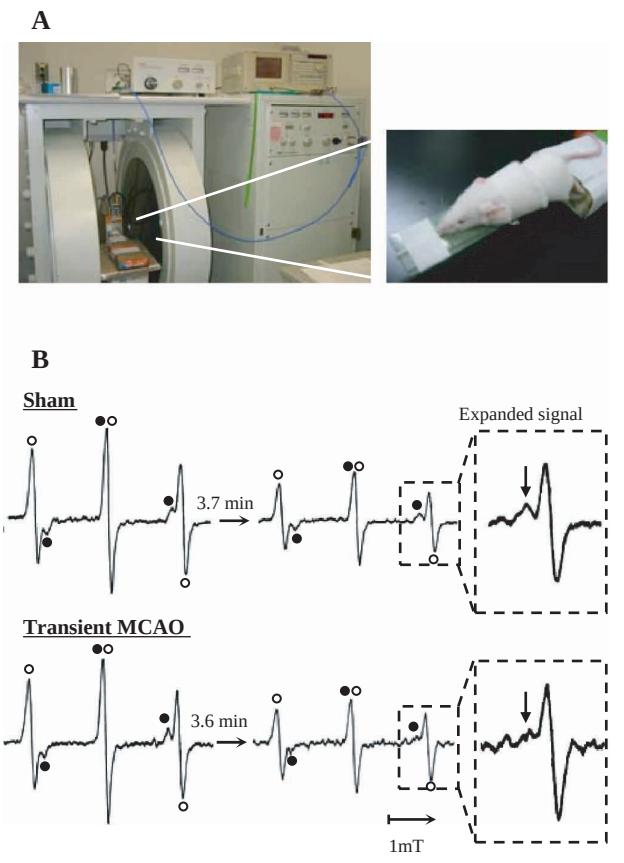

Figure 1. L-band ESR spectroscopy set-up (A) and typical ESR spectra of methoxycarbonyl-PROXYL obtained from the head of a sham-operated and a transient MCAO mouse after intravenous injection of the probe (B). The closed and open circles indicate the lipidic and aqueous components, respectively. Methoxycarbonyl-PROXYL (0.4 mmol/kg body weight) was administered intravenously to mice after 1 h of reperfusion, which followed a 1-h MCAO. Immediately after the injection, the ESR spectra at the head were measured at regular intervals using an L-band ESR spectrometer with a loop-gap resonator.

a hyperfine splitting (hfs) constant of 1.63 mT, indicating that the methoxycarbonyl-PROXYL was tumbling freely in a hydrophilic phase. The hfs constant for the lipidic component was 1.43 mT, which could be clearly separated from that of the aqueous component. The ratio of lipidic-to-aqueous components in the sham-operated mice was the same as in the transient MCAO mice just after injection of the spin probe (sham vs transient MCAO, 22.5 ± 2.3 vs $22.5 \pm 3.6\%$). However, the lipidic signal in the transient MCAO mice decreased more than that in the sham-operated mice at 3.6 min after injection, as indicated with arrows in the dotted square of Figure 1B.

The three points of the *in vivo* ESR spectra, which are the centre, and the aqueous and lipidic components in the head, decreased gradually with time after intravenous injection of the probe (Figure 2A–C). Figure 2 shows typical semi-logarithmic plots of the signal intensities as a function of time after methoxy carbonyl-PROXYL was injected into sham-operated (open circles) and transient MCAO (closed circles) mice. The ratio of lipidic-to-aqueous components shown in Figure 1 for a sham-operated mouse was 20.9%. We multiplied the signal decay rate of the lipidic and aqueous components by the ratio of each component, respectively. The total signal decay rate was ca. 0.0983/min, which was close to the signal decay rate of the centre peak. This result is consistent with the centre peak being composed of lipidic and aqueous components. For the transient MCAO mouse, using the ratio of lipidic-to-aqueous components (21.5%), the total signal decay rate was calculated to be ca. 0.114/min, which was also close to the signal decay rate of the centre signal for this mouse.

The signal decay rates of lipidic and aqueous components in the sham-operated and transient MCAO groups (1 h ischemia and 1 h reperfusion) are shown in Figure 2D. Significant enhancement of the signal decay was observed for the lipidic component of methoxycarbonyl-PROXYL when the mouse groups were compared; the signal decay rate of the transient MCAO group increased more than that of the sham-operated group. However, no significant difference in the signal decay rate was observed for the aqueous component.

In our earlier study, the signal decay rate of the centre peak of methoxycarbonyl-PROXYL did not change in transient MCAO rats compared with sham-operated rats [31]. Here, the signal decay rate of the centre peak in the transient MCAO mice was also not significantly enhanced. These results indicated that the amount of change in lipid component was too small to enhance the signal decay of center peak.

Inhibition of lipid peroxidation in brain homogenates and brain distribution of antioxidants

Trolox, a scavenger for lipid-derived radicals, has an inhibitory effect on *in vitro* lipid peroxidation that is virtually equal to that of vitamin E [41] and it blocks free-radical generation in the brain of transient ischemia rats [42]. Nitroxyl radicals are also known to inhibit lipid peroxidation [22]. In this study, antioxidant potency of methoxycarbonyl-PROXYL against copper-induced lipid peroxidation in rat brain homogenates was similar to that of Trolox (Figure 3A). We determined the brain-tissue concentration of these antioxidants at 5 min after intravenous injection, suggesting their presence in the brain (Figure 3B). These results indicate that methoxycarbonyl-PROXYL or Trolox promise to inhibit the lipid peroxidation in the brain.

Effect of Trolox on enhanced signal decay

We reported previously that the signal decay rate is enhanced in transient MCAO rats and the effect of antioxidants on the enhanced decay rate can be used

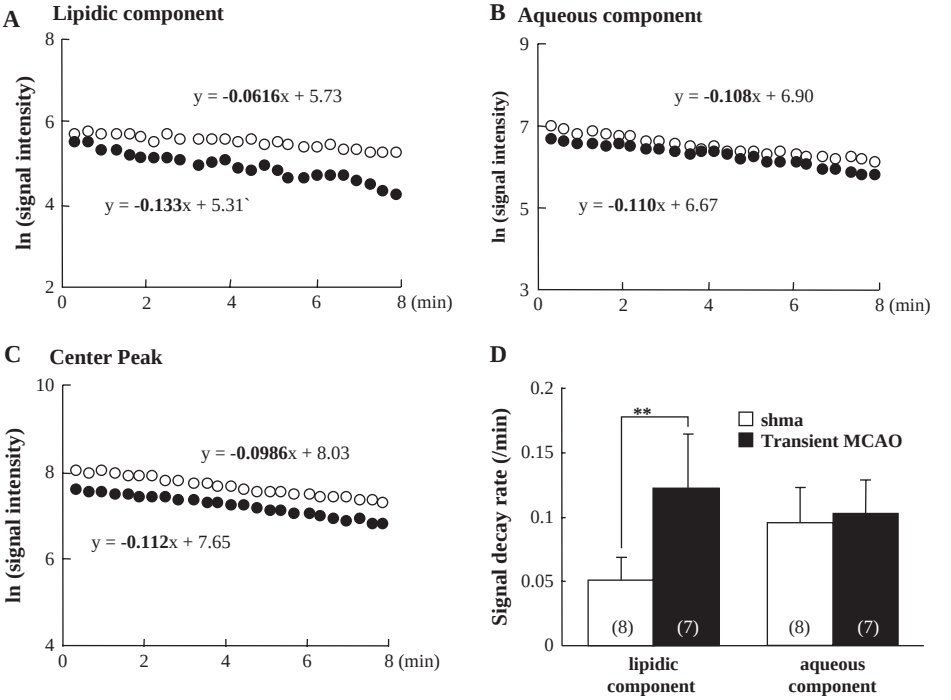

Figure 2. Typical signal decay curves of the lipidic component (A), the aqueous component (B) and the centre peak (C) of methoxycarbonyl-PROXYL in sham-operated and transient MCAO mice and the signal decay rates of the lipidic and aqueous components (D). The open and closed symbols indicate sham-operated and transient MCAO mice, respectively. Each value in (D) represents the mean ± SD. Values in parenthesis are the numbers of animals. ** $p < 0.005$.

to evaluate the role of *in vivo* free radical generation on the ESR signal decay [31]. Therefore, in the present study, we examined the selective effect of Trolox, a scavenger for lipid-derived radicals, on the enhanced decay rate of the lipidic component, to evaluate the generation of lipid-derived radicals. The simultaneous administration of Trolox with metho xycarbonyl-PROXYL completely suppressed the enhanced signal decay rate of the lipidic component (Figure 4A), while the signal decay rate of the aqueous component was unaffected (Figure 4B). This result indicates that the enhanced signal decay of the lipidic component of methoxycarbonyl-PROXYL can serve as an index of lipid-derived free radical generation. In our previous report, the enhanced signal decay of 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-l-oxyl (carbamoyl-PROXYL) was suppressed by SOD, while that of methoxycarbonyl-PROXYL was not (data not shown). Methoxycarbonyl-PROXYL is distributed to the membrane [31] and has good blood–brain barrier permeability [43] compared with carbamoyl-PROXYL. These different PROXYLs might monitor different reactive species.

In vivo *lipid peroxidation after transient MCAO*

The presence of methoxycarbonyl-PROXYL, which we used here to confirm *in vivo* lipid-derived radicals, might suppress the lipid peroxidation in transient

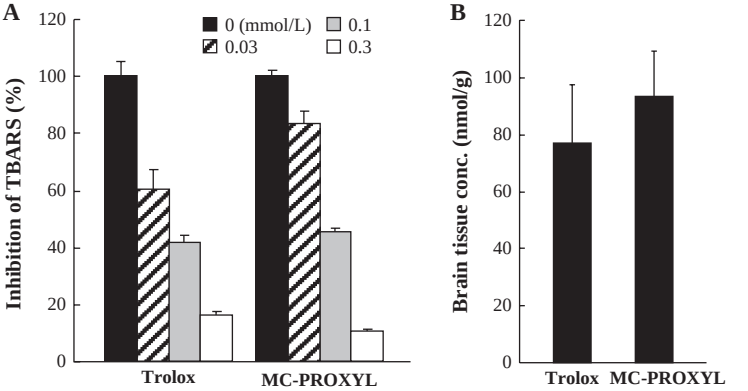

Figure 3. Inhibition of copper-induced lipid peroxidation by Trolox and methoxycarbonyl-PROXYL (A) and brain distribution of these antioxidants (B). Each value represents the mean ± SD. The data were obtained from three samples in (A) or three animals in (B).

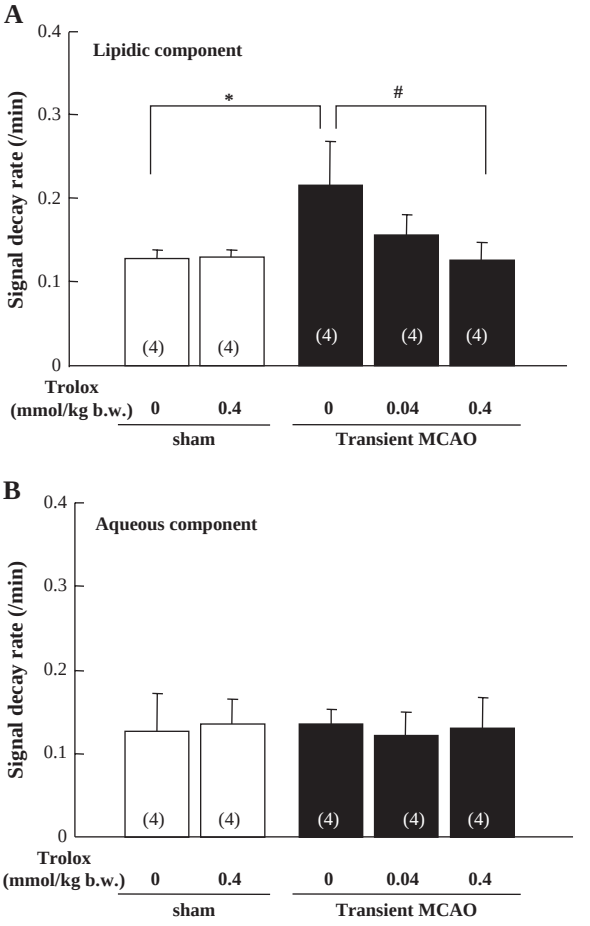

Figure 4. Effect of Trolox on the signal decay rates of the lipidic (A) and aqueous components (B). Each value represents the mean $\pm$SD of four mice. $^{*}p<0.05$ for comparisons of the sham-operated (open columns) and MCAO groups (closed columns); # $p<0.05$ for comparisons of the MCAO and Trolox groups.

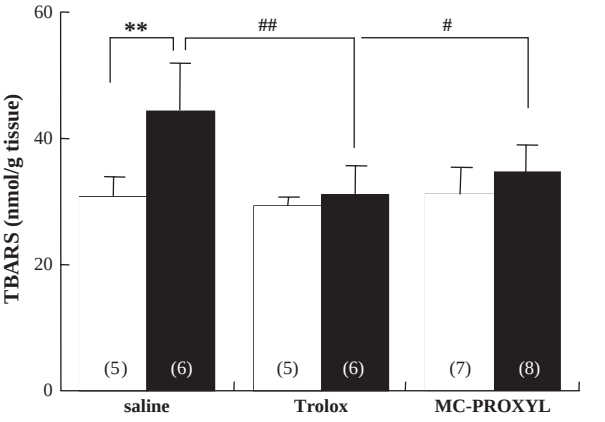

Figure 5. Effects of methoxycarbonyl-PROXYL and Trolox on the TBARS accumulation after transient MCAO. The antioxidants (0.4 mmol/kg body weight) were injected intravenously just after reperfusion following MCAO and the TBARS accumulation was evaluated as an index of lipid peroxidation. Each value represents the mean $\pm$SD. The values in parenthesis are the numbers of animals. $^{**}p<0.005$ for comparisons of the sham-operated (open columns) and MCAO (closed columns) groups; # $p<0.05$ and ## $p<0.005$ for comparisons of the MCAO and antioxidant groups.

MCAO mice. To examine this possibility, lipid peroxidation in the ischemic hemisphere from sham-operated and transient MCAO mice was evaluated in the transient MCAO mice by examining the accumulation of TBARS. The accumulation of TBARS was significantly increased after 6 h of reperfusion (Figure 5), although the level of TBARS was not greatly different between the sham-operated and transient MCAO group after 1 h of reperfusion (sham vs transient MCAO, 30.9 ± 4.7 vs 29.9 ± 4.6 nmol/g tissue). The contralateral hemisphere of transient MCAO mice after 6 h of reperfusion did not show a significant accumulation of TBARS (29.9 ± 3.7 nmol/g tissue). Methoxycarbonyl-PROXYL and Trolox suppressed the accumulation of TBARS. These findings confirm that the lipid-derived radical generation was inhibited by methoxycarbonyl-PROXYL. This observation is consistent with the results shown in Figure 4, in which the enhanced signal decay rate of methoxycarbonyl-PROXYL was inhibited by Trolox. These findings support the idea that the enhanced signal decay of the lipidic component represented the generation of lipid-derived free radicals in the ischemic hemisphere in the *in vivo* ESR experiments, even though the entire head was examined as the region of interest.

Discussion

In this report, we propose a new method for separately analysing free radical reactions in lipidic and aqueous compartments. We also demonstrated that fewer lipid-derived radicals were generated in the brain of transient MCAO mice treated with Trolox, scavengers of lipid-derived radicals.

The lipophilic nitroxyl radical methoxycarbonyl-PROXYL partitions into both aqueous and lipidic compartments *in vitro* and *in vivo* [31,32]. This unique feature allowed us to assess the free radical reactions in the lipidic and aqueous compartments individually and simultaneously in the mouse brain. In this study, lipidic and aqueous components were clearly observed in the *in vivo* spectrum of methoxycarbonyl-PROXYL after its intravenous injection into sham-operated and transient MCAO mice (Figure 1B). Under normal conditions, methoxycarbonyl-PROXYL is transported from the blood into the tissues, converted to its corresponding hydroxylamine by reducing agents such as ascorbic acid and redistributed throughout the body, as both lipidic and aqueous components. In addition to these reactions, the signal decay of methoxycarbonyl-PROXYL may be enhanced by local free radical generation in the lipidic component.

In fact, the lipidic signal of the methoxycarbonyl-PROXYL distinctly decreased at a faster rate than that in the aqueous phase in the transient MCAO

mouse 3.6 min after injection. This enhanced signal decay of the lipidic component was suppressed by Trolox, suggesting that lipid-derived radicals were generated in the transient MCAO mice. The mechanisms of steady-state signal decay are assumed to mainly involve reduction during *in vivo* ESR measurement. In addition, nitroxyl radicals react with alkyl radicals to form the corresponding alkyl radical adduct [17,18]. Hence, the product might also be generated in the transient MCAO mice. One limitation of the *in vivo* ESR technique is that the products generated by reactions between methoxycarbonyl-PROXYL and lipid-derived radicals are unclear, because they lose their paramagnetism. Further studies using more-specific methods are needed to determine the reaction products.

There was a distinct time lag between the free radical reaction and the accumulation of lipid peroxidation products. The accumulation of lipid peroxid ation products, which was evaluated by TBARS, was not apparent after 1 h of reperfusion following MCAO, whereas a significant increase in TBARS accumulation was observed after 6 h of reperfusion. Furthermore, Trolox or methoxycarbonyl-PROXYL suppressed the level of TBARS in the mouse brain after 6 h of reperfusion following 1 h of MCAO. These results indicate that the free radical reaction at lipidic compartments is associated with the accumulation of lipid peroxidation products. Our previous report suggested that the free radical generation at the interface of the cerebrovascular cell membrane was associated with the injury after ischemia reperfusion [31]. Thus, the enhancement of free radical reaction at lipidic compartments may be associated with the injury after reperfusion, because the lipid peroxidation of cellular membrane causes the brain injury [5–8].

In conclusion, we found that using methoxycarb onyl-PROXYL as a spin probe is possible to separate the free radical reactions of the lipidic compartment from those of the aqueous compartment. This study provides important information in support of a useful approach for detecting free radicals of lipidic compartment *in vivo*. The results of this separable analysis of free radical reactions suggest that lipid-derived radical scavengers might be considered as first-line antioxidants for treating the initial phase of ischemia reperfusion injury.

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