*Free Rad. Res. Comms.*, Vol. 19 Supplement, pp. S219-S225
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# *IN VIVO* EPR MEASUREMENT OF RADICAL REACTION IN WHOLE MICE – INFLUENCE OF INSPIRED OXYGEN AND ISCHEMIA – REPERFUSION INJURY ON NITROXIDE REDUCTION –

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*In vivo* EPR measurements were carried out with whole mice to evaluate the influence of inspired oxygen and ischemia-reperfusion injury on spin-clearance of the nitroxide radicals which were administered intravenously or intramuscularly. Nitroxide radicals in head, abdomen, or muscle domains were composed of sharp triplet lines. The peak heights decreased gradually with time. The reduction of nitroxide radicals depended both on the inspired oxygen concentration and on the domains. Femoral ischemia-reperfusion injury also affected spin-clearance of the nitroxide radical in the thigh. The results were discussed with regard to the generation of active oxygen species.

KEY WORDS: L-band EPR, nitroxide radical, active oxygens, ischemia-reperfusion, oxygen, *in vivo*.

## INTRODUCTION

Oxygen is a very important substrate in various biological systems, including energy generation in mitochondria and detoxification of xenobiotics by microsomes. Oxygen depletion causes a wide variety of disorders. On the other hand, hyperbaric oxygen also causes pathological conditions in various tissues. Active oxygen species participate in ischemia-reperfusion injury, and many efforts have been made to detect active oxygen species generated during ischemia-reperfusion. However, these reports were mainly of *in vitro* experiments, and there have been few *in vivo* investigations of the effect of oxygen on metabolism or the generation of active oxygen.

Recently, L-band EPR spectroscopy has developed and it makes possible to measure radical species in the whole animal non-invasively[1-5]. The *in vivo* EPR technique using the nitroxide radical would be very suitable to estimate radical reactions, since the nitroxide radical, which is susceptible to the local redox state and active oxygens, can be observed non-invasively in whole mice. We previously studied spin-clearance of nitroxide administered intravenously[6] and intramuscularly[7], and injected into the lung[8] in whole mice by using *in vivo* EPR. The results were reduction of the nitroxide radical in the hepatic, bladder, muscle and lung domains, and showed that the rate of reduction should depend similarly on the structure of the nitroxide compounds *in vivo* and *in vitro*.

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In the present paper, we examined the effects of inspired oxygen on the metabolism of the nitroxide radical in whole body by using *in vivo* EPR, and found that oxygen should be important to spin-clearance in various tissues. Moreover, we prepared ischemia-reperfusion injury in the femoral muscle of mice by occlusion and reperfusion of the femoral root with a thread. The reduction of nitroxide radicals in the femoral muscle with and without treatment of ischemia-reperfusion was investigated by *in vivo* EPR spectroscopy.

## MATERIALS AND METHODS

Spin labeled compounds, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (amino-TEMPO), 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (hydroxy-TEMPO), and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL), were purchased from Aldrich Chemical Co., and were dissolved in phosphate buffer (pH 7.4). Pentobarbital Sodium was purchased from Dainabot Co. Ltd.

Female ddY mice (3–4 weeks old, 15–20 g body weight) were used throughout this study. Mice were anesthetized by i.m. or i.p. injection of pentobarbital (80 or 125 mg/Kg) and fixed on a hand-made Teflon holder as described previously[6].

For the measurement under different oxygen conditions, a mouse fixed in a holder was placed in a resonator and exposed to an atmosphere of $N_2$-$O_2$ mixture (12, 20 and 80% $O_2$ in $N_2$) for 45 min before EPR measurement. The sterilized solution of spin-labeled compound (hydroxy-TEMPO or carbamoyl-PROXYL, 280 mM, 50 $\mu$l) was injected into a tail vein of the mouse, and immediately after the injection EPR spectra were measured.

The ischemia-reperfusion of a mouse thigh was carried out according to the modified method of Oyanagui *et al.*[9]. Occlusion was done by tieing the base of the femoral muscle with a thread for 20 min, and then followed by reperfusion. 50 $\mu$l of the solution containing spin labeled compound (amino-TEMPO or carbamoyl-PROXYL, 10 mM) were administered to the femoral muscle of mice either one minute after occlusion or one minute before reperfusion. Immediately after the injection, EPR spectra were successively observed with *in vivo* EPR spectroscopy.

EPR spectra from the abdomen and head were obtained with an *in vivo* EPR spectrometer (JEOL, JES-RE-1L or -3L) as described previously[6]. The frequency was 1.2–1.3 GHz and the power was 1.0–2.0 mW. The amplitude of the 100 KHz field modulation was 0.2 mT. The external magnetic field was swept between 44 and 54 mT at a scan rate of 5 mT/min.

## RESULTS & DISCUSSION

### *Effect of Inspired Oxygen on Radical Reaction in Whole Mice*

Figure 1a and b show typical EPR spectra from the abdomen and head of a mouse intravenously administered with hydroxy-TEMPO. Three sharp lines were observed in both domains and their hyperfine splitting was 1.60 mT. The line width ($\Delta H_{msl}$) was about 0.2 mT in both domains, and the oxygen concentration in inspired gas did not affect the line width, suggesting that the oxygen concentration in blood was not greatly affected by inspired oxygen concentration.

The intensity of the EPR signals of hydroxy-TEMPO decreased gradually during

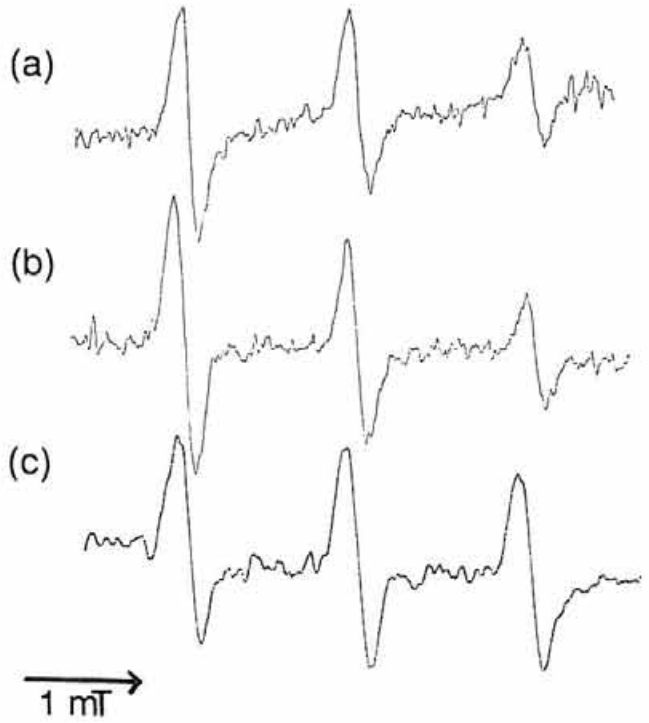


FIGURE 1 ESR spectra of hydroxy-TEMPO in the head (a) and in the abdomen (b) and amino-TEMPO in femoral domain (c) of mouse under 20% oxygen atmosphere. 280 mM of hydroxy-TEMPO or 10 mM of amino-TEMPO solution were intravenously or intramuscularly injected, and the spectra were observed with *in vivo* ESR spectrometer.

a field sweep. This is due to the rapid decay of the paramagnetism during the field sweep (2 min), as described previously[6]. Reduction curves of nitroxide radicals were determined from semilogarithmic plots of the peak heights of the EPR signal ($h(+1)$) in various oxygen concentrations, and clearance constants were calculated from the clearance curves (Table 1).

Most clearance curves of hydroxy-TEMPO were straight lines, indicating that the clearance in both domains should mostly obey first order kinetics. The clearance constants of hydroxy-TEMPO under 80% oxygen were not different from those under 20% oxygen in either domain (Table 1). Under 12% oxygen in the abdomen, however, the clearance constants were significantly larger than those under 20% oxygen ($p < 0.1$). These results suggest that clearance of hydroxy-TEMPO in the abdomen was influenced less by hyperoxia and more by hypoxia.

The spin-clearance constants of carbamoyl-PROXYL under 12% oxygen were significantly greater than that under 20% oxygen in both domains (in abdomen; $p < 0.05$, in head; $p < 0.001$) (Table 1). We previously reported that nitroxide radical loses its paramagnetism more rapidly by the treatment of microsomes under hypoxic condition[10]. In whole body of mice, a hypoxic condition may also favor reduction of nitroxide radicals.

Clearance constants of hydroxy-TEMPO and carbamoyl-PROXYL in the head

TABLE 1
Clearance Constants of Nitroxides under Various $O_2$ Concentration in Whole Mice (/min)

| | Hydroxy-TEMPO | | Carbamoyl-PROXYL | |
|---|---|---|---|---|
| | abdomen | head | abdomen | head |
| 12% $O_2$ | 0.95 ± 0.03 * | 0.69 ± 0.01 | 0.12 ± 0.01 ** | 0.10 ± 0.01 *** |
| 20% $O_2$ | 0.85 ± 0.01 | 0.71 ± 0.01 | 0.10 ± 0.02 | 0.07 ± 0.01 |
| 80% $O_2$ | 0.84 ± 0.02 | 0.71 ± 0.01 | 0.15 ± 0.02 *** | 0.07 ± 0.01 |

Clearance constants are presented as mean ± S.E. over 5 or 6 experiments.
* $p < 0.1$, ** $p < 0.05$, *** $p < 0.001$.

were significantly smaller than those in the abdomen under various oxygen concentrations, which suggests that the mechanism of reduction of nitroxide might differ between the brain and the liver or kidney. Nitroxide reduction systems in cells exist in mitochondria, microsomes, and plasma membrane[11-13]. Clark *et al.* investigated oxygen affinity of mitochondria[14] and demonstrated that the affinity of rat brain mitochondria was five times that of liver mitochondria. The high oxygen affinity of brain might contribute to slow reduction of hydroxy-TEMPO in the head under hypoxia.

It is notable that the clearance constant in the abdomen under 80% oxygen was significantly greater than that under 20% oxygen ($p < 0.001$). The following might be one explanation for this. Active oxygen species such as $O_2^-$, $\cdot OH$, and $H_2O_2$ are reported to be generated in the liver under hyperoxia[15], and the nitroxide radical might loss its paramagnetism by interaction with such active oxygen species, since nitroxide is reported to be reduced by active oxygen[16].

### *Effect of Ischemia-Reperfusion Injury on Radical Reaction in a Mouse Thigh*

Figure 1c shows a typical EPR spectrum of amino-TEMPO in the femoral muscle of a living mouse. The signal of amino-TEMPO decreased gradually with time. The logarithmic value of peak height at lower magnetic field was plotted against time (Figure 2a). Semilogarithmic plots of the peak heights in the thigh untreated, during ischemia, and after reperfusion were linear, suggesting that the clearance of nitroxide radical in femoral muscle should obey first-order kinetics. The clearance constants in the thigh under various conditions were calculated from the corresponding clearance curves (Figure 2b). The constant of amino-TEMPO was the largest in the femur after reperfusion, followed by untreated and ischemic one in this order.

No significant difference was, however, observed in the clearance constants of carbamoyl-PROXYL between the control and after reperfusion. During occlusion the signal hardly decreased (data not shown). Previously, we reported that TEMPO derivatives lose their paramagnetism more quickly than PROXYL derivatives, and that carbamoyl-PROXYL transfers easily from muscle to blood[6,7]. The present results suggest that spin clearance of carbamoyl-PROXYL in femoral muscle should arise mainly from transfer of the compound from muscle to blood, since stable signal was observed during occlusion of thigh.

The clearance constant of amino-TEMPO was the largest in the thigh treated with ischemia-reperfusion, indicating the possibility that amino-TEMPO may be susceptible to radical reaction during ischemia-reperfusion injury. However, the

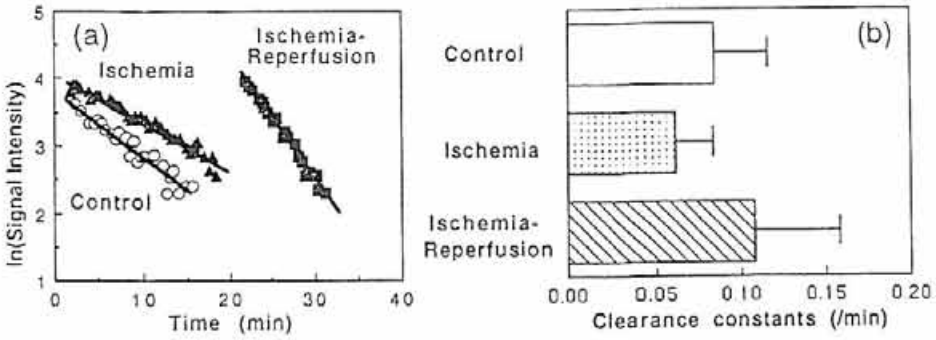


FIGURE 2 Clearance curve (a) and clearance constants (b) of amino-TEMPO in femoral muscle of a mouse with and without treatment of ischemia-reperfusion. (a) Symbols, ○, ▲, and ■ in Figure 2a, indicate the signal intensity of control, during ischemia, and after reperfusion.

difference between the control group and the treated one was not significant, since the deviation of the spin clearance of amino-TEMPO among mice was too large. Thus, we measured the influence of femoral ischemia-reperfusion on the spin clearance with an individual mouse. At first, 50 $\mu$l of amino-TEMPO solution were injected into the left thigh and then EPR spectra were measured till any signal became undetectable. Then the same amount of amino-TEMPO was injected into the right thigh with and without prior treatment of ischemia-reperfusion. Again, the clearance constant of the right thigh was measured, and the ratio of the clearance constant of the right thigh to that of the left one was used to estimate the effect of ischemia-reperfusion on the radical reduction. Table 2 shows the individual clearance constants and the average and S.D. of the ratio with and without treatment of ischemia-reperfusion. The ratio in the group treated with ischemia-reperfusion was significantly larger than that without ischemia-reperfusion ($p < 0.05$). The results suggest that the ischemia-reperfusion of the thigh increased the reduction rate of amino-TEMPO administered into femoral muscle of mice.

We did not confirm the occurrence of edema in the thigh after ischemia-reperfusion, although the femoral muscle was found to become inelastic. Tomasi *et al.* reported the generation of free radicals in femoral muscle of rat after occlusion of femoral vein and following reperfusion, using spin trapping technique[17]. We have not measured the generation of free radicals under the present experimental condition. However, if any free radical is produced in the muscle in our experiment, its reactions may contribute to the radical reduction of amino-TEMPO. We observed in an *in vitro* experiment that amino-TEMPO lost its paramagnetism very quickly by the action of hydroxyl radical and slowly by $O_2^-$ (data not shown). These facts imply that spin-clearance of amino-TEMPO in femoral muscle shown in this paper may reflect the degree of active oxygen generation. We are now studying the effect of antioxidants on the spin clearance of nitroxide radicals in femoral ischemia-reperfusion.

TABLE 2
Effect of Ischemia-Reperfusion in Femoral Muscle on Clearance Constants of Amino-TEMPO

| | Kinetic Constant (/min) | | |
|---|---|---|---|
| | Left | Right | Right/Left |
| Control | 0.149 | 0.124 | 0.832 |
| | 0.047 | 0.033 | 0.702 |
| | 0.115 | 0.045 | 0.391 |
| | 0.225 | 0.137 | 0.609 |
| | 0.245 | 0.112 | 0.457 |
| mean ± S.D. | 0.135 ± 0.089 | 0.084 ± 0.045 | 0.793 ± 0.503 |
| Ischemia Reperfusion | 0.060 | 0.112 | 1.867 |
| | 0.044 | 0.044 | 1.000 |
| | 0.033 | 0.053 | 1.606 |
| | 0.104 | 0.201 | 1.933 |
| | 0.086 | 0.109 | 1.267 |
| mean ± S.D. | 0.065 ± 0.029 | 0.104 ± 0.063 | 1.535 ± 0.397 |



## *Acknowledgements*

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, the Scientific Research Promotion Fund from Japan Private School Promotion Foundation, and Special Coordination Funds of the Science and Technology Agency of the Japanese Government.

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