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IN VIVO ESR STUDIES OF ANTIOXIDANT ACTIVITY ON FREE RADICAL REACTION IN LIVING MICE UNDER OXIDATIVE STRESS

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In vivo antioxidant activity seems to be quite complicate due to multiple interaction with biomaterials and differs from results by *in vitro* experiments. *In vivo* estimation of antioxidant activity is performed by measuring TBA reactive substances in blood or hydrocarbon gases in breath, but these systems do not measure free radical reaction but the final products of oxidative reaction. In the present study, we applied *in vivo* ESR to evaluate antioxidant activity by monitoring the redox reaction of nitroxide radical and clearly found that the nitroxide is very susceptible to oxidative stress *in vivo* and quite useful to evaluate antioxidant activity non-invasively.

KEY WORDS: L-band ESR, nitroxide radical, antioxidant, hyperoxia, *in vivo*, oxidative stress

INTRODUCTION

The effect of antioxidants *in vivo* is difficult to demonstrate, primarily because the appropriate indicator of oxidative stress is required *in vivo* under controlled conditions.[1] Therefore, the efficiency of antioxidant activity tends to be examined by *in vitro* experiments. *In vivo* ESR has been developed to observe free radicals non-invasively in whole body such as nitroxide radical.[2-6] This system is very suitable to investigate *in vivo* redox reaction, since the reduction rate of nitroxide is significantly influenced by oxidative stress such as ischemia-reperfusion,[7] radiation,[8] aging[9] and hyperoxia.[10] Then in this study, we applied *in vivo* ESR measurement to the estimation of antioxidant *in vivo*, and found that this system is very useful to evaluate the antioxidant activity in whole body, non-invasively and on real time.

MATERIALS AND METHODS

Anesthetised female ddY mice (3-4 weeks, 15-20 g body weight) were loaded with the solution of various antioxidants or physiological saline (0.2 ml, *i.p.*), and placed in a *in vivo* ESR resonator, followed by exposed to an atmosphere of N_2-O_2 mix-

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ture for 45 min, as described previously.[10] The sterilized solution of nitroxide compound (CPROXYL; 3-carbamoyl-2,2,5,5-tetramethyl pyrrolidine-1-yloxy, 280 mM, 50 μl) was injected into a tail vein of a mouse, and immediately after the injection ESR spectra were measured in abdomen.

RESULTS AND DISCUSSION

Three sharp lines were observed in abdomen (Figure 1a) and these signals decreased gradually, as described previously.[10,11] We reported that nitroxide radicals are reduced to the corresponding hydroxylamine *in vivo*, resulting in decrease of ESR signals.[9,11,12] It was because ESR signal of nitroxide in collected blood was recov-

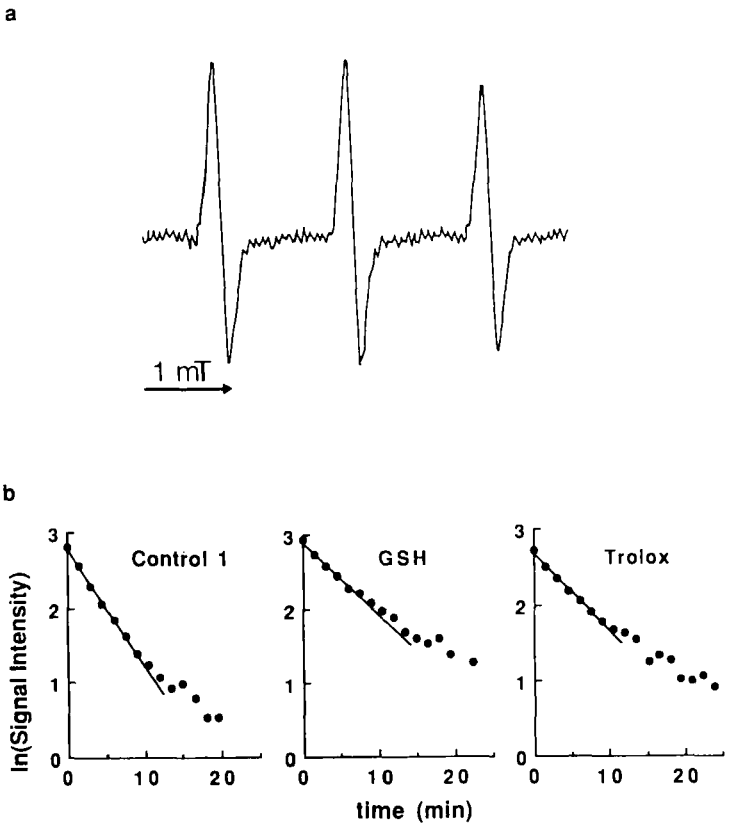

FIGURE 1 *ESR spectrum of CPROXYL in abdomen of a mouse and signal decay curves of CPROXYL under 80% O_2 in N_2 atmosphere.* An anesthctized female ddY mouse was loaded with antioxidant solution, placed in a resonator and exposed to an atmosphere of 80% O_2 in N_2. After 45 min, a solution of spin-labeled compounds (280 mM, 50 μl) was injected into a tail vein of a mouse and the ESR spectrum in abdomen was observed with an ESR spectrometer (JEOL, JES-RE-3L), which was equipped by a loop-gap resonator (33 mm i.d. × 24 mm long). The frequency was 1.3 GHz and the power was 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. **a**. ESR spectrum of CPROXYL in abdomen of a mouse. **b**. Signal decay curves (h(+1)) of CPROXYL in abdomen of mice.

ered by the addition of potassium ferricyanide, which oxidize hydroxylamine to nitroxide. Nitroxide in blood was also recovered by the potassium ferricyanide under hyperoxia (data not shown). Therefore, nitroxide signal decay was also due to reduction of nitroxide under hyperoxia. Signal decay curves of CPROXYL were obtained from semilogarithmic plots of the peak height of the ESR signal at the lower magnetic field (h(+I)) (Figure 1b).

Initial kinetic constants (k_1) were calculated from the slope of signal decay curves (Table I). In Control 1 and 2, the kinetic constants (k_1) under 80% oxygen atmosphere were greater than that under 20% oxygen atmosphere. The result was similar to that of our previous report.[10] It has been reported that hyperoxia induces the formation of oxygen radicals[13-15] and that nitroxide suppresses lipid peroxidation by interaction with oxygen radicals.[16] It is possible that the nitroxide radical should be reduced more rapidly due to the interaction with oxygen radicals under hyperoxia. The reduction rate of nitroxide in whole body was reported to be increased by other oxidative stress such as ischemia-reperfusion in femoral[7] and SOD or alloprinol suppressed the increase of nitroxide reduction rate due to reperfusion.

The effects of various chain-breaking antioxidants on kinetic constants were examined in both 80 and 20% oxygen atmosphere (Table 1). Uric acid, glutathione and ascorbic acid in saline solution were administered intraperitoneally with a dose of 10 mg/kg body weight. Trolox, a water-soluble vitamin E, was dissolved in saline containing ethanol (1.4% v/v) and administered with a dose of 1 mg/kg body weight. It should be mentioned that Trolox, uric acid and glutathione do not interact directly with nitroxide *in vitro* (data not shown).

Under 80% oxygen atmosphere, Trolox, uric acid and glutathione significantly decreased kinetic constants of CPROXYL. Trolox,[17-19] uric acid[17,20-22] and glutathione[17] have been reported to suppress the damage of tissue due to oxidative stress *in vivo* at the dose used for ESR experiments. The present work shows that Trolox, uric acid and glutathione are also capable of suppressing the effects of hyperoxia on the nitroxide reduction. Though ascorbic acid can reduce nitroxide quickly *in vitro*,[23] ascorbic acid administered intraperitoneally at the dose of

TABLE I
Effects of various antioxidants on kinetic constants of spin-clearance for CPROXYL in abdomen under normoxia and hyperoxia

	Dose (mg/kg)	(/min) 20% Oxygen	(/min) 80% Oxygen
Control 1[1]		0.102 ± 0.012	0.138 ± 0.014
Control 2[2]		0.105 ± 0.015	0.132 ± 0.012
Trolox	1	0.106 ± 0.010	0.114 ± 0.015[4]
Uric acid	10	0.109 ± 0.011	0.104 ± 0.021[5]
Glutathione	10	0.115 ± 0.009[3]	0.109 ± 0.014[6]
Ascorbic acid	10	0.105 ± 0.016	0.135 ± 0.008

Kinetic constants are presented as mean ± S.D. over 5 or 6 experiments.
[1] Saline (0.2 ml) administered.
[2] Saline containing ethanol (1.4% v/v, 0.2 ml) administered.
[3] $p < 0.1$, different from Control 1
[4] $p < 0.1$, different from Control 2
[5] $p < 0.01$, different from Control 1
[6] $p < 0.005$, different from Control 1

TABLE II
Effects of various antioxidants on *in vivo* formation of TBA-reactive substances in serum

Samples	Dose (mg/kg)	$Ab_{535\ nm}$ ± S.D.	Ratio
Control (80% O_2)		0.0491 ± 0.008	100
Trolox	1	0.0439 ± 0.011	89.4
Uric acid	10	0.0350 ± 0.009	71.3
Glutathione	10	0.0414 ± 0.008	84.3
Ascorbic acid	10	0.0415 ± 0.003	84.5
Control (20% O_2)		0.0268 ± 0.004[1]	54.6

Anesthetized mice were loaded with 0.2 ml of saline with antioxidants and exposed to 80% oxygen for 45 min. Blood was collected from carotid artery and the serum was obtained by centrifugation at 3000 rpm for 30 min after leaving at 4°C over night. 0.05 N HCl and 0.67% TBA solution were added to serum, and heated at 90°C for 30 min. The reaction mixtures were extracted with 4 ml of 15% methanol/n-butanol and the absorbance at 535 nm of n-butanol layer was measured. [1]$p < 0.005$, different from Control (80% O_2)

10 mg/kg body weight did not show any effect *in vivo* under both 20% and 80% oxygen atmosphere. These results of ascorbic acid agreed with that of liver damage by free radical injury.[17]

Under 20% oxygen atmosphere, antioxidants did not affect significantly on k_1 values, except for glutathione. Only glutathione significantly increased k_1 value in abdomen. Because glutathione injected to mice lung did not affect the reduction rate of nitroxide,[24] nitroxide injected to circulation should be reduced in different manner from that in pulmonary cells, in which nitroxide reduction system should exist in cell membrane.[12]

Table II summarizes the *in vivo* effects of various antioxidants on the formation of TBA-reactive substances in murine serum. Anesthetized mice were loaded with the solution of various antioxidants and exposed to 20% or 80% oxygen atmosphere for 45 min. Blood was collected from carotid artery and the serum was obtained by the centrifugation at 3000 rpm for 30 min after leaving at 4°C over night to complete clot reaction. TBA assay was carried out by the method of Naito and Yamanaka with a slight modification. The reaction mixture was extracted with 4 ml of 15% methanol/n-butanol and the absorbance at 535 nm in n-butanol layer was measured. The result suggested that hyperoxia significantly enhanced the formation of TBA-reactive substances in serum and that uric acid, glutathione, Trolox and ascorbic acid suppressed it. Most results of TBA assay coincided with those of *in vivo* ESR measurement, although ascorbic acid significantly depressed the generation of TBA-reactive substances. TBA assay must measure the final product of lipid peroxidation in serum, while *in vivo* ESR observes the radical reaction related to nitroxide reduction on real time, causing the distinction between the results.

In conclusion, *in vivo* ESR measurement has clarified that some chain-breaking antioxidants suppress really free radical reaction due to hyperoxia *in vivo*. The present system used *in vivo* ESR should be an excellent measurement system of oxidative stress *in vivo*, non-invasively and on real time, compared with the conventional indicator of lipid peroxidation *in vivo* such as the generation of hydrocarbon gases in breath or TBA-reactive substances in blood.

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