Free Rad. Res. Comms., Vol. 19 Supplement, pp. S171–S176
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DETECTION OF FREE RADICALS IN THE BLOOD OF ANIMALS USING EXTRACORPOREAL CIRCULATION

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A method was designed to detect free radicals in circulating blood of animals using extracorporeal circulation and electron paramagnetic resonance (EPR). An arterial-venous shunt was inserted into male Wistar rats between the distal abdominal aorta and the inferior vena cava. The polyethylene tube of the shunt was flowed through a quartz EPR cell. The circulation was supported by manual pumping and maintained at a rate of 3 ml/min. No signal was detected in the blood of untreated rats. After a bolus injection of four spin labels, 2,2,6,6-tetramethyl-piperidine-1-oxyl, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl, 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl, and 3-carbamoyl-2,2,5,5-tetramethyl-pyrroline-1-oxyl, signals from each were detected. The intensity of the signal decreased exponentially and the half time was 23.9, 21.6, 47.1, 42.4 sec, respectively. After administration of ascorbic acid, ascorbyl radical signals were detected. This was enhanced by ethylenediaminetetraacetic acid-Fe or diethylenetriaminepentaacetic acid-Fe but not desferrioxamine-Fe. This assay system is useful in detecting free radicals either administered or generated *in vivo*.

KEY WORDS: Extracorporeal circulation, Electron paramagnetic resonance, Spin labels, Ascorbyl radical.

INTRODUCTION

Free radicals are implicated in the pathogenesis of many diseases. In order to clarify the role of free radicals, it is important to detect them *in vivo*. Extracorporeal circulation is not strictly an *in vivo* condition, but it resembles the *in vivo* circulation to some extent. To enable the detection of free radicals in circulating blood of living animals, we designed a method that made use of electron paramagnetic resonance (EPR) and extracorporeal circulation in combination.

MATERIALS AND METHODS

Chemicals

2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPOL), and 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-oxyl (CPLDO) were purchased from Sigma (St. Louis, MO) and 3-carbamoyl-2,2,5,5-tetramethyl-pyrroline-1-oxyl (CPLNO) was purchased from Aldrich

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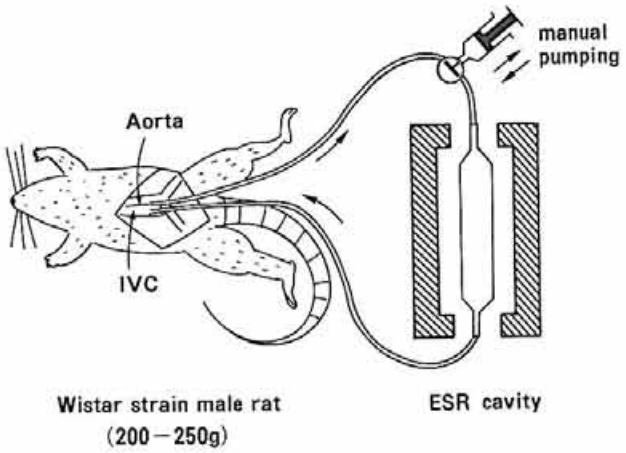

FIGURE 1 The procedure of extracorporeal circulation-EPR.

(Milwaukee, WI). Desferrioxamine mesylate (Desferal®) was purchased from Ciba Geigy (Basel). Other chemicals were obtained from Wako Pure Chemical (Osaka).

Extracorporeal Circulation

Male Wistar rats weighing 200–250 g were anesthetized with pentobarbital and heparinized (200 U/kg). A polyethylene tube was inserted between the distal portion of the abdominal aorta and the inferior vena cava. For EPR studies the shunt was connected to a flat quartz EPR cell, and the circulation was supported by manual pumping at the rate of 3 ml/min. The quartz cell was placed in the cavity of an EPR spectrometer (FR80, JEOL, Tokyo).

Detection of the administered free radicals

Solutions of four spin labels (TEMPO, TEMPOL, CPLDO, CPLNO) in physiological saline at a concentration of 1 mM were injected as a bolus (2.5 ml/kg) into the cervical vein. In some cases, the portal vein or renal arteries were ligated prior to injection. When monitoring the change in signal intensity, the EPR spectrometer was fixed at the peak of the middle signal of the triplet.

Detection of free radicals generated in vivo

The ascorbyl radical was detected in the blood after the administration of ascorbic acid, and the effects of chelated iron loading were examined. A solution of ascorbic acid (0.1 M) in physiological saline was injected into the cervical vein (2.5 ml/kg). The chelating agents used were ethylenediaminetetraacetic acid (EDTA), diethylenetriamine-pentaacetic acid (DTPA) and desferrioxamine. Chelated iron was made by mixing chelating agents and ferrous sulfate in PBS at 10 mM and adjusted the pH to 7.4. Under these conditions, iron is in the ferric state. The chelated iron solutions were then injected as a bolus into the cervical vein. When

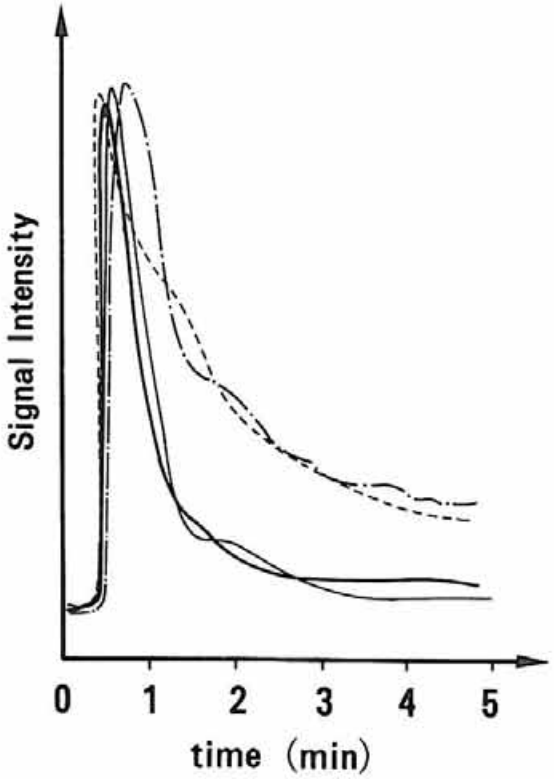

FIGURE 2 The change in signal intensity of the spin labels measured by extracorporeal circulation-EPR. A bolus of the spin labels was injected into the cervical vein. EPR was recorded by fixing the magnetic field at the peak of the middle signal of the triplet. The thick solid line: TEMPO, the thin solid line: TEMPOL, equally dotted line: CPNDO, unequally dotted line: CPLDO.

monitoring the change in the signal intensity, the field was fixed on the low field peak of the ascorbyl radical.

RESULTS

No signal was seen in the circulating blood of untreated rats. After the injection of the spin labels, their corresponding signals were detected. After a lag period, the signals increased rapidly then decreased exponentially (Figure 2). The half decrease times from the peak level of TEMPO, TEMPOL, CPLDO, CPLNO were 23.9 ± 4.3 ($n = 4$), 21.6 ± 0.6 ($n = 2$), 47.1 ± 12.8 ($n = 3$), 42.4 ± 15.8 ($n = 3$) sec (mean $\pm$ standard deviation), respectively. Ligation of the portal vein enhanced the maximal signal intensity several fold, but did not affect the half lives. Ligating the renal arteries affected neither.

The ascorbyl radical was detected only after the administration of ascorbic acid, and EDTA-Fe or DTPA-Fe enhanced the radical concentration but Desferal-Fe did not (Figure 3).

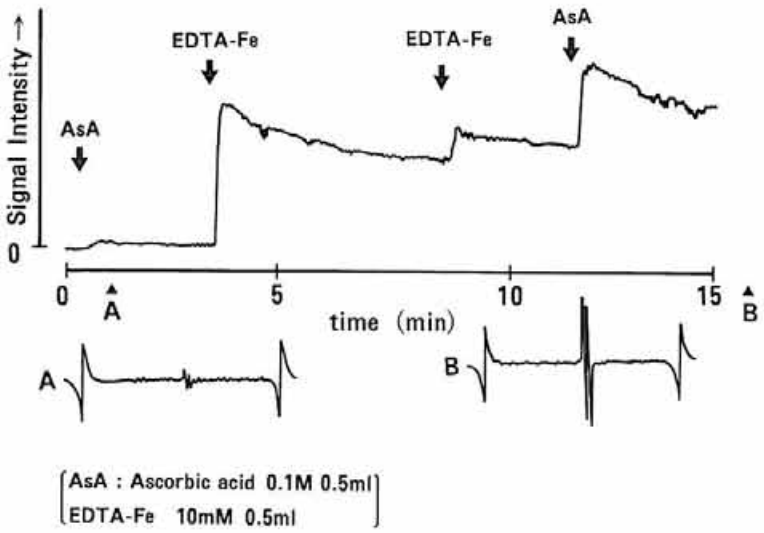

FIGURE 3 *Top:* The change in the signal intensity of the ascorbyl radical after injection of ascorbic acid (0.1 M, 2.5 ml/kg), and EDTA-Fe (10 mM, 2.5 ml/kg). *Bottom:* The EPR signal of extracorporeally circulating blood after the administration of ascorbic acid (left) and EDTA-Fe (right).

DISCUSSION

There are several ways to detect free radicals *in vivo*, including EPR[1]. Recent studies have mostly used L-band EPR[2-7]. In X-band EPR, the quantity of water in the cavity must be limited because of non resonant absorption of microwaves by water. Under these conditions extracorporeal circulation is an excellent method with which to detect free radicals in circulating blood.

Using extracorporeal circulation, we measured the radical concentration after the i.v. administration of stable spin labels. Although the decrease rates of the labels were relatively fast, it was simple to determine the half decrease time because continuous monitoring of the signal intensity was possible with this assay system. The reported half decrease times of spin labels in plasma or organs are longer (min – 10 min order) than in this study (less than 1 min for the four spin labels)[8,9]. There are many factors affecting the half time of aminoxyl radicals, such as blood reducing capacity, cellular uptake, cellular reducing capacity, and renal clearance. The major reason for this discrepancy could be due to the amount of the spin labels administered. We used relatively small amounts (2.5 μmol/kg) compared with other workers (more than 100 μmol/kg).

According to the reports of others, the decrease of these stable radicals *in vivo* is due mostly to reduction and not destruction[10-14]. Although some aminoxyl spin labels are excreted into the urine, this appeared not to be the major cause of the radical decrease in this study, because ligation of renal arteries had no effect on the half decrease time. This discrepancy could be due again to the difference in the amount of spin labels administered. The increase in the maximal signal intensity by ligation of the portal vein is probably due to the decrease in the distribution volume of the agents.

In addition to the administered radicals, the ascorbyl radical which was generated in the body, was also detected and the concentration was monitored. No radical was detected in the blood of untreated rats, but after the administration of ascorbic acid, the ascorbyl radical was found in the circulating blood, and it was enhanced by chelated iron (EDTA-Fe or DTPA-Fe).

It has been reported that the ascorbyl radical is present in human serum and that the level depends on the concentration of ascorbic acid[15]. The same group has also reported that the value for $[AFR]^2/[RAsA][DAsA]$ (where AFR, RAsA and DAsA indicates ascorbyl free radical, reduced form of ascorbic acid, and oxidized form of ascorbic acid, respectively), is constant in healthy people but increases in patients with various diseases[16,17]. Others have indicated that the level of the ascorbyl radical is a function of the rate of oxidation of ascorbic acid and the catalytic metal concentration, so it can be used as a probe for the presence of catalytic metals in buffers or in synovial fluid[18,19]. We speculate that the use of the ascorbyl radical can be used as an indicator of catalytic metals also in the circulating blood.

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