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Hepatic Damage Influences the Decay of Nitroxide Radicals in Mice—An *In Vivo* ESR Study

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To determine the role of the liver in the elimination of free radicals from the body, the clearance rate (K) of nitroxide radicals (Tempol) at the hepatic domain was compared with that at the pelvic domain of live mice, using L-band ESR spectroscopy. The reduction of Tempol in biopsy specimens (liver tissue and femoral muscle) and blood obtained from Tempol-treated mice was also monitored using X-band ESR spectroscopy. Results indicated that the reduction of nitroxide radicals was delayed in both the liver and peripheral tissues when the liver was damaged. The decrease in both blood supply and reductants in the damaged liver might be involved in delaying the reduction in the whole body, because the liver can reduce the radicals supplied via the blood from the peripheral tissues, and the reductants such as reduced glutathione in the peripheral tissues are supplied from the liver.

Keywords: Nitroxide radicals, in vivo ESR, glutathione, liver

Abbreviations: ESR, electron spin resonance; EPR, electron paramagnetic resonance; Tempol, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl; pO_2, partial pressure of oxygen; CCl_4, carbon tetrachloride; GSH, glutathione; ND-CTPO, deuterated 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxy

INTRODUCTION

Free radicals such as active oxygen radicals are thought to be involved in many diseases in which organs are under conditions of oxidative stress.[1] Active oxygen radicals are normally reactive and short-lived, but some relatively stable radicals and lipid peroxides that are secondary produced from active oxygen radicals can be transported to general tissues through circulation. Because the liver is a metabolic center in the body, it is speculated that these radicals may be eliminated by the liver and that the elimination of radicals may be disturbed in severe hepatic damage.

The recent development of L-band ESR (equivalent to EPR) spectroscopy has enabled us to measure free radicals in whole animals.[2–5] Nitroxide radicals such as Tempol are stable in nature. When they are administered to an animal,

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however, they are reduced to the corresponding hydroxylamines which are diamagnetic species and lose their ESR signals.[6] It has been reported that nitroxide radicals are effectively reduced in liver microsomes, mitochondria, and cytosol.[7–12] Other reports have indicated that this reduction was influenced by oxygen concentration.[13,14]

To estimate the role of the liver in the elimination of radicals in the whole body, the clearance of Tempol was measured at both the hepatic and pelvic domains of anesthetized mice using L-band ESR spectroscopy. Furthermore, to estimate the effect of oxygen concentration upon the clearance rate, the pO_2 of the liver and the femoral muscle was measured in vivo using an oxygen electrode.

MATERIALS AND METHODS

Reagents

Tempol was purchased from Wako Pure Chemical Industries, Ltd. Osaka, Japan. Pentobarbital was purchased from Dainabot Co., Ltd. Osaka, Japan. CCl_4, GSH reductase and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Nacalai tesque Co., Ltd. Kyoto, Japan. NADPH was purchased from Sigma Chemical Co., Ltd. St. Louis, U.S.A.

Animals

Male ddy mice weighing 18–20 g were obtained from Japan SLC Co., Ltd. Shizuoka, Japan. The mice were divided into five different groups, each containing at least four mice: 1) Control mice, which received the intraperitoneal injection of olive oil (10 ml/kg b.w.) 48 hours before the experiment; 2) CCl_4-treated mice, which received the intraperitoneal injection of a 20% solution of CCl_4 in olive oil (10 ml/kg b.w.) 48 hours before the experiment; 3) sham-operated mice, which received a midline incision on the abdominal wall; 4) portal vein-ligated mice, which received a 3-0 silk ligature of the portal vein proximal to the bifurcation after a midline incision on the abdominal wall; and 5) mice sacrificed by cervical dislocation. In group 3 and 4, mice were included in the experiment 30 min after the operation. Each mouse was anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), was laid prone and gently taped on a plastic board, and was then inserted into the cavity resonator. Tempol was dissolved in sterilized water at a concentration of 280 mM and Tempol (5 ml/kg) was administered through the tail vein. Maintenance of animals and experimental procedures were carried out in accordance with the guidelines of the Japan Council on Animal Care.

ESR Measurements

The ESR signals from the hepatic and pelvic domains of mice were obtained using an ESR spectrometer (FE2XG, JEOL, Japan) equipped with a hand-made L-band microwave power unit (1.2 GHz) and a loop gap cavity resonator (34 mm i.d. × 6.7 mm long). Typical conditions for the ESR measurements were as follows: Center of magnetic field, 460 gauss; scan speed, 50 gauss/60 sec; time constant, 0.1 sec. The modulation amplitude was adjusted to less than one-third of the line width. Figure 1-a shows the sensitivity curve of the L-band resonator used in present study and Figure 1-b shows the positions of hepatic and pelvic domains of a mouse.

The ESR signals from the liver tissue, muscular tissue, and blood were obtained using an X-band (9.4 GHz) ESR spectrometer (FE2XG, JEOL, Japan). Tissues were cut off from the liver and femoral muscle of anesthetized mice using a needle-like apparatus, soon after injection of Tempol via the tail vein. The blood was collected from the portal vein using an injector, soon after injection of Tempol via the tail vein. The tissue or blood was drawn into a glass capillary

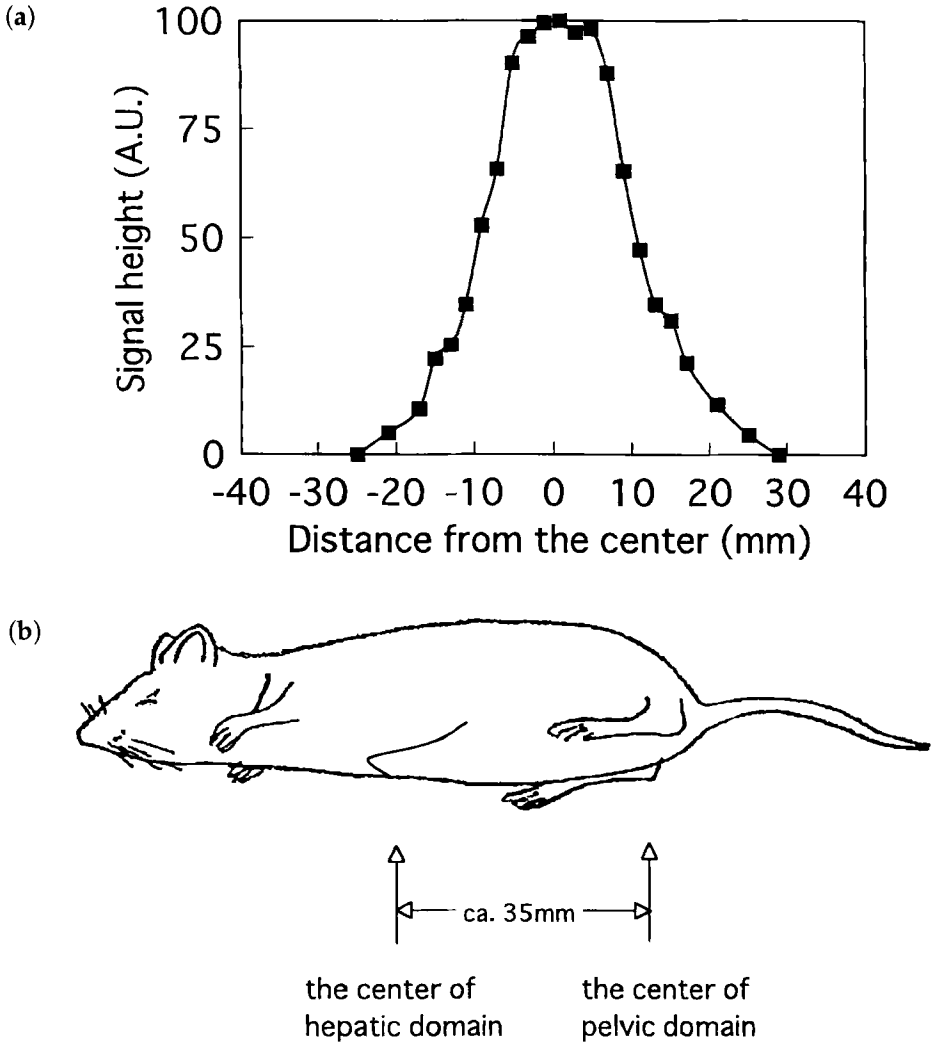

FIGURE 1 (**a**) Sensitivity curve of the L-band loop gap cavity resonator. This data was obtained by sliding a capillary tube, into which 1,1'-diphenyl-2-picrylhydrazyl (DPPH) was drawn at to a length of 1 mm, along a horizontal axis of the cavity. The highest sensitivity was obtained from the area of 10 mm in width. (**b**) The positions of hepatic and pelvic domains of a mouse. The center of hepatic domain was set on the level of xiphoid process and that of pelvic domain, on the level of the root of tail. The distance between the centers of two domains was about 35 mm. Considering the anatomy of a mouse and the sensitivity curve of L-band resonator, it was suspected that the signals from the hepatic domain were mainly originated in the liver, and that the signals from the pelvic domain were originated in femoral muscle, testis, bladder, a part of colon and so on.

tube (1.15 mm i.d.), which was inserted into a quartz ESR tube. The size of sample was fixed at ϕ 1.15 mm $\times$ 20 mm long. The measurement conditions were: Center of magnetic field, 3395 gauss; microwave power, 4 mW; modulation amplitude, 0.63 gauss; scan speed, 50 gauss/60 sec; time constant, 0.1 sec; temperature, 25°C.

pO_2 Measurements

The pO_2 of the liver and femoral muscle were measured using the polarographic oxygen electrode method (pO_2 monitor, INTER MEDICAL, Tokyo, Japan). Briefly, a miniature oxygen electrode was pulled from platinum wire (ϕ 0.2 mm) coated with polyurethane and epoxy. The elec-

trode tip was broken and coated with an oxygen permeable membrane. This oxygen electrode was used in conjunction with an Ag-AgCl reference electrode employing standard polarographic techniques. The oxygen and reference electrodes were calibrated in physiological saline solutions equilibrated with 21% O_2 or 100% N_2 gases, respectively. The oxygen electrode was inserted into the liver or femoral muscle, and the current flow signal was continuously recorded.

GSH Content Measurements

Total GSH contents of the liver and blood plasma were measured by oxidation of DTNB in a reaction mixture containing 0.25 mM NADPH, 0.1 U/ml glutathione reductase and liver homogenate or blood, using a spectrometer (UV-2100S, Shimadzu, Japan) at 405 nm.[15]

Statistical Analysis

Statistical analysis was performed by ANOVA. Differences were accepted as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

A typical ESR signal at the hepatic domain of a control mouse administered Tempol is shown in Figure 2-a. The ESR signal of Tempol, which is composed of three sharp lines of 1.55 mT hyperfine splitting, decreased in height and rapidly

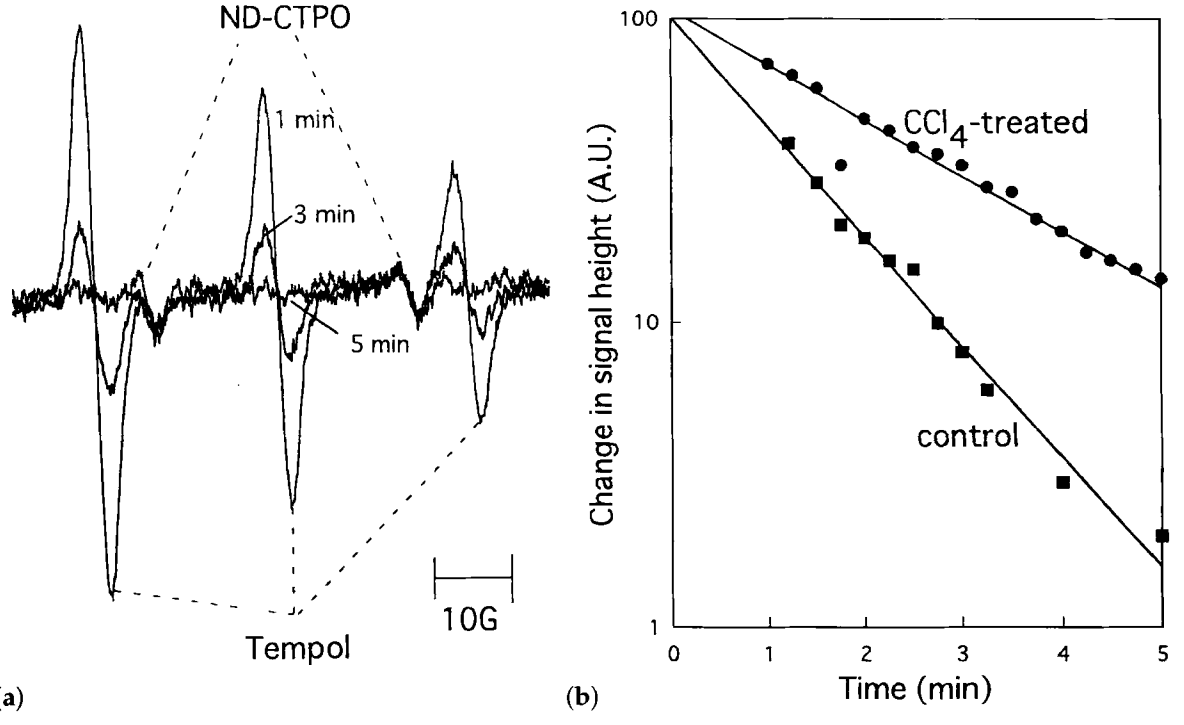

FIGURE 2 (**a**) Change in the ESR signal of Tempol at the hepatic domain of a control mouse. A solution of 1 mM ND-CTPO was drawn into a glass capillary tube that was then sealed at both ends and placed in the resonator as a marker. The ESR signal of ND-CTPO was composed of two sharp lines. The intensity of three lines of Tempol decreased gradually during field sweep. The number *indicates* the time (min) after injection of Tempol. (**b**) A typical decay of the ESR signal of Tempol at the hepatic domain of a control or a CCl_4-treated mouse. To obtain enough data to calculate the kinetic constant, only the middle signals of Tempol were repeatedly recorded.

disappeared. The decay of Tempol obeys first order kinetics, so that a semilogarithmic plot of the peak-to-peak heights of the middle ESR signals is a straight line (Fig. 2-b). The kinetic constant (K) was calculated from its slope.

The K at the hepatic domain was significantly smaller in CCl_4-treated, portal vein-ligated or sacrificed mice than in control or sham-operated mice (Fig. 3). CCl_4 is a well studied hepatotoxin which induces both necrosis of hepatocytes and the disturbance of microcirculation (hepatic ischemia) because of compression of the sinusoids by damaged and swollen cells.[16] Portal vein-ligation also induces hepatic ischemia/hypoxia, which seems to be involved in delaying the reduction of Tempol in the liver. The K at the pelvic domain was almost equal to the K at the hepatic domain in each group (Fig. 3), which indicates that the administered Tempol distributes quickly throughout the whole body and is reduced during the systemic circulation of the blood. In addition, the K at the pelvic domain was smaller in CCl_4-treated or portal vein-ligated mice than in control animals. These data indicate that hepatic necrosis and ischemia influence the reduction of Tempol in the whole body. Because the reduction takes place at the hepatic and pelvic domains of sacrificed mice, it is thought that the reaction doesn't necessarily require blood circulation or ATP.

As it was reported that oxygen concentration influenced the spin clearance of nitroxide radicals in whole mice,[14] the pO_2 of the liver and femoral muscle were measured (Table I). The pO_2 of femoral muscle was markedly higher than that of the liver in both control and CCl_4-treated mice. The pO_2 of the liver decreased but that of femoral muscle remained unchanged in CCl_4-treated mice. These results suggest that factors other than pO_2 may be involved in delaying the reduction at the pelvic domain in CCl_4-treated mice.

The *ex vivo* decay of Tempol in the biopsy specimen and blood which were not influenced by blood circulation or oxygen supply was then

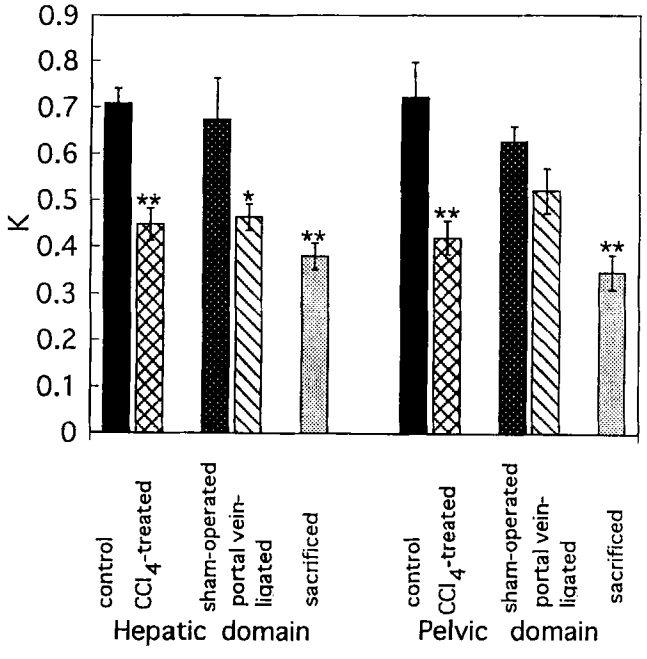

FIGURE 3 The clearance rates (K) of Tempol at the hepatic domain and the pelvic domain of mice *in vivo*. Data are presented as mean ± SEM where N = 4–5. *$p < 0.05$, **$p > 0.005$, compared with the each control value. (CCl_4-treated or sacrificed vs. control, portal vein-ligated vs. sham-operated)

TABLE I The pO_2 of the liver and femoral muscle of mice

	control	CCl4-treated
liver (Torr)	13.2 ± 1.75	3.8 ± 1.72*
femoral muscle (Torr)	52.3 ± 3.86#	52.1 ± 5.25#

Data are presented as mean ± SEM where N = 5 – 10.
*$p < 0.05$, compared with the control value (liver).
#$p < 0.001$, compared with each liver value.

examined (Fig. 4). Tempol was reduced not only in the liver and femoral tissue but also in blood. The K of the liver tissue was higher than that of the femoral muscle in controls, but this difference was not significant. The K of the liver tissue significantly decreased in CCl_4-treated mice, which suggests that the volume of reductants in the liver may decrease when the liver is damaged. Because the K of the blood was about 1/20–1/30 of the K of the liver and femoral muscle, the blood seemed to be involved in part with the reduction of Tempol in the whole body. The K of the blood significantly decreased in CCl_4-treated mice, which suggests that the volume of reductants may decrease in the blood.

Some previous reports have shown that sulfhydryl compounds, such as GSH and cysteine, and ascorbate play an important role in reducing radicals in biological systems.[1,17] Our past study with rat isolated hepatocytes suggested that the decay rate of nitroxide radicals was dependent on the cytosolic water-soluble antioxidant content.[18] Because the liver is the major source of plasma GSH for other organs,[19] the depletion of hepatic GSH may result in GSH depletion in other tissues. In fact, the present study showed that the GSH content significantly decreased in the liver and blood plasma of CCl_4-treated mice (Table II). Furthermore, when 1 μ mol of reduced GSH was added to 1 ml of 2.8 mM Tempol, the Tempol signal was immediately reduced (data not shown). These findings suggest that the decrease in GSH may be

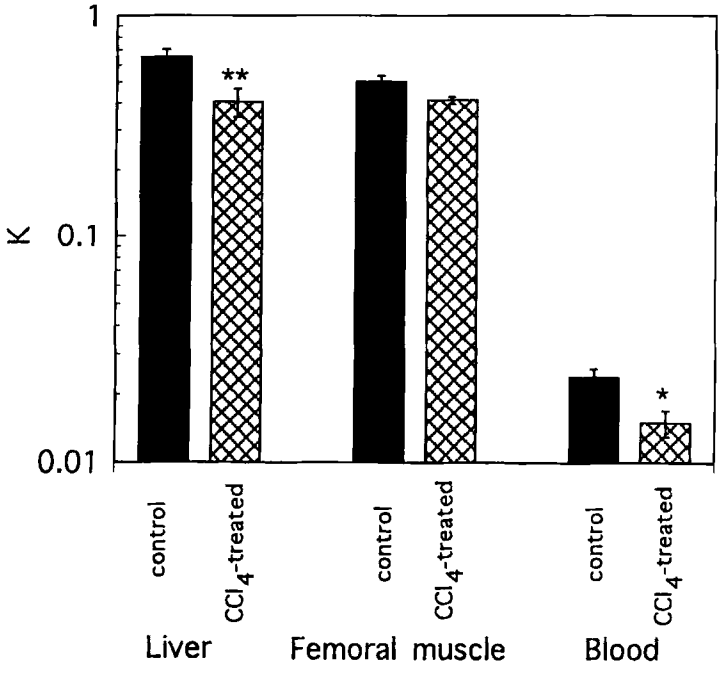

FIGURE 4 The clearance rates (K) of Tempol in biopsy specimens and blood of mice. Data are presented as mean ± SEM where N = 4–6. *$p < 0.05$, **$p < 0.005$, compared with the each control value.

involved with the delay of the reduction in both the liver and the blood.

In conclusion, the present *in vivo* ESR study clearly revealed that the nitroxide radical was able to be reduced not only in the liver but also in peripheral tissues of mice, and that the reductions in both the liver and peripheral tissues were delayed in animals with liver damage. One explanation for the delay in the reduction in peripheral tissues (organs) of liver-damaged animals is that the decrease of hepatic blood flow (hepatic ischemia) might diminish the supply of Tempol to the liver from peripheral tissues. Alternatively, the decrease in hepatic reductants might diminish the supply of reductants such as GSH to peripheral tissues from the liver.

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