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Original Contribution

EFFECT OF HYDROGEN PEROXIDE IN REDOX STATUS ESTIMATION USING NITROXYL SPIN PROBE

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Abstract—A procedure for estimating in vivo redox status using EPR and a hydrogen peroxide (H_2O_2)-dependent spin probe method is described. The mechanism of decreasing spin clearance in the selenium-deficient (SeD) rat is discussed. The in vivo decay constant of the nitroxyl spin probe in the liver region of SeD rats appeared to be slightly lower that of the selenium-adequate control (SeC) group, and was significantly smaller than that of normal rats. Bile H_2O_2 levels in normal rats were significantly lower than those in SeD rats. The in vivo decay constant of the spin probe in SeD rats depended on the bile H_2O_2 level. Furthermore, H_2O_2 was detected in the bile in all SeD rats, whereas bile H_2O_2 could be detected in only half of the normal rats. It was found that the in vivo decay constant of the spin probe in normal rats also depended on whether bile H_2O_2 was detected or not. In vivo decay constants were smaller in rats subjected to the surgical operation than in the nonoperated groups. The EPR signal of the nitroxyl radical in the liver homogenate was increased by addition of H_2O_2, which was administered 30 min before the rat was killed. It appears that H_2O_2 can oxidize the hydroxylamine formed following reduction of the spin probe in the liver.

Keywords—Spin clearance, Nitroxyl radical, Electron paramagnetic resonance, Selenium deficiency, Free radicals

INTRODUCTION

Reactive oxygen species (ROS) such as hydroxyl radical ($^{\bullet}OH$), the superoxide anion radical ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2) can cause several disorders such as inflammatory disease, cancer, cardiovascular disease, Alzheimer's disease, and aging. However, the detection of ROS and free radicals in the living body is difficult because ROS and/or other free radicals are highly reactive. In vivo EPR spin probe methods using nitroxyl spin probes to measure redox status in vivo are currently being investigated [1], due to the long half-life of these probes which allows for their detection using EPR.

The first in vivo EPR experiment done by Feldman et al. [2] used TEMPOL as the spin probe and an X-band EPR with helical coil-type detector surgically implanted into the rat liver. After development of the L-band in vivo EPR spectrometer/imager [3], nitroxyl radicals were widely applied to redox monitoring of living mice and rats [4–8]. When the nitroxyl spin probe is administered to a living animal, in vivo EPR signal intensity of the probe is decreased by one-electron reduction of the probe to the corresponding hydroxylamine. The decay rate of EPR signal intensities in a part of an animal is affected by several factors such as distribution, excretion, enzymatic/chemical one-electron reduction of nitroxyl radical to the corresponding hydroxylamine, and reoxidation of the hydroxylamine to the nitroxyl radical [1]. The in vivo decay constant of the nitroxyl radical was found to increase with additional oxidative stress such as ischemia–reperfusion [9], X-ray irradiation [10], streptozotocin-induced diabetes [11], iron overload [12], and radical generation in the lung by diesel exhaust particles [13]. Thus, increase in the in vivo decay constant is attributed to the generation of ROS such as $O_2^{\bullet -}$ and $^{\bullet}OH$. However, the in vivo decay constant decreased in animal models of aging [14] and selenium (Se) deficiency [15].

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In those studies, the decrease has been attributed to depletion of the total reducing capacity in those models rather than to the ROS generated.

We found that the in vivo decay constant of the nitroxyl spin probe in Se-deficient rats significantly decreased compared with normal rats [15]. We also reported that the bile H_2O_2 levels of Se-deficient rats (8 weeks old, male) were significantly higher than those of normal rats [16]. Se is the active center of glutathione peroxidase. Therefore, Se deficiency decreases the ability to reduce H_2O_2 and, consequently, increases the amount of H_2O_2 in the bile. It is believed that Se deficiency exposes the animal to oxidative stress by H_2O_2.

Under oxidative conditions, the hydroxylamines, i.e., reduced forms of nitroxyl radicals, are reoxidized to the paramagnetic state and can be detected by EPR [17]. Although H_2O_2 does not directly reoxidize hydroxyl amines, H_2O_2–mediated oxidation to nitroxyl radical was observed in the presence of transition metal ions such as Cu^{2+} and Fe^{3+} [18]. Se deficiency should result in reoxidation of once-reduced nitroxyl spin probes and, consequently, will decrease the rate of decay of the in vivo EPR signal. In this study, the decay rates of the in vivo EPR signal of a nitroxyl spin probe are measured and compared among groups of rats with different bile H_2O_2 levels. A H_2O_2-dependent spin probe method is described to provide evidence for the increased rate of reoxidation resulting from accumulation of excess H_2O_2 caused by Se deficiency in rats.

MATERIALS AND METHODS

Materials

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was purchased from Labotec Company, Ltd. (Tokyo, Japan), and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (carbamoyl-PROXYL: 3CP) from Sigma Chemical Company (St. Louis, MO, USA). Other materials used were of analytical grade. The Se-deficient powder diet was purchased from Oriental Yeast Company, Ltd. (Tokyo, Japan). The Se content of the Se-deficient diet was reported in our previous paper as 0.017 mg/kg by instrumental neutron activation analysis [19]. Deionized, ultrapure water (deionization by the Milli-Q system) was used in all experiments.

Animals

Wistar rats were obtained from Japan Laboratory Animals, Inc. (Tokyo, Japan). Eight week old male (body weight (mean ± SD) = 222.3 ± 7.4 g, $n = 28$) rats were used as normal rats in the present experiments. Mother rats that were in their 15th day of pregnancy were fed the Se-deficient diet and ultrapure water. Baby rats were fed by their own mothers until weaning at 4 weeks of age. After weaning, they were fed the Se-deficient diet and ultrapure water until commencement of the experiments. Eight week old male (127.8 ± 20.5 g, $n = 49$) rats were used in experiments on Se-deficient (SeD) rats. Se control (SeC) rats were bred identically to SeD rats except that Na_2SeO_4 (0.1 ppm of Se) was added to their ultrapure water. Eight week old male rats (136.2 ± 19.8 g, $n = 21$) were also used for these experiments.

Measurements of liver selenium content

Rats were anesthetized with Nembutal (50 mg/kg body wt, intraperitonealy) according to previous articles [15,16]. The rats were then sacrificed by bleeding from the abdominal aorta. Subsequently, livers were perfused with ice-cold physiological saline (0.9% NaCl), removed, and homogenized with a fourfold volume of physiological saline. Rat livers were freeze-dried and then pulverized. One hundred milligrams of the pulverized liver was sealed in a quartz tube for analysis. Standard Reference Material 1577 (bovine liver) obtained from the National Institutes of Standards and Technology (Gaithersburg, MD, USA), was used to check analyses of inorganic elements. Contents of selenium and other inorganic trace elements in dried rat liver samples were assayed by means of instrumental neutron activation analysis. Neutron irradiation was carried out in the D pipe of the JRR-4 nuclear reactor at Japan Atomic Energy Research Institute (Tokai, Japan). γ-Ray spectra of irradiated samples were measured with a high-purity germanium semiconductor detector equipped with a multichannel analyzer (Seiko EG&G Co., Ltd., Matsudo, Japan). Se content of rat liver was analyzed by the photopeak at 264.7 keV [19].

Measurements of liver glutathione peroxidase (GSH-Px) activity

Rats were killed by decapitation. The livers were perfused with ice-cold physiological saline, removed, and homogenized with a fourfold volume of ultrapure water. GSH-Px activity in liver homogenate was measured based on the method described by Paglia and Valentine with some modifications [20].

Measurements of in vivo spin clearance

Rats were anesthetized with Nembutal (50 mg/kg body wt, intraperitoneally). Then, 0.3 μmol/g body wt of a 300 mM aqueous solution of 3CP was injected into the tail vein of rats, and EPR signals of the spin probe were measured in the upper abdomen (liver part) with a low-frequency (300 MHz) in vivo EPR spectrometer (JEOL, Tokyo, Japan) [15]. The central peak of the triplet was observed repeatedly for 10 min with 14 to 15 s intervals. EPR conditions were as follows: microwave frequency,