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Electron paramagnetic resonance decay constant and oxidative stresses in liver microsomes of the selenium-deficient rat

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Abstract

The free radical-reducing activity and the membrane fluidity of liver microsomes from selenium-deficient (SeD) rats were examined by means of electron paramagnetic resonance (EPR) spin label method using nitroxyl-labeled stearic acids. Our findings show that the membrane fluidity and lipid peroxidation levels in SeD rat liver microsome were relatively unchanged compared with normal rat. In contrast, SeD caused the induction of liver microsomal cytochrome P-450 activity. The nitroxyl spin probes are substrates for reduction-relating cytochrome P-450. Previous in vivo studies suggested that the total liver free radical reduction activity in SeD rat was decreased. In contrast, SeD caused the induction of liver microsomal cytochrome P-450 activity, and the reduction rate of nitroxyl radical existing at shallow depth in membrane was increased. Selenium-deficient rats experienced an increase in hydrogen peroxide (H_2O_2) due to a pronounced loss of glutathione peroxidase (GSH-Px) activity. This masked the overall reduction rate of the nitroxyl spin probe by reoxidation of the hydroxylamine form. Although the SeD condition caused induction of liver cytochrome P-450 and chronic increased H_2O_2, this did not result in oxidative liver damage. An increased level of glutathione in SeD liver was also evident, likely due to the absence of GSH-Px activity. Using the EPR spin label method, we have shown that SeD causes complicated redox changes in the liver, notably, alterations in the levels of cytochrome P-450 and GSH-Px systems.

Keywords: Nitroxyl radical; Order parameter; Cytochrome P-450; Glutathione; Glutathione peroxidase; Hydrogen peroxide

1. Introduction

Selenium (Se) is an essential metal derived solely from dietary sources that forms the active center of several important enzymes, and 25 selenoproteins have been identified [1]. Se in mammals is in, primarily, glutathione peroxidase (GSH-Px), which reduces hydrogen peroxide (H_2O_2) using glutathione (GSH) as the cofactor. Feeding rat on a Se-deficient (SeD) diet reduces tissues' Se levels, notably in liver, in accordance with the Se contents of the diet [2] and/or the feeding period [3]. Selenium-deficient diet results in decreased GSH-Px activity and oxidative stress due to the increasing level of tissues' H_2O_2 [4]. Selenium-deficient diet exposure from a pregnant mother gives her children markedly low Se level from a relatively young age [5,6]. Severe SeD might influence not only GSH-Px, but also other selenoproteins with unknown functions.

In a previous work, the decay constant of a nitroxyl spin probe determined by means of in vivo electron paramagnetic resonance (EPR) spectroscopy in the SeD rat upper abdomen (liver region) decreased compared with the normal rat [7]. The decay constant was concluded as a decreased total reducing capacity by enzymatic free radical reducible systems. However, the decrease of the in vivo EPR decay constant of the nitroxyl spin probe was complicated by the reoxidation of hydroxylamine to the original nitroxyl radical due to increased levels of H_2O_2 [8]. The contribution of enzymatic free radical reducible systems to the in vivo EPR decay constant of the nitroxyl spin probe is still under investigation.

H_2O_2 is a membrane-permeable molecule and a relatively strong oxidant, in particular, the membrane lipids. The peroxidation of the membrane phospholipids results in the formation of double bond on the carbon chain of the lipid. Generally, the increasing number of double bond on the phospholipids' carbon chain activates the microdynamics

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and/or movement of membrane phospholipids' carbon chain by decreasing the order of the lipid carbon chains. A similar effect can be obtained by incorporating cholesterol molecules into the membrane. Oxidative stress in the cell membrane could cause the lipid peroxidation and increase micromovement of membrane phospholipids.

The purpose of this paper is (a) to clarify the relation of Se deficiency to in vivo and in vitro EPR signal decay of nitroxyl spin probe and (b) to determine the vestiges of in vivo H_2O_2 generation in the liver. Electron paramagnetic resonance spin probe method using nitroxyl spin-labeled stearic acid can estimate the membrane fluidity as obtaining order parameter and/or correlation time [9] simultaneously with the reduction of the nitroxyl radical. The EPR spectrum of the spin-labeled stearic acid incorporated in lipid membrane gives anisotropic EPR spectrum depending on the restriction of carbon chain movement. In this study, we examined the reducing activity and the membrane fluidity of SeD rat liver microsome using nitroxyl-labeled stearic acids and correlated the lipid peroxidation.

2. Materials and methods

2.1. Materials

Phosphatidylcholine (PC) was purchased from Funakoshi (Tokyo, Japan). 5-(*N*-Oxyl-4′,4′-dimethyloxazolidine)-stearic acid (5-SLS), 12-(*N*-oxyl-4′,4′-dimethyloxazolidine)stearic acid (12-SLS) and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (3-CP) were purchased from Sigma (St. Louis, MO). Selenium-deficient powder diet was purchased from Oriental Yeast (Tokyo, Japan). Deionized water (deionization by the Milli-Q system) was used for all experiments. Other materials were in analytical grade.

2.2. Animals

Wistar rats (15th day after pregnancy) were fed with Se-deficient diet and ultrapure water. Newly born rats have been kept with their own mother rat for 4 weeks. Then, the young rats were weaned and fed with SeD diet and ultrapure water (Milli-Q water) until the start of the experiments (SeD group). Healthy Wistar rats purchased from Japan Laboratory Animals (Tokyo, Japan) were used as the normal group. Six-week-old female rats were used for experiments. The body weight of SeD and normal group at 6 weeks of their age is 81.6±18.5 g (n=25) and 119.4±4.4 g (n=10), respectively. (Values are indicated as mean±S.D., and n indicates the number of rats.) The animal experiments were carried out in compliance with the *Guidelines for Animal Care and Use* at Showa Pharmaceutical University (2001) and approved by the Ethical Committee for Animal Care and Use of Showa Pharmaceutical University.

2.3. Preparation of microsome suspension

Five rats from each group were fasted for 24 h before the experiment and sacrificed by decapitation. The liver was perfused with ice-cold physiological saline (0.9% NaCl) until the blood was sufficiently removed. The liver was removed and homogenized with a fourfold volume of 1.15% KCl in ice. After precentrifugation (9000×g for 15 min, twice) to remove nuclear and mitochondrial fraction, a microsomal fraction was separated by ultracentrifugation (100,000×g for 60 min, twice) of the supernatant. The microsomal fraction was suspended by a twofold volume of PBS (pH 7.4) and kept in −80°C until the start of the experiment. The protein concentration of the microsomal suspension was measured using Bio-Rad Protein Assay Kit (Bio-Rad laboratories, Hercules, CA). The protein concentration of the microsomal suspension was adjusted to 0.27 mg/ml before the experiment.

2.4. Preparation of PC suspension

A 1.0 ml *chloroform* solution (7.5 mM) of PC was added into two *glass* test tubes and evaporated until dryness. One test tube was flushed by nitrogen gas, air sealed tightly and kept in a −30°C refrigerator. Other tubes were kept at room temperature with an open lid overnight in the dark to mildly peroxidize the PC (PC-OOH). PBS (500 μl, pH 7.4) was added into both test tubes and vortexed hardly until lipid layer completely comes off the surface of the test tubes. The PC suspension was immediately used for the EPR experiment below.

2.5. Electron paramagnetic resonance spin label spectroscopy

An aliquot (40 μl) of 0.65 mM methanol solution of each 3-CP, 5-SLS and 12-SLS was added into a *polyethylene* microtube and then evaporated until dry. The 3-CP was used only for time course study with microsomal sample. Rat liver microsomal suspension or PC suspension (30 μl) was added into the microtube. The microtube was vortexed hardly for at least 2 min to incorporate the spin-labeled stearic acid into lipid membrane. Twenty microliters of the spin-labeled PC suspension was drawn into glass capillary tube, and the EPR spectra were measured by X-band (9.4 GHz) EPR spectrometer (RE-1X, JEOL, Akishima, Tokyo, Japan). For microsomal sample, 5 μl of 0.1 mM NADPH was added into spin-labeled microsomal suspension, the tube was vortexed, the microsomal suspension was drawn into glass capillary and the X-band EPR spectra were measured repeatedly with 30~60 s time interval. Order parameter (S, unitless) and correlation time (τ, in nanoseconds) were calculated for 5-SLS- and 12-SLS-labeled samples, respectively. The methods to calculate S and τ were well summarized in a text book [10]. For microsomal sample, time course of center signal height (logarithmic value) was plotted with time, and the decay constant was estimated. The EPR conditions were as follows: microwave frequency was 9.4 GHz, microwave power was 10 mW, magnetic field modulation frequency was 100 kHz, field modulation width was 0.1 mT, field sweep ratio was 5 mT/min and time constant was 0.3 s.

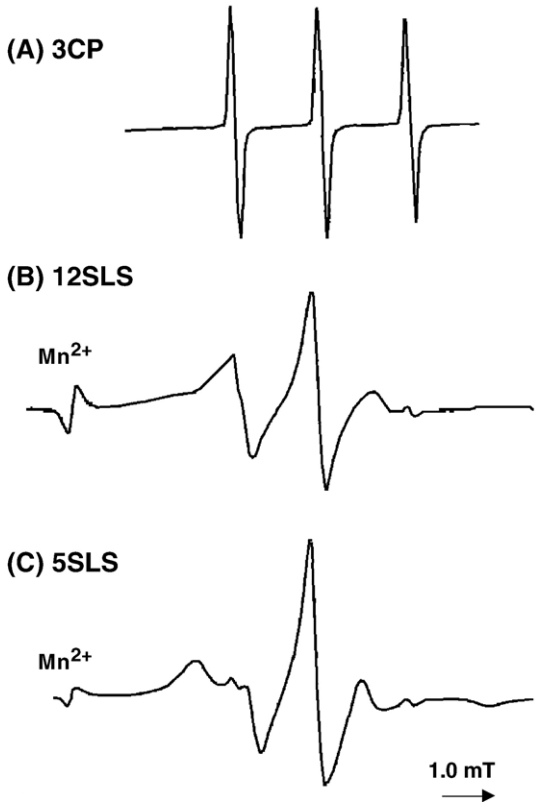

Fig. 1. X-band EPR signal of several nitroxyl spin probes in a microsomal suspension. (A) 3-CP, (B) 12-SLS and (C) 5-SLS. Electron paramagnetic resonance conditions are as follows: microwave frequency, 9.4 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation width, 0.1 mT; field scan rate, 5 mT/min; time constant, 0.03 s.

2.6. Cytochrome P-450 activity

The cytochrome P-450 activity in the microsome was measured according to a method described by Omura and Sato [11]. The cytochrome P-450 activities were standardized with protein concentrations and expressed as nanomoles per milligram protein. The protein concentration of the microsomal suspension was measured using Bio-Rad Protein Assay Kit (Bio-Rad laboratories).

Table 1
Order parameter (S) and correlation time (τ) of 5-SLS and 12-SLS spin probes incorporated in liposome or rat liver microsome and TBARS level of each lipid assembly

	S of 5-SLS	τ of 12-SLS	TBARS (nmol/mg PC or nmol/mg protein)
PC liposome	0.47±0.00 (4)	1.31±0.11 (5)	0.39±0.52 (3)
PC-OOH liposome	0.49±0.06 (3)	1.27±0.08 (4)	1.93±0.90 (3)
Normal rat liver microsome	0.54±0.00 (2)	1.73±0.02 (2)	954±180 (3)
SeD rat liver microsome	0.56±0.01 (2)	1.57±0.01 (2)	955±32 (3)

Values are indicated by mean±S.D. Values enclosed in parentheses are the number of measurements. The order parameter (S) is unitless. The correlation time (τ) is expressed in nanoseconds.

2.7. Glutathione peroxidase activity and GSH content

A portion (50 μl) of the liver homogenate was used as a sample. The liver homogenate was diluted by water until 250- to 1250-fold. The GSH-Px activity in liver homogenates was determined by a modified version of the method of Paglia and Valentine [12]. The GSH amount in liver homogenates was determined by the method of Griffith [13] with some modification.

2.8. TBARS levels

The TBARS levels of the liver homogenates, the liver microsome and the liposome suspensions were determined according to the method of Ohkawa et al. [14] with some modifications.

3. Results

The 3-CP in microsomal suspension showed an isotropic triplet line EPR signal (Fig. 1A). The 12-SLS (Fig. 1B) in microsomal and PC liposomal suspension showed weak anisotropic, and the 5-SLS (Fig. 1C) showed relatively large anisotropic EPR signal of nitroxyl radical. The EPR signal intensities of those spin probes were decreased in microsomal suspension with time after addition of NADPH. Notable line width change was not observed during time course measurements for any nitroxide probes.

Little sharp triplet EPR lines of free 12-SLS and 5-SLS were overlapping with the anisotropic broad lines of entrapped probes in the microsomal membrane (Fig. 1B and C). In the case of 12-SLS, the sharp triplet was disturbing the spectral analysis. Therefore, the spectrum was carefully replotted, subtracting contribution of sharp triplet of free 12-SLS on the squared chart paper (data not shown). Then, the spectra of 12-SLS were analyzed for the correlation time.

Table 1 shows results of membrane fluidity. No difference between the order parameter of 5-SLS in PC and PC-OOH liposome was observed. The correlation time of 12-SLS in PC-OOH liposome appears to be lower, whereas no significance was obtained. The membrane fluidity of the reconstructed liposome with the PC-OOH appeared to increase at relatively deep position compared with fresh PC liposomes, whereas the peroxidation of PC was very mild. Similarly, the membrane fluidity of the liver microsome

Table 2
Reduction rate constants of several spin probes in rat liver microsome and cytochrome P-450 activity in the microsome

	3-CP (min^{-1})	5-SLS (min^{-1})	12-SLS (min^{-1})	P-450 (nmol/mg protein)
Normal	1.51±0.35	2.21±0.17	1.98±0.34	0.597±0.027
SeD	1.71±0.03	2.87±0.25*	2.48±0.01	0.914±0.072*

Values are indicated by mean±S.D. The number of measurement was three for all experiments.

* Significance between normal and SeD groups with $P<.05$.

Table 3
Glutathione peroxidase activity, GSH contents and TBARS measured in the liver homogenate

	GSH-Px (U/mg protein)	GSH[a] (mmol/mg protein)	TBARS (nmol/g liver)	TBARS/P (nmol/mg protein)
Normal	653.67±52.47	0.115±0.028	511.3±39.5**	4.19±0.40
SeD	8.24±0.84***	0.183±0.046*	342.5±55.4	5.18±0.41**

Values are indicated by mean±S.D. of six rats for GSH-Px and GSH, and five rats for TBARS. Each sample was measured by a duplicate experiment.
[a] Result of 8-week-old rats.
* Significance between normal and SeD groups with $P<.05$.
** Significance between normal and SeD groups with $P<.01$.
*** Significance between normal and SeD groups with $P<.001$.

fraction of SeD rat appeared to increase at deep position compared with that of normal rat, whereas no significance was obtained. The TBARS levels measured were 0.39± 052 nmol/mg PC for PC liposome and 1.93±0.90 nmol/mg PC for PC-OOH liposome. This fact suggests that the hepatic cell membrane of the SeD rat have not experienced or experienced a very weak lipid peroxidation.

Decay constant of nitroxyl spin probes in the SeD rat liver microsomal suspension was faster than that of normal rat (Table 2). The decay constants of 5-SLS show significance between normal and SeD rat, whereas no significance was obtained for the decay constants of other spin probes.

Cytochrome P-450 activity in the SeD rat liver microsome was significantly increased compared with normal rat (Table 2). Nitroxyl spin probes are substrates for cytochrome P-450 relating to reduction. The reduction rates depend on the location and/or position of the nitroxyl radical in the membrane.

Table 3 shows GSH-Px activity, GSH contents and *TBARS level* in rat liver. The GSH-Px activity was significantly low in the SeD rat. On the other hand, GSH content was significantly increased in the SeD rat compared with the normal rat. TBARS level was significantly lower in the SeD rat than the normal rat, whereas TBARS/P level was significantly higher in the SeD rat compared with the normal rat.

4. Discussion

The reduction of nitroxyl radical, that is, EPR signal decay, depends on oxygen concentration in the reaction mixture [15]. The initial oxygen concentration in all samples might be equilibrated to air by several vortexing processes. Notable line width change was not obtained during time course measurements for any nitroxyl spin probes. The oxygen concentration in the reaction mixture was almost constant during the measurement.

Glutathione peroxidase consumes H_2O_2 using GSH as a cofactor. The liver GSH-Px activity of our SeD rat was extremely low throughout their lifetime [16], resulting in an increased level of H_2O_2 [4,8]. The resulting increase in H_2O_2 leads to a chronic oxidative stress. Furthermore, the increased presence of H_2O_2 caused reoxidation of the nitroxyl probe radical to the hydroxylamine form, masking the contribution of reduction related to cytochrome P-450 activity [9]. Reduction is mediated by an NADPH-cytochrome P-450 oxidoreductase, not directly by a cytochrome P-450 [17,18]. However, substantial activity of microsomal electron transport system is generally regulated by an activity of cytochrome P-450. Therefore, the reduction rate of 5-SLS, where the nitroxyl radical on its carbon chain (0.5 nm depth in the membrane) is relatively near to the active center of cytochrome P-450 (0.7–0.8 nm depth in the membrane) [9], was significantly increased in the SeD rat liver microsome. Other nitroxyl spin probes also showed higher values of reduction rate in the SeD rat liver microsome compared with the normal rat liver microsome, whereas no significances were obtained. 3-CP can go into the membrane lipid phase slightly, but most of the compound exists in hydrophilic phase. 12-SLS has its nitroxyl radical at 1.35 nm depth in the membrane [9]. The in vivo reduction rate of nitroxyl radicals can also be enhanced by the increased level of GSH, which can work as an H donor in the reduction of nitroxyl radicals [19,20]. Nevertheless, the net effect of H_2O_2 was to make the in vivo decay of nitroxyl radical slower [8]. Our findings show that the nitroxyl radical-reducing activity was increased by Se deficiency-induced cytochrome P-450 activity.

The increases in liver damage indices in SeD rats were not dramatic compared with the lack of GSH-Px activity, suggesting that oxidative damage caused by the Se deficiency may be relatively weak. Selenium-deficient rats can live more than 50 weeks (unpublished data). Therefore, in the absence GSH-Px, another substitutive factor may protect cell and/or cell membranes from the chronic H_2O_2-induced oxidative stress. Oxidative damage (TBARS) in the liver of the SeD rat liver homogenate and microsome was small (Tables 1 and 3). Membrane peroxidation was quite mild compared with the CCl_4-induced liver damage [9] because the damage in the SeD rat liver microsome could not affect the membrane fluidity (Table 1).

The relationship between cytochrome P-450 induction and reactive oxygen species and/or other free radical species is unclear. Oxidative stress caused by H_2O_2 itself was a possible trigger of cytochrome P-450 induction. In vivo EPR experiments have shown that the EPR signal decay constant of nitroxyl spin probe decreased in rat liver when the H_2O_2 level in the bile was increased [8]. However, this H_2O_2 generation may not be a sufficiently injurious level in the SeD rat (Table 1). H_2O_2 can be reduced by excess GSH, which can be elevated due to decreased consumption by GSH-Px. Glutathione level in the SeD rat liver was increased (Table 3), in agreement with several earlier reports with blood and plasma [21–23]. The consumption of H_2O_2, GSH and transition metal ions may produce unknown complicated redox reactions. Such reactions may have some

relations with triggering induction of the cytochrome P-450 activity consistent with other hepatic stressors [24,25]. Which cytochrome P-450 may be induced is under investigation.

Regular purchasable Wistar rats were used as a control of comparison with the SeD rats in a series of our reports [3–8,16], although the SeD diet supplemented with Se should be used as a control. Se contents in the normal diet (CE-2, CLEA Japan, Tokyo, Japan) and the SeD diet (Oriental Yeast) were 0.86±0.03 and 0.017±0.002 mg/kg (mean±S.D. of three samples) as determined by the instrumental neutron activation analysis [5,6]. Differences between the normal and the SeD diets were, however, not only Se contents but also that the contents of several minerals and vitamins were relatively low in the SeD diet, the exception being vitamin E was 2.5-fold higher than the normal diet. Addition of Se in such a low nutritional status can make additional oxidative stresses, which may make the results more complicated to interpret. Therefore, protocol for the control to the SeD diet, that is, Se supplement with SeD diet, is in progress.

Se deficiency caused the induction of liver microsomal cytochrome P-450-mediated reduction activity. An increased probe reduction rate in SeD rat liver microsomes can be expected. Concomitantly, increased H_2O_2 levels due to the loss of GSH-Px activity resulted in decreasing in vivo EPR decay constant of a nitroxyl spin probe in SeD rat due to the ability of H_2O_2 to reoxidize hydroxylamine to form nitroxyl radical. The oxidative damage in SeD rat may be small as demonstrated by the relatively normal level of lipid peroxidation in SeD rat liver microsomes and a greater than normal level of reduced GSH. The cytochrome P-450 may be induced by the oxidative stress without significant tissue damage. Although enzymatic radical-reducing activity in liver microsome was increased in SeD rat liver, increased H_2O_2 level masked in vivo reduction rate of nitroxyl radical. In addition, increased GSH level may be a masked oxidative injury by H_2O_2.

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