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Bioavailability of selenium from selenotrisulfides in primary cultured neuronal cells

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Summary

Selenium plays vital roles as a defense against oxidative stress in the central nervous system. This essential micronutrient is transported to the brain in the form of selenoprotein P. Additionally, small molecular-mass selenium compounds are also suggested to participate in supplying selenium to the brain, although its definitive transport pathways to the brain still remain unclear. Selenotrisulfide (–S–Se–S–, STS) is a metabolic intermediate of selenium and can react with free cysteine (Cys) thiols in proteins through the thiol-exchange reaction (R–S–Se–S–R' + R"–SH → R–S–Se–S–R" + R'–SH). These reactions of free Cys thiols in human hemoglobin (Hb) and serum albumin (HSA) with STS are involved in the selenium metabolic and/or transport pathway in red blood cells. In this study, rat dorsal root ganglion (DRG) neurons are supplemented with STS species including STSs bound to HSA and Hb to determine the selenium utilization efficiency from STS species. After incubation with STS species for 72 h, the cellular selenium concentration and activity of selenium-dependent glutathione peroxidase in DRG neurons increased as well as those incubated with selenious acid. Selenium from STS is thought to be absorbed and utilized for the selenoprotein synthesis in neurons.

Key words: selenium, selenotrisulfide, neuron, brain, protein thiol

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Introduction

Selenium plays vital roles in the brain [1, 2]. Relatively higher amounts of lipids and vigorous oxygen consumption in the brain are supposed to cause numerous amounts of peroxides including phospholipid peroxides. Because excess amounts of peroxides could cause various diseases, such as cerebrovascular diseases and neurodegenerative diseases [3–5], antioxidative selenoproteins, such as glutathione peroxidases (GPxs) and thioredoxin reductases, are suppose to be important in the brain. The importance of selenium in the brain is also suggested from the fact that the selenium concentration in the brain is less vulnerable compared to other peripheral organs/tissues though the mechanism of selenium retention has not been fully

elucidated [6, 7]. Selenoprotein P (SelP) in blood is reported to play a key role in the systematic delivery of selenium to the brain [8–11]. However, there is a possibility that small molecular-mass selenium species are involved in the SelP-independent selenium transport pathway to the brain.

Selenotrisulfide (R–S–Se–S–R', STS) is thought to be one of the key metabolic intermediates of selenium [12]. STS can react with protein by the free cysteine (Cys) thiols through the thiol-exchange reaction (R–S–Se–S–R' + Protein-Cys–SH → Protein-Cys–S–Se–S–R + R'–SH). We have developed a comprehensive method to detect STS reactive protein from the organ/tissue of rodents using penicillamine selenotrisulfide (PenSSeSPen) and MALDI TOF mass spectrometry [13–16]. With this methodology, liver fatty acid binding protein, cystatin-12 precursor and myoglobin were identified to bind selenium via selenotrisulfide bonds in the liver, brain and heart of rat, respectively. Generally, STS species with small molecular-mass thiols, such as glutathione, are chemically unstable under physiological conditions. However, selenium bound to protein via STS is relatively more stable compared to STS with small molecular-mass thiols [17, 18]. There is a possibility that these STS reactive proteins can participate in the transport and/or metabolic pathways of the selenium in biological systems.

In this study, primary cultured dorsal root ganglion (DRG) neurons of rats were incubated with PenSSeSPen and protein bound STS to elucidate the involvement of STS species in the selenium transport to neuronal cells. DRG neurons can be easily obtained from adult rats and have the same biological features as the brain neurons. We previously demonstrated that selenium from selenious acid (SA) and seleno-L-methionine (SeMet) was absorbed into DRG neurons and utilized to increase the cellular GPx activity [19]. Human hemoglobin (Hb) and human serum albumin (HSA) were used as models of the STS reactive protein. Hb has free Cys thiols in its β-subunits and mediates the selenium metabolism in red blood cells (RBCs) [17, 20]. Free Cys thiol in HSA promotes the efflux of selenium from RBCs to plasma [18]. These proteins seemed to have suitable characteristics as STS reactive proteins, such that they can react with STS and stably retain selenium in its molecules. Their large molecular mass also made it easy to modify these proteins with small molecular-mass STS compounds. In addition, the selenium absorption character in DRG neurons from STS compounds was compared to human hepatoma cells.

Materials and Methods

Materials

Selenious acid (SA), seleno-L-methionine (SeMet), glutathione in the reduced form (GSH) and hydrogen peroxide were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). L-Penicillamine (Pen), human serum albumin (HSA, 66,400 Da), human hemoglobin (Hb, 64,500 Da) and matrix-assisted laser desorption ionization time of flight-mass spectrometry (MALDI TOF-MS)-grade sinapinic acid were purchased from the Sigma Co. (St. Louis, MO, U.S.A.) and 2,3-diaminonaphthalene (DAN) was from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) and glutathione reductase (GR) were from the FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The water used throughout this study (>18 MΩ cm) was generated by a Milli-Q Reference system (Millipore Corp., Billerica, MA, U.S.A.). All other chemicals were of commercial reagent or special grade and used as received.

Preparation of selenotrisulfide compounds

Thiol can react to form selenotrisulfide (STS) by the Painter reaction [6, 12]. According to this reaction procedure, L-penicillamine selenotrisulfide (PenSSeSPen) was synthesized as follows. An equal volume of 1 mM SA solution and a 4 mM Pen solution were mixed and allowed to react with stirring for 2 h at room temperature and left overnight at 4 °C. The resulting snow-white precipitate was isolated and carefully washed twice with cold water and methanol. The obtained material was dried in a desiccator at room temperature for 24 h or longer, then stored at −20 °C until used. Elemental analysis (%), calcd for $C_{10}H_{20}N_2O_4S_2Se$: C 31.97, H 5.33, N 7.46, Se 21.04, found: C 31.13, H 5.86, N 7.24, Se 21.89. $[\alpha]_D$, +7.07. λ_{max} in deionized water 266 nm (ε_{mM}: 1.47). MALDI TOF-MS (positive ion mode), calcd for $C_{10}H_{20}N_2O_4S_2{}^{80}Se$ 376.2, found *m/z* 376.3.

An equal volume of 3 mM HSA and 6 mM dithiothreitol (DTT) were mixed in 66 mM phosphate buffer (pH 7.4) and allowed to react for 90 min at room temperature. The reaction mixture was dialyzed overnight against 6.6 mM phosphate buffer (pH 7.4) at 4 °C to remove any unreacted DTT using the dialysis tube Spectra/Por Membrane (regenerated cellulose, molecular mass cutoff; 6–8 kDa, Spectrum Laboratories, Inc.). Reduced HSA was incubated with PenSSeSPen for 30 min followed by dialysis under the same conditions. STS containing HSA (HSA-SSeSPen) was concentrated and dried by lyophilization. Hb was

reduced with 12 mM DTT, and STS containing Hb (Hb-SSeSPen) was prepared by the same procedure as the HSA-SSeSPen.

Ultrafiltration

Ultrafiltration of HSA-SSeSPen and Hb-SSeSPen was performed by an Amicon Ultra Ultracel-30K [molecular-mass cut-off (MMCO) 30 kDa, Millipore Corp.]. After centrifugation at 7,500 *g*, 4 °C for 20 min, the selenium concentration in the filtrate was determined.

MALDI TOF mass spectrometry of selenotrisulfide conjugated protein

The HSA samples were digested with trypsin (0.2 mg/mL in water) at 37 °C for 1 h before the mass spectrometric analysis. An excess amount of sinapinic acid was suspended in 34% (v/v) acetonitrile and 0.067% trifluoroacetic acid for preparation of the matrix solution. After centrifugation at 7,500 *g* for 3 min, the obtained supernatant was mixed with the HSA and Hb samples in a 3 to 1 ratio by volume, and an aliquot was applied to an AnchorChip target (Bruker Daltonics, Inc., Bremen, Germany). Mass spectra were acquired in the linear positive ion mode using an Ultraflex III MALDI TOF/TOF-MS (Bruker Daltonics, Inc.).

Animals

Three-week old male Wistar rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). The rats were housed on a 12-h light/ 12-h dark schedule at 25 ± 2 °C and 60% relative humidity, and were freely given deionized water and a regular CE-2 diet (CLEA Japan, Inc.). All experiments with the live animals were performed in compliance with the guidelines of the Nagasaki University on Animal Care and Use, and the institutional committee approved the experimental protocols.

Cell culture

Rat dorsal root ganglional neurons were excised from 3–6 week-old Wistar rats according to a previously described procedure [19]. Briefly, the spinal column was excised from the rat under isoflurane anaesthesia. The dorsal root ganglia (DRG) from the spinal cord were cut into small pieces in an ice cold Hanks balanced salt solution and incubated with 0.15% collagenase (from *Streptomyces parvulus*, 400–500 units/mg, Wako Pure Chemical Ind.) for 2 h, then with a mixture of 0.05% trypsin (from porcine pancreas, 1000–2000 units/mg, Nacalai Tesque, Inc.) and 0.02% EDTA for 1 h. After washing twice with medium, the treated DRG were plated into poly-L-lysine coated dishes. The DRG neurons were cultured in Dulbecco's modified Eagle medium (DMEM, FUJIFILM Wako Pure Chemical Corporation) containing 10% fetal bovine serum (FBS, Sigma Co.) supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin (FUJIFILM Wako Pure Chemical Corporation) at 37 °C and 5% CO_2 with humidity for 7–10 days before use until the dendrites of the neurons well developed. The selenium compounds, including the synthesized STS species, were dissolved in the medium to make the final selenium concentration of 1.0 μM. After incubation for the indicated period, the DRG neurons were washed 3 times with Hanks balanced salt solution followed by treatment with 0.25% trypsin. The detached DRG neurons were collected and lysed by sonication. Human hepatoma (HepG2) cells were purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank. The HepG2 cells were cultured and treated under the same conditions as the DRG neurons except for using phosphate buffered saline (PBS, calcium and magnesium free) instead of Hanks balanced salt solution.

Determination of cellular glutathione peroxidase activity

The cellular GPx activity was measured by monitoring the absorbance at 340 nm due to the NADPH [21]. The cell lysate was combined with sodium azide (1 mM), a GR solution (1 unit/mL), a reduced GSH solution (1 mM) and an NADPH solution (0.2 mM) in 66 mM phosphate buffer (pH 7.4). The reaction was initiated by the addition of a hydrogen peroxide solution (0.25 mM). Absorbance at 340 nm was recorded every 1 min just after mixing by inversion. The GPx activity was calculated using the following equation (1) as μmoles of NADPH oxidized per minute, where ΔA_{SAM} is the decrease in the absorbance at 340 nm of the sample solutions between 15 and 75 s after the addition of the substrates, ΔA_{BLK} is the decrease in absorbance at 340 nm per minute of the solutions using the 66 mM phosphate buffer instead of the sample solutions, 10 is the dilution factor, ε_{mM} is the extinction coefficient for the 1 mM NADPH solution (6.22 mM^{-1} cm^{-1}), and c is the protein content (mg/mL).

$$\text{GPx activity} = (\Delta A_{SAM} - \Delta A_{BLK}) \times 10/\varepsilon_{mM}/c \quad (1)$$

Determination of selenium and protein thiol concentrations

After acid digestion with a 1 : 4 mixture by volume of perchloric acid and nitric acid, the selenium concentrations in the specimens were fluorometrically determined using DAN [22]. The selenium standard solution for atomic absorption spectrometry (1000 mg/L in 0.1 mol/L nitric acid, Kanto Chemical Co., Inc., Tokyo, Japan) was used as the standard material for preparation of the calibration curve (1-10 pgSe/mL). An FP-6600 fluorometer (JASCO Corporation, Tokyo, Japan) was used for measurement of the fluorescence intensity (Ex: 375 nm, Em: 520 nm). The protein concentrations in the specimens were measured by the Lowry method using bovine serum albumin (Nacalai Tesque, Inc.) as the standard [23]. The protein thiol concentrations were colorimetrically determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma Co.) [24]. An equal volume of a 1 mM DTNB solution in a 66 mM phosphate buffer (pH 7.4) was added to the sample solution, then incubated for 30 min. The absorbance at 450 nm was recorded by a V-660 spectrometer (JASCO Corp.). GSH was used as the standard compound for preparation of the calibration curve.

Statistical analysis

All data are presented as the mean and standard deviation (SD). Statistical analyses were performed using PRISM 4 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Statistically significant differences between the controls and selenium-treated groups were determined by one-way ANOVA using the Tukey method. Comparisons were considered to be statistically significant at $P < 0.05$.

Results and discussion

PenSSeSPen is a model compound of GSSeSG, which was an important intermediate in the metabolism of selenious acid [12, 25], and efficiently used to investigate the selenium metabolic pathway and selenium reactive proteins [13-16, 20, 26]. Because PenSSeSPen is also effective in restoring the organ/tissue selenium concentration and hepatic GPx activity of dietary selenium deficient mice [6], there was a possibility that PenSSeSPen was absorbed and utilized as the selenium source compound in cultured cells. Both HSA and Hb can participate in the thiol-exchange reaction with PenSSeSPen to form the asymmetric STS (HSA-Cys34-SSeSPen and Hb-βCys93-SSeSPen, respectively) [17, 18]. One HSA molecule contains 35 Cys residues and 34 of them were involved in the 17 intramolecular disulfide bonds. Only one Cys residue at amino acid position 34 (Cys34) is free and can participate in the thiol-exchange reaction with selenotrisulfide to form HSA-Cys34-SSeSR. HSA with the free Cys thiol promotes efflux of the selenium from red blood cells (RBC) in a thiol concentration dependent manner. Selenium bound to HSA via STS can be transported to isolated rat hepatocytes [18]. On the other hand, Hb has one free Cys residue in each β subunit (Cysβ93). These free Cys thiols can also react with STS and form Hb-Cysβ93-SSeSR. Selenium bound to Hb is transferred to the free Cys thiol in anion exchanger-1 in the RBC membrane [26, 27]. These reactions of the free Cys thiols in Hb are involved in the release of selenium from the RBC. Because both HSA and Hb can bind selenium and participate in the RBC membrane transport of selenium, these proteins appeared to be suitable as model proteins transferring selenium to cultured cells in this study.

Since Cys34 in HSA is usually oxidized, reduction with DTT was carried out before the treatment with PenSSeSPen. The reduction of HSA was confirmed by determination of the thiol content in HSA [**Figure 1 (a)**]. The protein thiol content in HSA increased from 0.22 to 1.0 in the 1 HSA molecule after reduction with DTT. For further confirmation of the reduction of Cys34 of HSA, HSA before and after reduction with DTT was digested with trypsin followed by a MALDI TOF mass spectrometric analysis. The mass spectral peaks of the free-Cys containing fragments were specified by the molecular mass gain

Table 1. Selenium content in PenSSeSPen treated HSA or Hb and selenium filtration rate with MMCO 30 kDa membrane after 72-h incubation in PBS.

	Se (mol/mol-protein)	Se filtered (%)
HSA	0.56 ± 0.06	35.5 ± 8.6
Hb	0.59 ± 0.02	28.3 ± 6.6

Data are mean ± SD (n = 4).

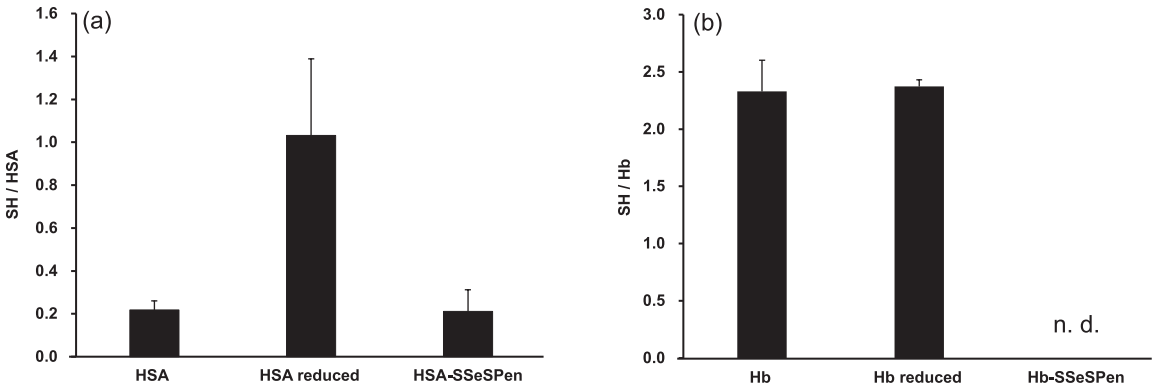

Figure 1. Thiol content in HSA (a) and Hb (b). HSA and Hb were reduced with DTT followed by treatment with PenSSeSPen. Data are mean ± SD (n = 3), n. d., not determined.

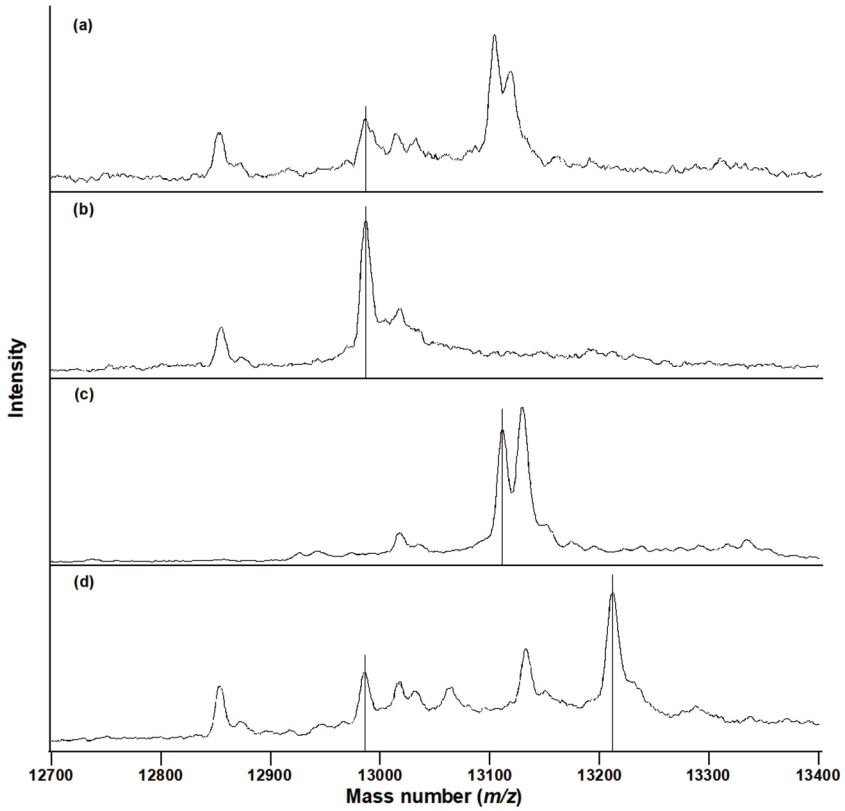

Figure 2. MALDI TOF mass spectrometric analysis of Cys34 fragment of HSA digested with trypsin. (a) non-treated HSA, (b) DTT-reduced HSA, (c) NEM-treated HSA and (d) PenSSeSPen-treated HSA. Solid lines were drawn at *m/z* 12,986 (Cys34 fragment, reduced form), *m/z* 13,112 (NEM-adduct), *m/z* 13,212 (SeSPen-adduct).

in *m/z* by 125 after alkylation with the thiol-specific *N*-ethylmaleimide (NEM) in comparison to the reduced HSA [**Figures 2 (b) and (c)**]. After digestion with trypsin, the fragment peak containing Cys34 could be detected at *m/z* 13,105 and *m/z* 12,986 in the mass spectrum of the non-reduced HSA and DTT reduced HSA, respectively [**Figures 2 (a) and (b)**]. The difference in these molecular masses of 119 was near the molecular mass of Cys (Mw: 121.16). These facts suggested that the reduction with DTT was effective to reduce thiol in Cys34 and the non-reduced HSA used in this study might be oxidized with Cys. After PenSSeSPen treatment of the reduced HSA, the thiol content in HSA decreased to 0.21 while selenium bound to HSA was calculated to be 0.56 in 1 molecule of HSA [**Figure 1 (a), Table 1**]. In the case of the PenSSeSPen-treated HSA, the molecular mass of the Cys34 containing fragment shifted by 226 (*m/z* 13,212) which corresponded to the molecular mass of selenenyl

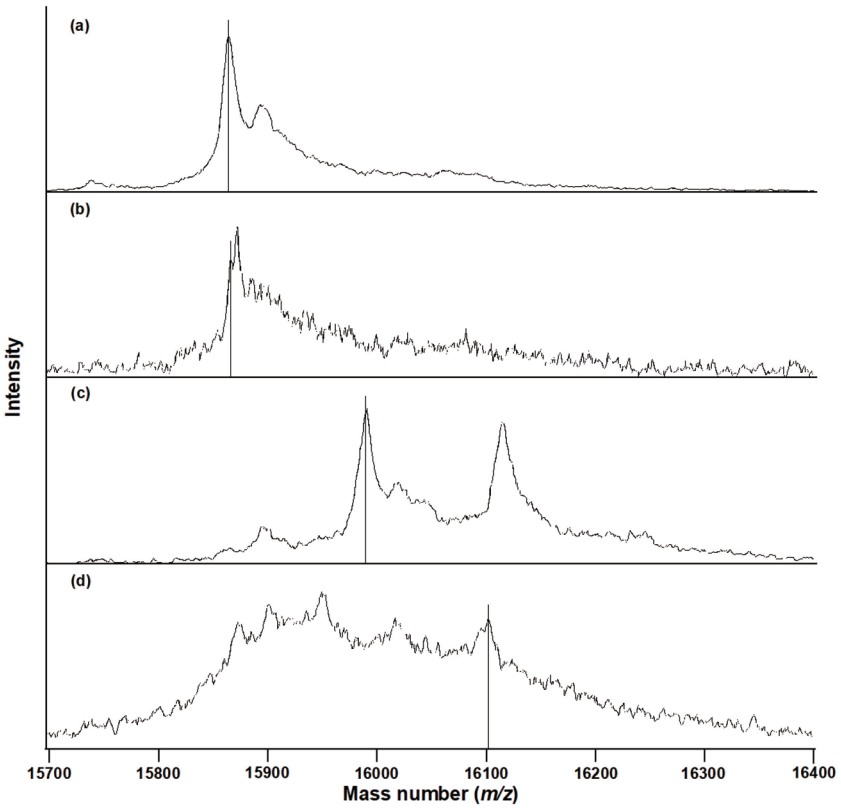

Figure 3. MALDI TOF mass spectrometric analysis of Hb β-subunit. (a) non-treated Hb, (b) DTT-reduced Hb, (c) NEM-treated Hb and (d) PenSSeSPen-treated Hb. Solid lines were drawn at *m/z* 15,864 (Hb β-subunit, reduced form), *m/z* 15,990 (NEM-adduct), *m/z* 16,099 (SeSPen-adduct).

penicillamine (−Se−S−Pen) [**Figure 2 (d)**]. These results indicated that a new STS bond was formed in the Cys34 of HSA through the thiol-exchange reaction (PenSSeSPen + HSA-Cys34−SH → HSA-Cys34-SSeSPen + Pen−SH). According to the thiol determination and mass spectrometric analysis of the PenSSeSPen-treated HSA, a considerable amount of unreacted free thiol remained in the HSA after the PenSSeSPen treatment [**Figures 1 (a) and 2 (d)**]. Additionally, the peak shift at *m/z* 13,133 in the mass spectrum of the PenSSeSPen-treated HSA suggested the formation of a disulfide bond in Cys34 with Pen (HSA-Cys34-SS-Pen). These facts seemed to contribute to the difference in the thiol content of the reduced HSA (1.0 mol/mol-HSA) and selenium content in the PenSSeSPen-treated HSA (0.56 ± 0.06 mol-Se/mol-HSA).

Human Hb-SSeSPen was also prepared by a procedure similar to the HSA-SSeSPen. Because Hb is composed of double sets of α- and β-subunits, there are 2 free Cys thiols in 1 Hb molecule (Cysβ93). Reduction of Hb with DTT did not affect the thiol content in Hb [**Figure 1 (b)**]. These data coincided with the result of the mass spectrometric analysis of the non-reduced and DTT reduced Hb, in which peaks corresponding to the Hb β-subunit were observed at *m/z* 15,864 in the mass spectra of both the non-reduced and reduced Hb [**Figures 3 (a) and (b)**]. The mass spectral peak of the Hb β-subunit at *m/z* 15,864 was specified by the molecular mass gain in *m/z* by 125 after treatment with NEM [**Figure 3 (c)**]. Thiols in Hb seemed to be less susceptive to oxidation compared to those in HSA. After treatment with PenSSeSPen, the thiol content in Hb decreased and could not be determined by the DTNB method [**Figure 1 (b)**]. However, the selenium bound to Hb was 0.59 ± 0.02 mol-Se/mol-Hb, which suggested only 30% of the thiol in the Hb molecules participated in the binding of selenium (**Table 1**). A peak shift corresponding to the −Se−S−Pen moiety was observed at *m/z* 16,099 in the mass spectrum of the PenSSeSPen-treated Hb [**Figure 3 (d)**]. These results demonstrated the occurrence of the thiol-exchange reaction (PenSSeSPen + Hb-Cysβ93−SH → Hb-Cysβ93-SSeSPen + Pen−SH). The formation of a disulfide bond with Pen was also suggested by the peak shift at *m/z* 16,019 in the mass spectrum of the PenSSeSPen-treated Hb and this seemed to be related to the lower selenium content than that which was estimated from the thiol content of Hb (2.0 mol/mol-Hb).

The stability of the STS-bound HSA and Hb was evaluated by ultrafiltration. Generally, STS compounds with small molecular-

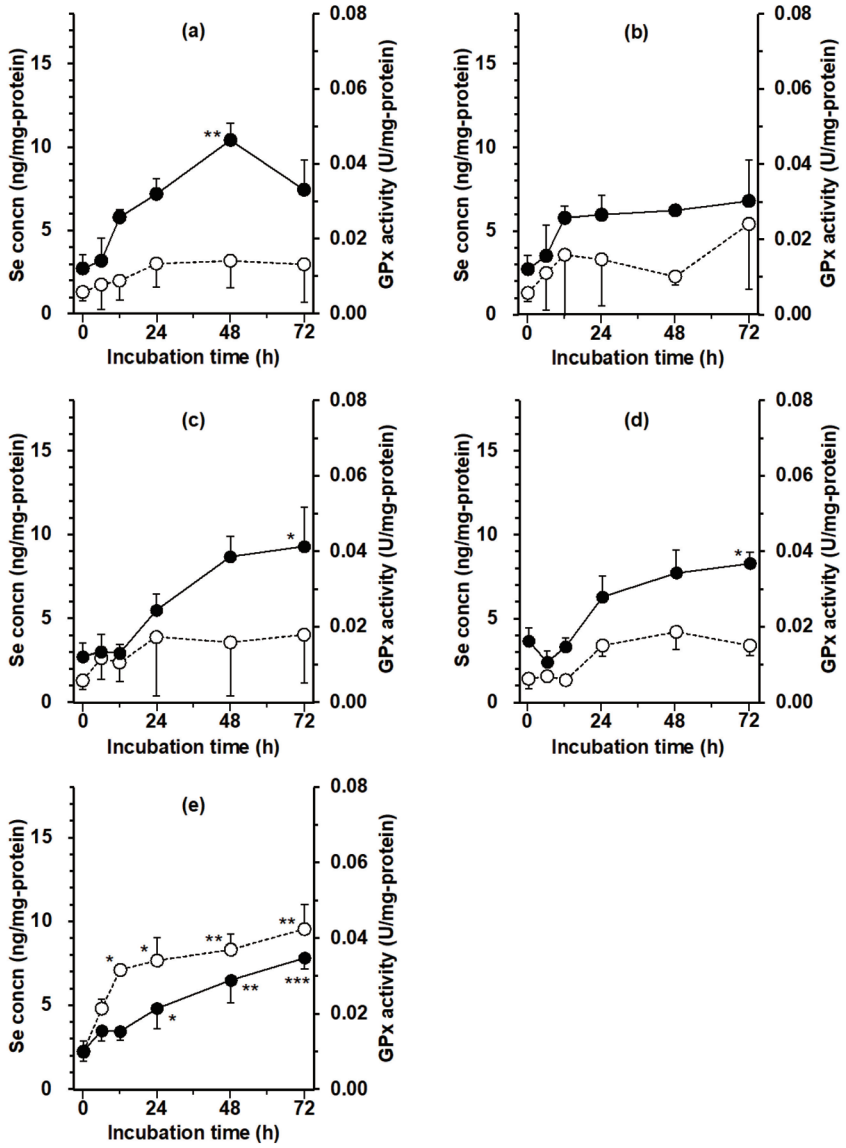

Figure 4. Changes in cellular selenium concentration (open circles) and GPx activity (closed circles) of DRG neurons incubated with SA (a), SeMet (b), PenSSeSPen (c), HSA-SSeSPen (d) and Hb-SSeSPen (e). Selenium concentrations in the culture media were 1.0 μM. Data express mean ± SD (n = 3). *, **, ***, significantly different from the value at time 0 h with $P < 0.05$, < 0.01, < 0.001 (ANOVA with a Tukey test).

mass thiols are liable to decompose and become red elemental selenium at physiological pH *in vitro.* The prepared HSA-SSeSPen and Hb-SSeSPen were each dissolved in PBS and incubated for 72 h followed by ultrafiltration with membrane MMCO 30 kDa. Selenium released from the HSA-SSeSPen and Hb-SSeSPen was calculated as 35.5 and 28.3% of the total selenium bound to the protein before incubation, respectively (**Table 1**). Because most of the selenium was proved to remain on the membrane, the protein bound STS appeared to be stable during the incubation time.

To evaluate the selenium absorption and utilization efficiency in cultured cells, the PenSSeSPen and protein bound STS were added to the medium for culture of the DRG neurons to make the final selenium concentration 1.0 μM, then incubated for 6-72 h. SA and SeMet were utilized as the standard selenium compound. Changes in the cellular selenium concentration and cellular GPx activity depended on the incubation time and the types of selenium species supplemented to the culture media. During the incubation time of 6–24 h, an increase in the cellular selenium concentration along with the incubation time was observed in all the DRG neurons supplemented with the selenium species (**Figure 4**). Although it was not significant, incubation with the selenium species other than Hb-SSeSPen kept the cellular selenium concentration at about 3–4 ng-Se/mg-protein during the incubation time of 24–72 h. A significant increase in the cellular selenium concentration was observed only in the Hb-SSeSPen-

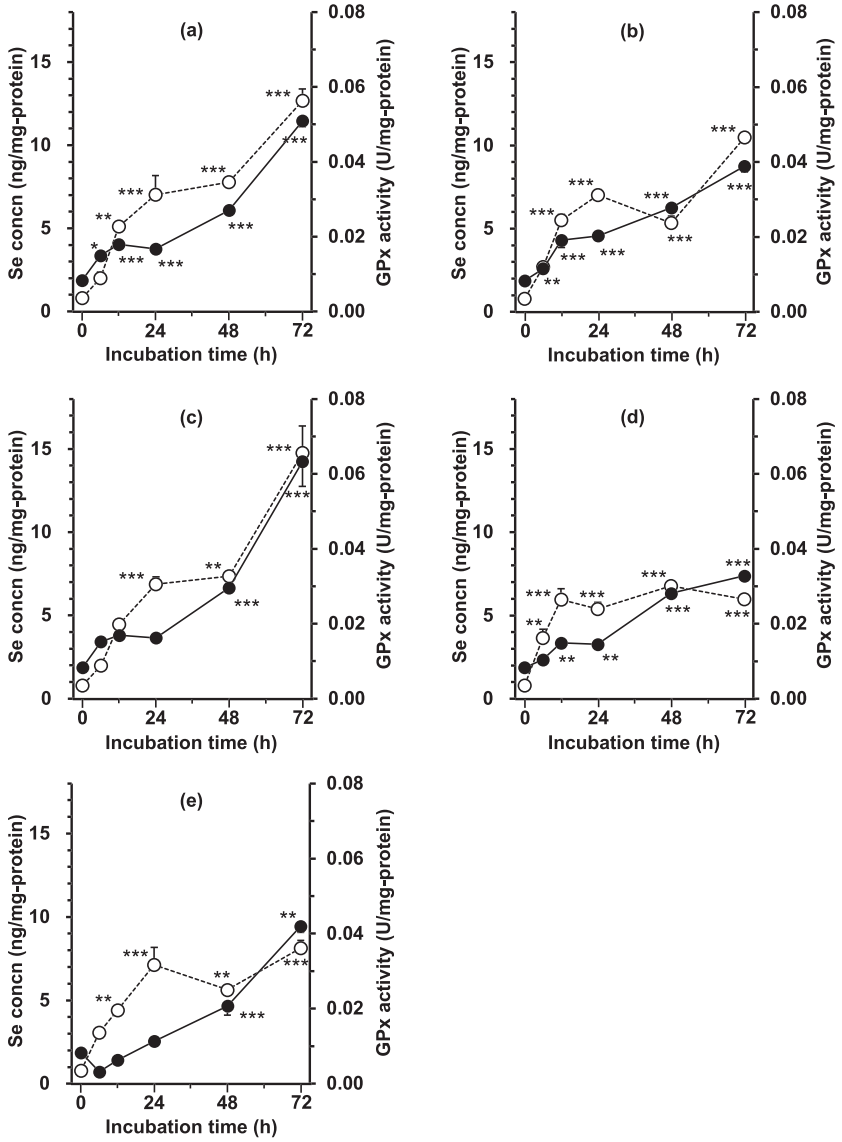

Figure 5. Changes in cellular selenium concentration (open circles) and GPx activity (closed circles) of HepG2 cells incubated with SA (a), SeMet (b), PenSSeSPen (c), HSA-SSeSPen (d) and Hb-SSeSPen (e). Selenium concentrations in the culture media were 1.0 μM. Data express mean ± SD (n = 3). *, **, ***, significantly different from the value at time 0 h with $P < 0.05$, < 0.01, < 0.001 (ANOVA with a Tukey test).

supplemented DRG neurons incubated for more than 24 h [**Figure 4 (e)**]. These data indicated that selenium from STS could be absorbed into the DRG neurons as well as SA and SeMet. The cellular GPx activity in the DRG neurons supplemented with the STS species increased in a time dependent manner. After incubation with the selenium for 72 h, the cellular GPx activity of the DRG neurons supplemented with PenSSeSPen or HSA-SSeSPen was higher than those with SA, SeMet or Hb-SSeSPen. On the other hand, the GPx activity of the DRG neurons supplemented with SA or SeMet increased faster and indicated higher values at the incubation time of 12 h. In the DRG neurons incubated for 72 h, selenium from PenSSeSPen and HSA-SSeSPen was presumed to be more effectively utilized for the synthesis of the GPx than the other selenium species. On the other hand, Hb-SSeSPen seemed to be less effective to raise the GPx activity in the DRG neurons although a remarkable increase in the cellular selenium concentration compared to the other 4 selenium species was observed. This might be due to the adhesion of Hb to the cell surface since the DRG neuron adhered the petri dish looked slightly reddish after 3 washings with Hanks balanced salt solution (data not shown). There was a possibility that selenium from Hb-SSeSPen nonspecifically bound to cell surface was not effectively absorbed into the DRG neurons and utilized for the GPx synthesis. Consequently, selenium from the STS species absorbed into the DRG neurons was suggested to be utilized for the synthesis of selenoproteins. Only the SeMet treatment did

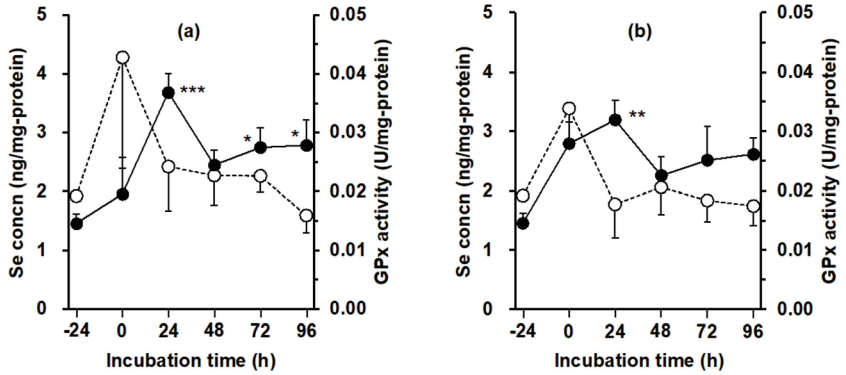

Figure 6. Changes in cellular selenium concentration (open circles) and GPx activity (closed circles) of DRG neurons incubated with PenSSeSPen (a) and HSA-SSeSPen (b). DRG neurons were incubated in 10% FBS-containing DMEM during time 0 to 96 h subsequent to 1.0 μM selenium supplemented medium during time −24 to 0 h. Data express mean ± SD (n = 3). *, **, ***, significantly different from the value at time 0 h with $P < 0.05$, < 0.01, < 0.001 (ANOVA with a Tukey test).

not cause a significant increase in the cellular GPx activity of the DRG neurons during the 72-h incubation. A similar tendency was observed when other cell lines were incubated with SeMet [28, 29]. Because SeMet is misincorporated into proteins instead of methionine and cannot be directly utilized for selenoprotein biosynthesis [30], the bioavailability of SeMet was presumed to be lower than that of the SA and STS species.

Human hepatoma (HepG2) cells were incubated with selenium species under the same conditions as the DRG neurons to examine the effect of the cell type on the selenium bioavailability from the STS species. HepG2 cells that originated from liver cancer and immortalized might have different selenium absorption and metabolic pathways from the primary cultured DRG neurons. The selenium concentration of all the HepG2 cells incubated with selenium species significantly increased after the 24-h incubation and the values were higher than that of the DRG neurons supplemented with the same selenium species except for Hb-SSeSPen (**Figure 5**). The HepG2 cells incubated with SA and PenSSeSPen exhibited a similar increasing pattern of the cellular selenium concentration and higher cellular selenium concentration [**Figures 5 (a) and (c)**]. On the other hand, the cellular selenium concentration of the HepG2 cells supplemented with HSA-SSeSPen and Hb-SSeSPen was lower than that of the HepG2 cells incubated with other selenium species including PenSSeSPen [**Figures 5 (d) and (e)**]. These data suggested that selenium absorption mechanisms from the STS species in the DRG neurons and HepG2 cells might be different. In animals, the liver is the main organ to synthesize SelP; plasma selenoprotein transports selenium from the liver to the brain and other peripheral organs [8, 9]. Because the HepG2 cells are reported to synthesize and secrete SelP [31, 32], they might be able to absorb a greater amount of selenium compared to the DRG neurons. While PenSSeSPen-supplemented HepG2 cells indicated the highest cellular GPx activity after the 72-h incubation, the increase in the cellular GPx activity of the HepG2 cells supplemented with HSA-SSeSPen and Hb-SSeSPen was lower than that of the PenSSeSPen or SA supplemented cells. This might be caused by the lower level of selenium in the protein bound STS supplemented HepG2 cells. Although the efficiency of the selenium absorption and utilization was different, selenium from the STS species appeared to be absorbed and utilized to increase the cellular GPx activity in the HepG2 cells as well as the DRG neurons. A future study will be needed to reveal the cause of these differences in the selenium utilization efficiency between the DRG neurons and HepG2 cells.

In our previous research, the DRG neurons incubated with SA or SeMet for 24 h could retain the increased cellular GPx activity for the following 96 h in the SA or SeMet free media [19]. This characteristic selenium retention behavior of the DRG neurons may be associated with the homeostatically maintained store of selenium in the brain at the whole-body level. In this study, we examined whether selenium from the STS species would be retained in the DRG neurons. DRG neurons were first incubated in the PenSSeSPen or HSA-SSeSPen-supplemented media prepared to make the selenium concentration 1.0 μM for 24 h, then incubated in STS free media for the following 96 h. After a 24-h incubation (at incubation time 0 h) with the STS species, both the DRG neurons supplemented with PenSSeSPen and HSA-SSeSPen showed an increased cellular selenium concentration (**Figure 6**). The highest cellular GPx activity was observed at 24 h after the removal of the STS species from the media in both

DRG neurons. While the selenium concentration in the DRG neurons rapidly decreased to the basal level (at incubation time −24 h), the cellular GPx activity was maintained at a higher level than that before the STS species supplementation. These facts suggested that selenium from PenSSeSPen and HSA-SSeSPen was retained in the DRG neurons and utilized to maintain the cellular GPx activity. The cellular selenium concentration in the DRG neurons after removal of the STS species was extremely low, but the GPx activity was kept at a higher level. This was probably because the amount of selenium utilized for the GPx activity was much lower than that stored in the DRG neurons. This also corresponded to the fact that there was not a correlation between the increase in the cellular selenium concentration and GPx activity in the DRG neurons (**Figure 4**). Actually, the SA supplementation at 0.1 μM was sufficient to raise the cellular GPx activity to the same level as that of the DRG neurons supplemented with 1.0 μM SA in our previous study [19]. The increase in the extremely small amount of cellular selenium was presumed to be sufficient to raise the GPx activity in the DRG neurons for at least 96 h.

In conclusion, we prepared the protein-bound STS species, HSA-SSeSPen and Hb-SSeSPen, in this study. Although selenium absorption and utilization mechanisms were thought to be different between the cell types and among the selenium source compounds, selenium from PenSSeSPen, HSA-SSeSPen and Hb-SSeSPen was absorbed into both the DRG neurons and HepG2 cells and utilized for increasing the cellular GPx activity. STS compounds including the STS bound to protein were proved to be utilized as a selenium source for the selenoprotein synthesis in both cells.

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