Effect of Se-methylselenocysteine on the Antioxidant System in Rat Tissues

Ho-Sang Shin[*] and Eun-Mi Choi[†]

Department of Chemistry, University of Incheon, Incheon 406-772, Korea

Abstract

We assessed the effect of Se-methylselenocysteine (MSC) treatment, at a dose of 0.75 mg/rat/day for 1 or 2 weeks, on the activities of antioxidant systems in Sprague-Dawley rat tissues. Significant changes in glutathione and antioxidant enzyme activities, with different patterns among tissues, were evidenced. Glutathione content and its reduction state in the liver, lung, and kidney were elevated upon MSC treatment, whereas they were significantly lowered in the spleen. Among the tissues exhibiting glutathione increase, there were different enzymatic responses: γ-glutamylcysteine ligase activity, the rate-limiting enzyme in the glutathione synthesis pathway, was increased in the liver, whereas the activities of the enzymes associated with glutathione recycling, namely, glutathione peroxidase, glutathione reductase, and glucose 6-phosphate dehydrogenase, were significantly increased in the lung and the kidney. The superoxide dismutase activity was decreased in all tissues upon MSC treatment, whereas catalase activity was increased in all tissues but the liver. Lipid peroxidation level was transiently increased at 1 week in the lung and the kidney, whereas it was persistently increased in the spleen. The increase was not evident in the liver. The results indicate that the MSC treatment results in an increase in the antioxidant capacity of the liver, lung, and kidney principally via an increase in glutathione content and reduction, which appeared to be a result of increased synthesis or recycling of glutathione via tissue-dependent adaptive response to oxidative stress triggered by MSC. The spleen appeared to be very sensitive to oxidative stress, and therefore, the adaptive response could not provide protection against oxidative damage.

Key words: Se-methylselenocysteine (MSC), antioxidants, glutathione, oxidative stress, Sprague-Dawley rats

INTRODUCTION

Selenium (Se) is an essential micronutrient in mammals, and its deficiency has been implicated in a range of diseases including cardiovascular disease, immune dysfunction, and cancer (1-3). Additionally, supranutritional Se supplementation has been reported to be effective in the reduction of cancer incidence (4-7) and improvement of diseased states such as inflammation (8,9). Recently, protection of normal tissue from a variety of chemotherapeutic agent-induced toxicities and increase in the therapeutic index of chemotherapeutic agents by Se have also been reported (10,11). Both organic and inorganic Se compounds have been reported to have a protective effect in cells and animal models. In the diet, Se exists principally in organic forms, mostly in the form of selenoaminoacids such as selenomethionine, selenocysteine, and Se-methylselenocysteine (MSC).

The mechanism by which Se exerts its beneficial effects is not yet fully understood. However, it is suggested that many selenoproteins and low molecular weight Se metabolites play important roles (12). Most of the organic and inorganic forms of Se appear to be used to produce selenoproteins via generation of hydrogen selenide (H₂Se), which either functions as a precursor for selenoprotein synthesis or undergoes stepwise methylation to generate mono-, di-, and trimethylated forms of Se (13). In the studies of the chemopreventive effect of Se, it has been demonstrated that MSC is more effective than other organic and inorganic forms of Se and also does not exert some of the toxic effects associated with inorganic Se. MSC accumulates in plants such as broccoli, garlic, and onion (14,15). Unlike selenomethionine, MSC is not incorporated into proteins, but is readily hydrolyzed by β-lyase to generate methylselenol, an active Se metabolite suggested to be responsible for the anti-carcinogenic effect (16,17).

The antioxidative role of Se was proposed because it is a component of glutathione peroxidase (GPx) and because other Se-containing proteins are involved in the antioxidant metabolism. However, effective doses of Se necessary for the protective effect appear to exceed those regarded as necessary for supporting maximal expression of most selenoproteins (6,12,18). This suggests that the antioxidative function of Se might include more general metabolic phenomena. Our previous report demonstrated

[*]Present address: BioInfra, Seoul 110-799, Korea
[†]Corresponding author. E-mail: eunmi@incheon.ac.kr
Phone: +82-32-835-8233, Fax: +82-32-835-0762

that MSC pretreatment prevented alloxan-induced hyper-glycemia in rats, and the prevention appeared to be associated with the increased antioxidant activities in the pancreases of the MSC-treated rats (19).

The antioxidant system is composed of low-molecular mass agents and antioxidant proteins. Glutathione (GSH) is the primary small molecular thiol antioxidant. Glutathione is synthesized by a series of enzyme reactions, among which γ-glutamylcysteine ligase (γ-GCL) is the rate-limiting enzyme. Glutathione removes hydrogen peroxide at the expense of its oxidation to glutathione disulfide (GSSG), which is catalyzed by GPx. Glutathione can also remove reactive oxygen species (ROS) by direct nonenzymatic reaction. Therefore, the ratio of GSH/GSSG in cells is lowered under the oxidative stress, unless the GSSG is reduced by glutathione reductase (GRd), which utilizes NADPH produced by glucose 6-phosphate dehydrogenase (G6PD) as reducing agent, to regenerate GSH or is secreted from cells. Glutathione can also be consumed to form protein mixed-disulfides under oxidative stress. Therefore, the cellular content and redox status of glutathione indicate not only the antioxidant capacity of cells but also the degree of oxidative stress being experienced by the cells. Superoxide dismutase (SOD) and catalase (CAT) are major ROS-scavenging antioxidant enzymes which remove superoxide anion and hydrogen peroxide by converting them to hydrogen peroxide and water, respectively.

Despite the proposed antioxidative roles of Se, the systemic effects of MSC on the antioxidants in normal tissues have not been reported. In the present study, we determined that the supranutritional MSC administration increased activities of the antioxidant system in various rat tissues, and the patterns of the responses were significantly different among tissues.

MATERIALS AND METHODS

Animals and treatments

Male Sprague-Dawley rats (180~250 g) used in this study were obtained from Orient Bio Inc. (Seongnamsi, Gyeonggido, Korea). They were fed on a standard laboratory diet and water *ad libitum* and were maintained under a 12-hr light/dark cycle. The treatment group rats received MSC hydrochloride (Sigma, St. Louis, MO, USA) dissolved in water by oral gavage at a dose of 0.75 mg/rat/day, equivalent to 4 μmol Se/rat/day, for one or two weeks. The dose was chosen based on our previous results (19). Control rats were given only water. During the period of the treatment, no differences in the physical appearance and body weight change between the MSC- and control-group were exhibited. After one

or two weeks of MSC treatment, the rats were fasted overnight and sacrificed by CO_2. The tissues, namely, liver, lung, kidney, and spleen, were removed, washed, and immediately frozen by clamping with dry ice-cooled tongs. The tissues were then stored at -80°C until analysis. All procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee at University of Incheon.

Measurement of glutathione

Glutathione in the tissues was analyzed via the HPLC method of Reed et al. (20). In brief, 5% perchloric acid (PCA) extract of the tissue was derivatized with 2,4-dinitrofluorobenzene, and reduced (GSH) and oxidized (GSSG) glutathione were separated by HPLC on a Spherisorb NH_2 column (particle size 5 μm, 25 cm×4.6 mm; Waters, Milford, MA, USA). To avoid the effects of artifactual oxidation of glutathione during the sample treatment process, duplicate extracts with 5% PCA containing 50 mM N-ethylmaleimide (NEM-PCA) were used to determine GSSG. GSH was determined by the difference between the amount of total glutathione measured from the PCA extract and the GSSG measured from the NEM-PCA extract. Total glutathione was expressed as GSH equivalent to the sum of GSH and GSSG, that is, GSH+2 GSSG, per mg protein. The redox status of glutathione was presented as a GSH/GSSG ratio.

Enzyme assays

Supernatants from the tissue homogenates with enzyme-specific buffers were utilized for enzyme assays. Homogenates for SOD and CAT were prepared with buffers containing 1% Triton X-100.

The activity of γ-GCL was measured by monitoring oxidation of NADH at 340 nm in reaction mixtures containing 140 mM Tris-HCl (pH 8.2), 10 units/mL lactate dehydrogenase, 10 units/mL pyruvate kinase, 75 mM KCl, 25 mM $MgCl_2$, 10 mM ATP, 5 mM L-glutamate, 10 mM α-amino-L-butyrate, 0.2 mM NADH, 0.2 mM EDTA, and 1 mM phosphoenolpyruvate (21). GPx activity was measured by monitoring NADPH oxidation at 340 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 unit/mL GRd, 1 mM GSH, and 0.25 mM H_2O_2. The reaction mixture without H_2O_2 was incubated for 5 min, and the reaction was initiated via the addition of H_2O_2 (22). GRd activity was measured by monitoring NADPH oxidation in reaction mixtures containing 100 mM potassium phosphate (pH 7.4), 2 mM GSSG, 0.6 mM EDTA, and 0.5 mM NADPH (23). G6PD activity was measured by monitoring the reduction of $NADP^+$ at 340 nm in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 2 mM

NADP$^+$, and 4 mM glucose 6-phosphate (24). SOD activity was measured by monitoring the autoxidation of epinephrine at 480 nm in reaction mixtures containing 50 mM sodium carbonate (pH 10.2), 0.1 mM EDTA, and 0.4 mM epinephrine (25). One unit of SOD indicated the quantity of enzyme required to induce a 50% inhibition of the oxidation. CAT activity was measured by monitoring the removal of H_2O_2 at 240 nm in reaction mixtures containing 50 mM potassium phosphate (pH 7.0) and 10 mM H_2O_2 (26).

Measurement of lipid peroxidation

The extent of lipid peroxidation in the tissues was measured by the method of Ohkawa et al. (27) that measures the thiobarbituric acid-reactive substances (TBARS) in samples. The thiobarbituric acid-malondialdehyde (TBA-MDA) adduct was measured by fluorescence at an excitation wavelength of 515 nm and an emission wavelength of 553 nm and was calibrated with 1,1,3,3-tetraethoxypropane, which was converted quantitatively to MDA in the reaction procedure.

Statistical analysis

Data are expressed as the means ± standard deviations (SD). The significance of the differences between the experimental and control groups was determined using the Student's t-test. P values of <0.05 were considered significant.

RESULTS

Effect of MSC on the antioxidant system in liver

In the liver, glutathione content was increased twofold by MSC treatment for 2 weeks, whereas its redox status (GSH/GSSG ratio) was not significantly affected (Fig. 1A). Among the glutathione-metabolizing enzymes, γ-GCL and GRd activities were increased significantly by MSC treatment, whereas those of GPx and G6PD were decreased (Fig. 1B). The ROS-scavenging SOD and CAT activities were also decreased significantly by MSC treatment (Fig. 1C). Therefore, MSC treatment appeared to cause an increase in glutathione content in the liver principally by stimulating its synthesis. The absence of statistical difference in the redox status of glutathione was probably attributable to the inverse changes in GRd and G6PD activities, the two enzymes that participate in the reduction of GSSG to GSH.

Effect of MSC on the antioxidant system in lung

In the lung, both the glutathione content and GSH/GSSG ratio were significantly increased by MSC treatment (Fig. 2A). However, unlike in the liver, the γ-GCL activity was not affected, whereas the enzymes involved in the glutathione recycling, namely, GPx, GRd, and G6PD, exhibited significant increases in their activities during the 2 weeks of MSC treatment (Fig. 2B). The results suggest that the MSC-induced increase in the glu-
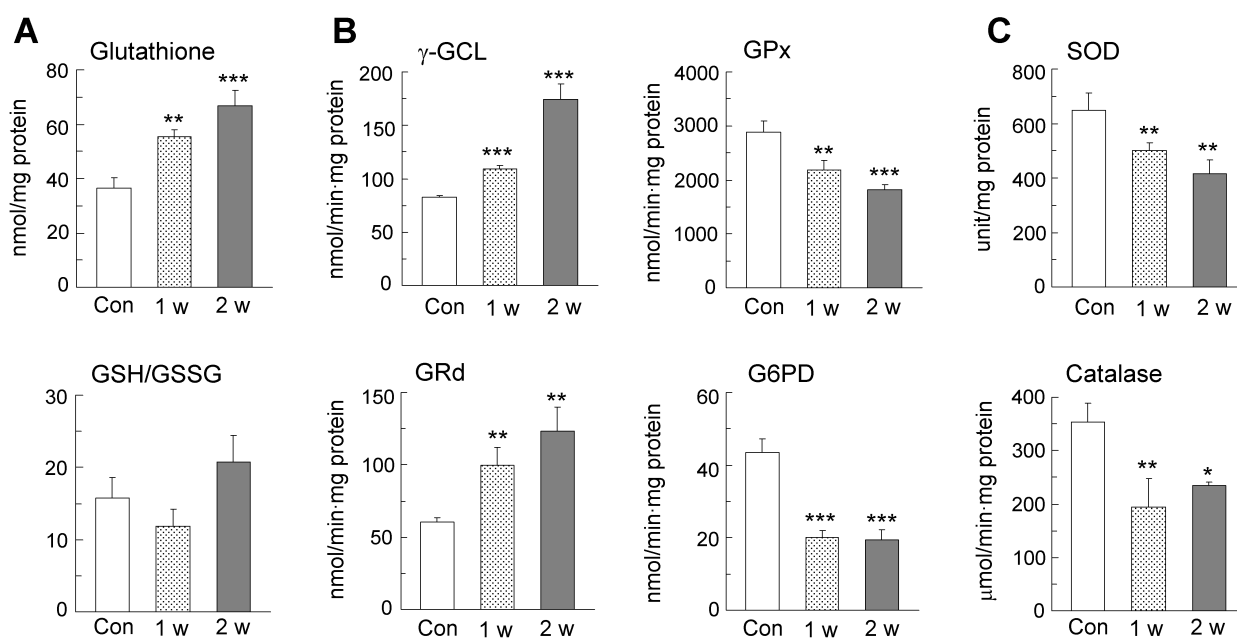

Fig. 1. Effect of Se-methylselenocysteine (MSC) treatment on the antioxidant system in rat liver. The rats received MSC for one or two weeks at a dose of 0.75 mg/rat/day. Total glutathione content and GSH/GSSG ratio (A) activities of enzymes involved in glutathione metabolism, i.e., γ-glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), glutathione reductase (GRd) and glucose 6-phosphate dehydrogenase (G6PD) (B); and activities of ROS scavenging enzymes, i.e., superoxide dismutase (SOD) and catalase (CAT) (C) in the liver were measured. Results are expressed as means ± SD (n=5). *p$<$0.05, **p$<$0.01, ***p$<$0.001.
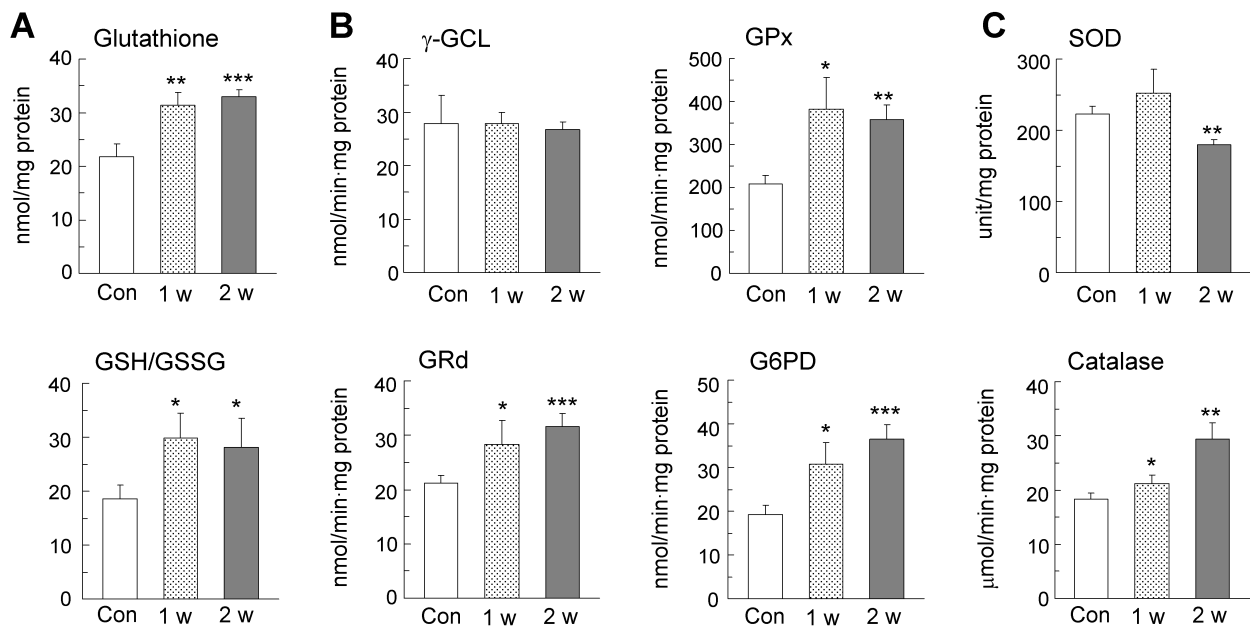
Fig. 2. Effect of Se-methylselenocysteine (MSC) treatment on the antioxidant system in rat lung. The rats received MSC for one or two weeks at a dose of 0.75 mg/rat/day. Total glutathione content and GSH/GSSG ratio (A) activities of enzymes involved in glutathione metabolism, i.e., γ-glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), glutathione reductase (GRd) and glucose 6-phosphate dehydrogenase (G6PD) (B); and activities of ROS scavenging enzymes, i.e., superoxide dismutase (SOD) and catalase (CAT) (C) in the lung were measured. Results are expressed as means ± SD (n=5). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

tathione content in the lung was not due to the stimulation of its synthesis. Instead, it appeared to be due to the stimulation of glutathione recycling, that is, reduction of GSSG, which is produced in the process of ROS removal via GPx reaction, by GRd with utilizing G6PD-generated NADPH as reducing agent. The active recycling of glutathione prevents it from being depleted due to the secretion of GSSG, eventually leading to the increase in the glutathione content. The activity of CAT was increased by MSC treatment, whereas that of SOD was decreased (Fig. 2C).

Effect of MSC on the antioxidant system in kidney

The kidney exhibited antioxidative response similar to the lung. The glutathione content and GSH/GSSG ratio were increased during the 2 weeks of MSC treatment (Fig. 3A). The activity of γ-GCL was not increased in the kidney, whereas those of GPx, GRd, and G6PD were increased. It was notable that the increase in the G6PD activity was transient, that is, it was increased at 1 week and then reverted to the basal level at 2 weeks of the treatment (Fig. 3B). Therefore, like in the lung, the elevation of glutathione content and GSH/GSSG ratio in the kidney appears to be attributable to the increased recycling of glutathione. The CAT activity was transiently increased at 1 week of MSC treatment, whereas SOD activity was decreased after receiving 2 weeks of the treatment (Fig. 3C).

Effect of MSC on the antioxidant system in spleen

Unlike the other three tissues, the spleen exhibited declines in the glutathione content and GSH/GSSG ratio at 2 weeks of MSC treatment (Fig. 4A). Among the glutathione-metabolizing enzymes, GPx activity was significantly increased during the 2 weeks of MSC treatment, and G6PD activity was transiently increased at 1 week (Fig. 4B), whereas γ-GCL and GRd activities were not affected. CAT activity was increased during the 2 weeks of MSC treatment, whereas SOD activity exhibited no statistically different change (Fig. 4C).

Effect of MSC on the lipid peroxidation in the tissues

Lipid peroxidation, a marker for oxidative stress, was assessed to probe for the relationship of oxidative stress with the antioxidative response in the tissues upon MSC treatment (Fig. 5). Lipid peroxidation level was significantly elevated not only in the spleen but also in the lung and the kidney. The lipid peroxidation levels in the lung and the kidney were transiently elevated, exhibiting decline at 2 weeks of the MSC treatment, whereas its level in the spleen continued to elevate up to 2 weeks. The liver did not exhibit increase in the lipid peroxidation level.

DISCUSSION

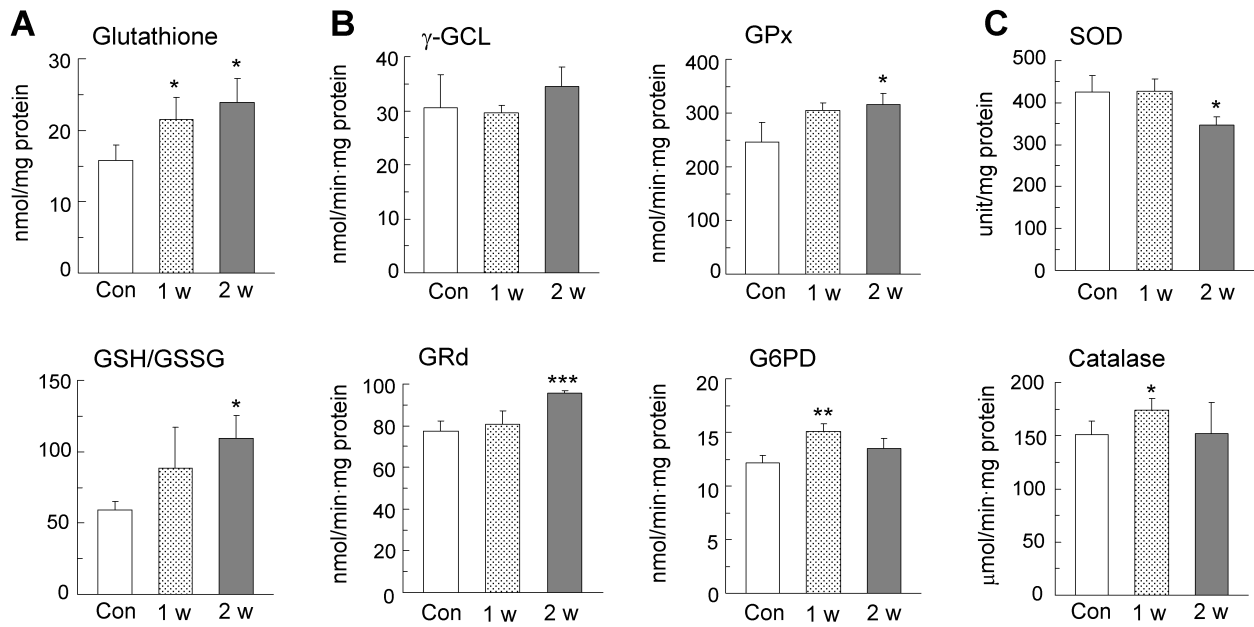The cell protective effect of Se has been demonstrated

Fig. 3. Effect of Se-methylselenocysteine (MSC) treatment on the antioxidant system in rat kidney. The rats received MSC for one or two weeks at a dose of 0.75 mg/rat/day. Total glutathione content and GSH/GSSG ratio (A) activities of enzymes involved in glutathione metabolism, i.e., γ-glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), glutathione reductase (GRd) and glucose 6-phosphate dehydrogenase (G6PD) (B); and activities of ROS scavenging enzymes, i.e., superoxide dismutase (SOD) and catalase (CAT) (C) in the kidney were measured. Results are expressed as means±SD (n=5). *p<0.05, **p<0.01, ***p<0.001.
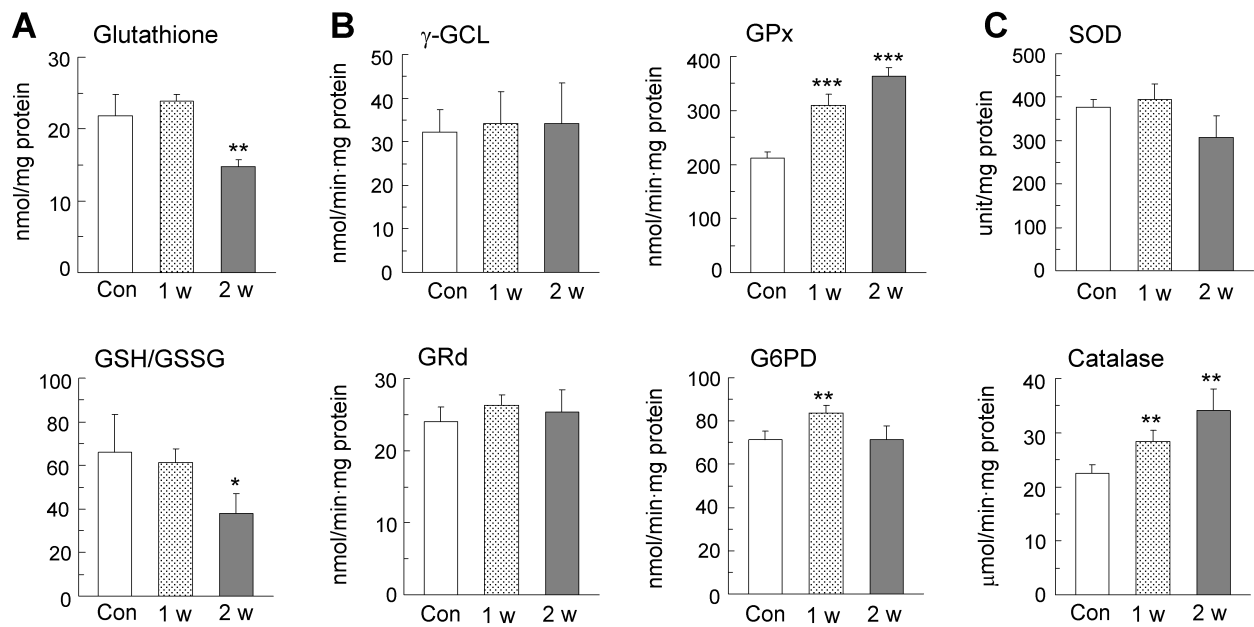

Fig. 4. Effect of Se-methylselenocysteine (MSC) treatment on the antioxidant system in rat spleen. The rats received MSC for one or two weeks at a dose of 0.75 mg/rat/day. Total glutathione content and GSH/GSSG ratio (A) activities of enzymes involved in glutathione metabolism, i.e., γ-glutamylcysteine ligase (γ-GCL), glutathione peroxidase (GPx), glutathione reductase (GRd) and glucose 6-phosphate dehydrogenase (G6PD) (B); and activities of ROS scavenging enzymes, i.e., superoxide dismutase (SOD) and catalase (CAT) (C) in the spleen were measured. Results are expressed as means±SD (n=5). *p<0.05, **p<0.01, ***p<0.001.

in various studies, and a role for the Se as an antioxidant has been suggested. In this report, we determined the systemic difference in the mode of antioxidative re-sponse among tissues to the supranutritional admin-istration of MSC (0.75 mg/rat/day). The dose of MSC used in this study was chosen based on the previous
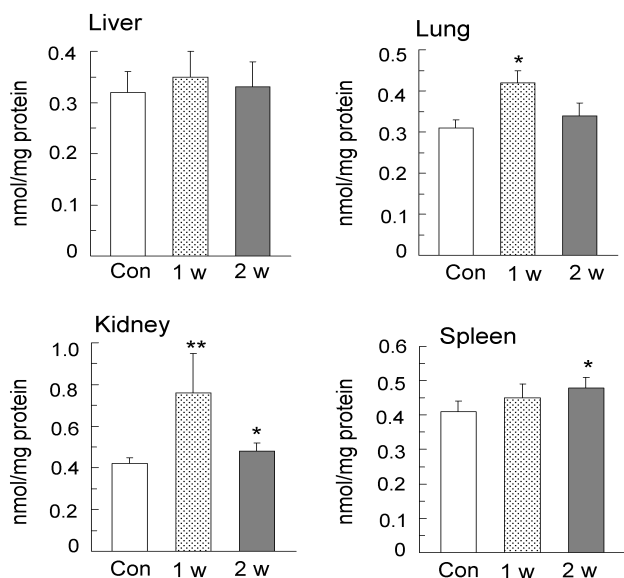
Fig. 5. Effect of Se-methylselenocysteine (MSC) treatment on lipid peroxidation level in rat tissues. The rats received MSC for one or two weeks at a dose of 0.75 mg/rat/day. Lipid peroxidation levels in the tissues of the rats were analyzed by measuring malondialdehyde (MDA) as thiobarbituric acid-eactive substances (TBARS). Results are expressed as means$\pm$SD (n=5). $^*p<0.05$, $^{**}p<0.01$.

reports. It was reported that LD_{50} of sodium selenate in mice was 3.6 mg/kg body weight, which is equivalent to about 20 μmol Se/kg body weight (28). Azrak et al. (10) reported that tissue and plasma selenium concentration was MSC dose-dependent up to 0.2 mg/mouse (body weight 20~25 g)/day. Also, our previous study demonstrated that 2-week MSC pretreatment of rats, at a dose of 0.75 mg/rat/day, prevented alloxan-induced hyperglycemia (19). In the current experiments, rats did not show any apparent sign of stress due to the MSC administration. The dose-dependency of MSC treatment was not examined in this study.

The results from the current study demonstrated several findings, the first of which was that MSC treatment resulted in an increase in glutathione contents in the liver, lung, and kidney. An increase in the GSH/GSSG ratio was also exhibited in the lung and the kidney. The results indicate that the MSC treatment increased antioxidant capacity of those tissues. On the contrary, the MSC treatment induced a decrease in the glutathione content and the GSH/GSSG ratio in the spleen, which indicates that the spleen was under increased oxidative stress due to the MSC treatment. The second observation was that, among the tissues exhibiting an MSC-induced increase in glutathione content, only the liver displayed increased γ-GCL activity. γ-GCL is the rate-limiting enzyme in the glutathione synthesis pathway. In the other two tissues, the lung and the kidney, activities of the enzymes

associated with glutathione recycling, that is, GPx, GRd, and G6PD, were significantly increased instead. Thirdly, differences were also noted among tissues in the response of SOD and CAT, the two major ROS-scavenging enzymes. The SOD activity was decreased in all tissues, while CAT activity was increased in all tissues except for the liver. The negative correlation between SOD, which catalyzes dismutation of superoxide to hydrogen peroxide and molecular oxygen, and GPx and CAT, which scavenge hydrogen peroxide, may imply different roles of these enzymes and the relative importance of hydrogen peroxide removal for antioxidant protection. The imbalance between SOD and CAT or GPx was also evidenced in previous studies with human (29) and rats (30).

The tissue-dependent nature exhibited in the mode of antioxidative response to the MSC treatment may be related to the function of each tissue in glutathione metabolism. Glutathione is the most important non-protein thiol in living systems. It is a fairly ubiquitous substance in aerobic life forms and generally exists in millimolar concentrations. Participation of glutathione in detoxifying the xenobiotic conjugation reaction as well as peroxide reduction is essential for cell survival. Glutathione participates in ROS removal by either GPx reaction or nonenzymatic direct reaction. Liver and kidney play a major role in the homeostasis of glutathione. Once synthesized, glutathione can undergo transport across biological membranes, to become part of a complicated interorgan transport network. The liver is the major organ for glutathione synthesis and export. The efflux of glutathione from the liver contributes >90% of total glutathione inflow into the circulation (31). The plasma glutathione can be used through the γ-glutamyl cycle in extra-hepatic tissues rich in γ-glutamyltranspeptidase (γ-GT). The kidney is the most important organ for excretion and homeostasis, and is a common target for a variety of toxic agents due to its metabolic capacity for concentrating toxicants and/or metabolites during the excretory process. The kidney can take up glutathione through either γ-GT-mediated breakdown of γ-glutamyl bonds and subsequent resynthesis inside the cell or intact glutathione uptake by active transport. The kidney contains the highest activity of γ-GT, which makes it the most important tissue for turnover of plasma glutathione (32). The lung is directly exposed to higher levels of oxygen than most other tissues. The healthy lung is efficiently protected and buffered against exogenous oxidants (33). The lung epithelium has also been shown to have higher level of γ-GT activity, and it utilizes extracellular glutathione from plasma and alveolar lining

fluid (34). The notion suggested from our results, that is, MSC-induced increase in glutathione content in the liver is principally associated with stimulated glutathione synthesis, whereas that in the lung and kidney is associated with stimulated recycling, appears to correlate with the assumed function of the respective tissues in antioxidative glutathione metabolism.

The spleen exhibited a unique response among the tissues, in which both the glutathione content and GSH/GSSG ratio decreased significantly by 2 weeks of MSC treatment. These data suggest that the spleen was under increased oxidative stress. The increase in lipid peroxidation levels in the tissues of MSC-treated rats supports the role for MSC as a prooxidant. The prooxidant function of Se has been reported in many studies regarding cancer chemoprevention (35) and toxicity (36) of Se compounds. Furthermore, our results suggest that the antioxidant induction by Se administration might be closely associated with its prooxidant nature. Although the MSC itself is not an oxidizing agent, methylselenol generated from MSC via β-lyase reaction is known to produce ROS through the reaction with O_2 (37). The increase in the antioxidant capacity exhibited in the liver, kidney, and lung appears to be a result of adaptive response to the oxidative stress provoked by MSC. The transient nature of the lipid peroxidation increase shown in the lung and the kidney, that is, reversion of the lipid peroxidation at 2 weeks of MSC treatment, evidenced the consequence of the increased antioxidant capacity. In that sense, the spleen provides an example for the organs that have low oxidative damage threshold, while the liver provides an example of the other extreme, meaning it has a superb capacity of inducing antioxidant activities, particularly with glutathione metabolism, and, therefore, shows no accumulation of lipid peroxidation during the course of MSC treatment. The spleen is the largest organ of the lymphatic system. Cells of the immune system are particularly sensitive to changes in the antioxidant status because they carry out important functions through the generation of a large amount of ROS. Moreover, the cells of the immune system have high content of polyunsaturated fatty acids in their plasma membranes (37). The characteristics of the spleen suggest that it might be particularly sensitive to the oxidative stress condition. Even in the spleen, however, the induction of antioxidant enzymes such as GPx, G6PD, and CAT was evidenced, but their levels were not enough to protect the cells from oxidative damage provoked by MSC.

In conclusion, our study showed that supranutritional MSC administration induced increases in the antioxidant system in the tissues of rats, and that this effect appeared to be tissue-specific. The increased antioxidant capacity exhibited in the liver, lung, and kidney appeared to be a result of the adaptive response to oxidative stress provoked by MSC, which is evidenced by the transient increases in the lipid peroxidation level. Although the increase in the antioxidant enzymes were also exhibited in the spleen, the decrease in glutathione and persistent increase in the lipid peroxidation indicate that the spleen was under increased oxidative stress provoked by MSC treatment at the dose used in this study. Therefore, different tissues might require different levels of MSC to acquire antioxidative protection. Our results suggest that the tissue difference should be an important factor when the Se compounds are considered as normal cell-protective agents. An appropriate, tissue-specific dose could be found to support normal cell protection while fortifying abnormal cell toxicity of Se chemotherapeutic agents.

ACKNOWLEDGEMENTS

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