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Effect of Selenium on Rat Thioredoxin Reductase Activity

Increase by Supranutritional Selenium and Decrease by Selenium Deficiency

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ABSTRACT. Thioredoxin reductase is a newly identified selenocysteine-containing enzyme that catalyzes the NADPH-dependent reduction of the redox protein thioredoxin. Thioredoxin stimulates cell growth, is found in dividing normal cells, and is over-expressed in a number of human cancers. Redox activity is essential for the growth effects of thioredoxin; thus, thioredoxin reductase could be involved in regulating cell growth through its reduction of thioredoxin. In rats fed a selenium-deficient diet (<0.01 ppm) for up to 98 days, thioredoxin reductase activity was decreased, compared with that of rats fed a normal selenium diet (0.1 ppm), in lung, liver, and kidney, while thioredoxin reductase activity in the spleen and prostate was unaltered. Rats fed a high selenium diet (1.0 ppm) exhibited a 1.5-fold increase in kidney and a 2.0-fold increase in lung thioredoxin reductase activity that began to return to control values after 20 and 69 days, respectively. Liver showed a 2.1-fold increase in thioredoxin reductase activity at 20 days only. Thioredoxin reductase protein levels measured by western blotting using an antibody to human thioredoxin reductase were decreased in rats fed the selenium-deficient diet and did not increase in rats fed the high selenium diet. Rat thioredoxin reductase was shown to incorporate ^{75}Selenium. Thus, in some tissues at least, the increase in thioredoxin reductase activity of rats fed a high selenium diet appears to be due to an increase in the specific activity of the enzyme, possibly caused by increased selenocysteine incorporation without an increase in thioredoxin reductase protein synthesis. BIOCHEM PHARMACOL **57**;2:187–193, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. thioredoxin reductase; glutathione peroxidase; selenium

Se† is a biologic trace element that is necessary for cell growth [1]. Animal studies have shown that supranutritional doses of dietary Se decrease the incidence of spontaneous, viral, and chemically induced tumors [2]. Epidemiologic studies in humans have shown a consistent trend for populations having a low Se intake to have an increased incidence of a variety of cancers [3, 4]. A recent landmark human intervention study [5] that was double-blind, placebo-controlled, and randomized, involving a total of 1312 patients with a mean follow-up of over 6 years, found that oral administration of 200 μg Se/day, which is between 3 and 4 times the recommended daily allowance, results in a significant reduction in total cancer mortality and an over 50% decrease in the incidence of lung, prostate, and colon cancer.

Mechanisms that have been suggested to explain the cancer preventive activity of Se include stimulation of the immune system [6], alterations in carcinogen metabolism [7], an increase in GSSG leading to inhibition of protein synthesis [8], the formation of cytotoxic methylated metabolites of Se [9], the induction of apoptosis due to altered polyamine metabolism [10], and protection by glutathione peroxidase against oxidative damage [11]. Glutathione peroxidase is a Se-containing enzyme that decreases the levels of reactive oxygen species, and it was suggested that a Se-dependent increase in glutathione peroxidase activity might prevent reactive oxygen species-induced DNA damage and, thus, protect against the development of cancer [11]. However, animal studies revealed that cytosolic glutathione peroxidase activity was already increased maximally in tissues of animals fed a normal Se diet, where Se is below the levels necessary for cancer prevention [12–14]. Another Se-containing enzyme, type 1 deiodinase, is also maximally active at normal levels of dietary Se [12]. These findings led some investigators to conclude that selenoproteins are unlikely to be involved in the cancer preventive activity of Se [15].

There are two ways Se can be incorporated into proteins. First, it can be incorporated nonspecifically as selenomethionine from the diet, which is not distinguished by mammals from methionine [16]. This incorporation is random, only occurs at high levels of selenomethionine intake, and has no known Se-related biologic function. Second, Se can be incorporated specifically in the form of

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† *Abbreviations:* Se, selenium; and SeCys, selenocysteine.

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the amino acid SeCys [17]. All SeCys-containing enzymes identified to date catalyze oxidation-reduction reactions in which SeCys forms part of the active site [17]. Mammalian SeCys-containing proteins that have been characterized are cellular and plasma glutathione peroxidases, phospholipid hydroperoxide glutathione peroxidase, type 1, 2, and 3 deiodinases, and selenoproteins P and W of unknown function [17]. The most recently identified selenoenzyme is thioredoxin reductase [18].

Thioredoxin reductase is a SeCys-containing flavoprotein that catalyzes the NADPH-dependent reduction of the redox protein thioredoxin [19]. Thioredoxin is necessary for embryonic development [20] and is found at high levels in dividing normal cells.* It is also over-expressed by a number of human primary tumors [21, 22 ,*]. Experimental studies have shown that thioredoxin offers a growth advantage to tumors by suppressing apoptosis [23]. Redox activity is necessary for the growth-stimulating activity of thioredoxin [24]. The only known way for thioredoxin to be reduced is by thioredoxin reductase [25], so that thioredoxin reductase could play a role in regulating the redox activity of thioredoxin and, thus, could indirectly affect cell growth.

We have reported previously that Se increases the activity of thioredoxin reductase in human cancer cell lines by increasing the incorporation of SeCys, leading to an increase in the specific activity of the enzyme, and, to a lesser extent, by increasing thioredoxin reductase protein levels [26]. The increase in thioredoxin reductase activity occurred over a range of Se concentrations from <0.01 to 10 μM, which encompasses the range of Se concentrations found in human serum (between 1 and 5 μM) [27]. We now report the effects of Se at both below and above normal dietary levels on thioredoxin reductase protein and activity in rat tissues. The demonstration of an increase in thioredoxin reductase activity at supranutritional levels of dietary Se, where cancer prevention occurs, could provide correlative evidence to support the hypothesis that thioredoxin reductase is involved in the cancer preventive activity of Se.

MATERIALS AND METHODS

Animals

Torula yeast, which is low in Se, was used as the base for the Se modified diets [28]. Diets were prepared by Harlan Teklad based on their TD 92163 Se-deficient Torula yeast diet with additional Se added as sodium selenite. Male 6-week-old Sprague-Dawley rats were fed one of three diets: a low Se diet with <0.01 ppm Se (TD 92163); a normal Se diet with 0.1 ppm Se; and a high Se diet with 1.0 ppm Se. Groups of four rats fed the diets were killed at 0, 20, 40, 69, and 98 days. Blood was collected by cardiac puncture into heparinized tubes, and plasma was prepared and stored frozen at −80°. The liver, lung, kidney, spleen, and prostate were removed rapidly and frozen in liquid N_2 until assay.

For studies of ^{75}Se incorporation into thioredoxin reductase, a 200 g male rat fed a normal 0.1 ppm Se diet was injected i.p. daily for 2 days with 100 μCi ^{75}Se (sp. act. 1.95 mCi/mg of Se) obtained from the University of Missouri Research Reactor Facility. The animal was killed at 48 hr, and the liver, lung, and kidney were removed. All animal studies were IACUC approved by the University of Arizona Animal Care Committee.

Assays

Tissues were homogenized for 1 min in 4 vol. of 50 mM HEPES buffer, pH 7.6, 5 mM EDTA using a PowerGen model 700 biohomogenizer (Fisher Scientific), and the mixture was centrifuged at 110,000 *g* for 1 hr to provide a supernatant fraction. To measure thioredoxin reductase accurately in tissue supernatants, we found that it was necessary to first remove endogenous thioredoxin and other small molecular weight reductants. To do this, 0.2-mL aliquots of supernatant were mixed with 0.2 mL of fresh adenosine 2′,5′-diphosphate coupled-agarose beads (ADP-agarose) (Sigma Chemical Co.) for 1 hr at 4°.The beads were washed by recentrifugation at 1000 *g* with 2 × 1 mL of 0.1 M NaCl, and then thioredoxin reductase was eluted with 0.5 mL of 1.0 M KCl. Preliminary studies showed that all of the thioredoxin reductase activity in the supernatant was recovered by this procedure. Thioredoxin reductase activity was measured spectrophotometrically, by a modification [29] of the method of Holmgren [30], as the oxidation of NADPH at 339 nm, using 5 μM human recombinant thioredoxin [22] as substrate and oxidized insulin as the final electron acceptor. Supernatant glutathione peroxidase activity was measured spectrophotometrically at 339 nm as the NADPH/glutathione reductase-linked H_2O_2-dependent oxidation of glutathione [31]. Both thioredoxin reductase and glutathione peroxidase activities were expressed as nanomoles NADPH oxidized per minute per milligram of supernatant protein.

Western Blotting

A rabbit polyclonal antiserum (M2098) against a synthetic peptide to an amino acid protein sequence of human thioredoxin reductase, Phe^{155}-Leu-Ileu-Ala-Thr-Gly-Gly-Arg-Pro^{163}, was used for western blotting [32]. The same amino acid sequence is found in rat thioredoxin reductase [33], and the antiserum recognized a protein present in rat tissues with the same size, 55 kDa, as human thioredoxin reductase. The amino acid sequence is not found in glutathione reductase, which was not recognized by the antiserum. Western blots of tissue supernatants were visualized using the Renaissance chemiluminescence system (DuPont NEN) and quantitated using a PhosphorImager (Molecular Dynamics). Standards of human thioredoxin reductase [29] were run on each blot and used to quantify the levels of rat immunoreactive protein.

* Grogan T, Fenoglio-Priser C, Zaheb R, Bellamy W, Vela E, Richter L and Powis G, Manuscript submitted for publication.

^{75}Se Incorporation

To measure the incorporation of ^{75}Se into rat thioredoxin reductase, 0.5 mg of supernatant protein from the liver, lung, and kidney of the ^{75}Se-treated rat was mixed for 4 hr at 4° with either 0.2 mL of ADP-agarose beads or with M2098 rabbit human thioredoxin reductase antibody bound to protein A-agarose beads (Sigma Chemical Co.). The beads were washed five times with 20 mM Tris buffer (pH 8.0), 137 mM NaCl, 10% glycerol, 0.1% Triton X-100, and then heated at 100° for 10 min in 0.5 M Tris buffer (pH 6.8), 10% SDS, 20% glycerol, 0.1% bromophenol blue, and 3% β-mercaptoethanol to release and denature attached proteins prior to SDS 7.5% PAGE. Blots were transferred to a polyvinylidene difluoride membrane, and radioactivity was measured by autoradiography. Thioredoxin reductase was measured by western blotting using M2098 rabbit polyclonal antibody to human thioredoxin reductase as described above.

Selenium Assay

Serum Se was measured by atomic absorption spectroscopy on a Zeeman 3030 spectrometer (Perkin–Elmer).

RESULTS

Serum Selenium and Body Weight Changes

Feeding rats either a low (<0.01 ppm) Se diet or a high (1 ppm) Se diet had no effect on the increase in body weight compared with rats fed the control (0.1 ppm) Se diet (Fig. 1A). Selenium in the serum of rats fed the low Se diet was decreased from 2.8 to 0.7 μM by day 40, while rats fed the high Se diet showed no increase in serum Se over the time course of the study (Fig. 1B).

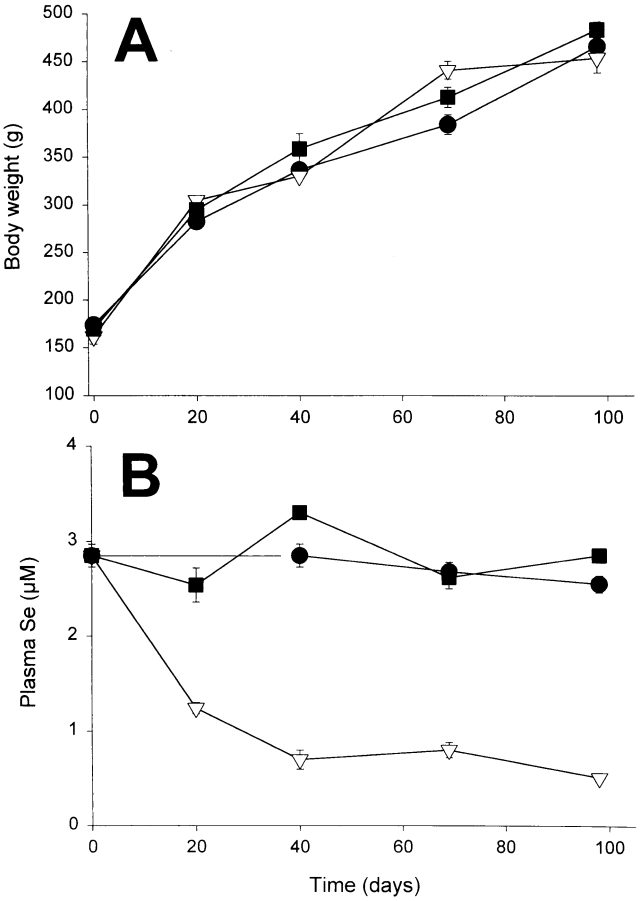

FIG. 1. Body weight (A) and plasma Se (B) of male rats fed different Se-containing diets: (▽) < 0.01 ppm Se-deficient diet, (●) 0.1 ppm control Se diet, and (■) 1.0 ppm Se-supplemented diet. Each point is the mean ± SEM of four rats.

Tissue Thioredoxin Reductase

Thioredoxin reductase activity in tissues of rats fed the Se-deficient diet fell to a level of about 1–2 nmol/min/mg irrespective of the control level of thioredoxin reductase activity (Fig. 2). The time taken for thioredoxin reductase activity to fall to this level was dependent on the level of thioredoxin reductase activity in rats fed a control Se diet. In kidney, which had the highest control levels of thioredoxin reductase activity (28.1 nmol/min/mg), the fall took 98 days; in liver, which had a control level of 15.8 nmol/min/mg, the fall took 40 days; and in lung, which had a control level of 2.3 nmol/min/mg, the fall took only 20 days. Prostate and spleen activities were measured only at 98 days, and in both cases the activity in animals fed the Se-deficient diet was not significantly different ($P > 0.05$) from the control diet values (Table 1).

Feeding rats a diet containing 1.0 ppm Se gave significant maximal increases, compared with rats fed the control diet, in thioredoxin reductase activity in rat kidney and lung of 1.5- and 2.0-fold, at days 20 to 40 and 69, respectively (Fig. 2). Thereafter, thioredoxin reductase activity declined, and in the kidney had returned to almost control values by 98 days. There was a transient increase in thioredoxin reductase activity in liver of 2.1-fold at 20 days, which had returned to control diet values by 40 days. Spleen and prostate tissue of rats fed a 1.0 ppm Se diet were measured only at 98 days. Spleen thioredoxin reductase activity showed a significant 1.4-fold increase compared with rats fed a control diet, but there was no increase in the prostate thioredoxin reductase activity (Table 1).

Thioredoxin Reductase Protein

Thioredoxin reductase tissue protein levels were measured by quantitative western blotting, and the results are shown in Table 2. Thioredoxin reductase protein levels in the lung, liver, and kidney of rats on a control Se diet varied by up to 3-fold. Thioredoxin reductase activities in the same tissues varied by up to 12-fold. Animals fed a Se-deficient diet showed significant decreases in the levels of thioredoxin protein compared with animals fed a control diet, which was most marked by 98 days. Animals fed a high Se diet did not show a significant increase in thioredoxin

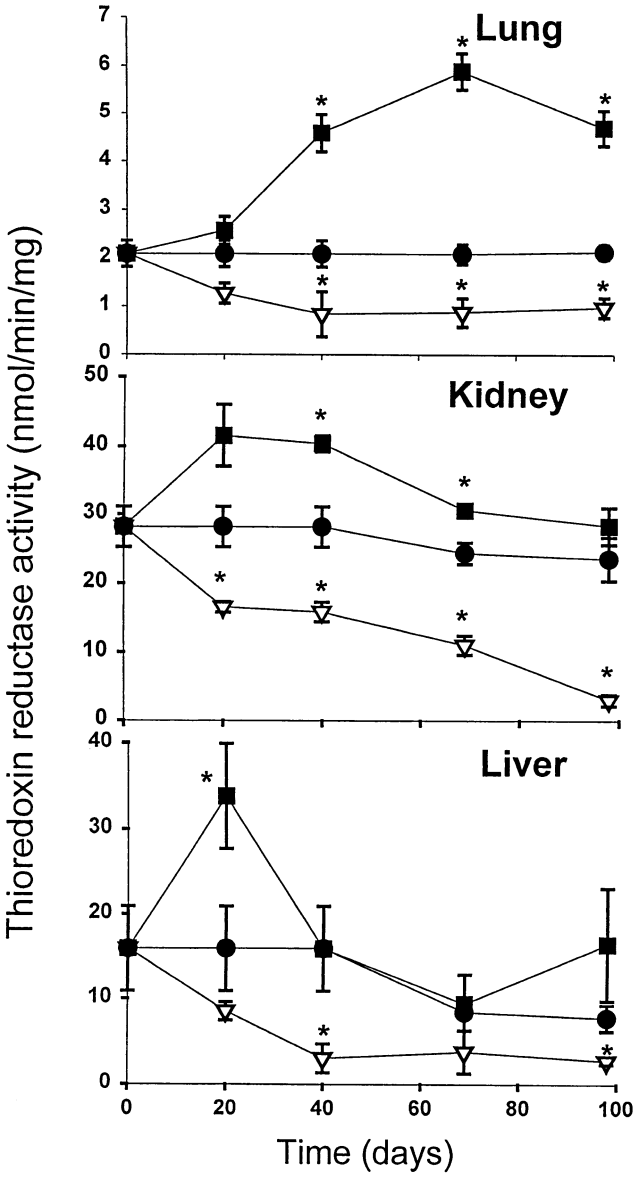

FIG. 2. Thioredoxin reductase activity in liver (A), lung (B), and kidney (C) of rats fed different Se-containing diets: (▽) <0.01 ppm Se-deficient diet, (●) 0.1 ppm control Se diet, and (■) 1.0 ppm Se-supplemented diet. Each point is the mean ± SEM of four rats. Key: *P < 0.05 compared with control (0.1 ppm) Se diet.

reductase protein in liver and kidney at 20 days, which was the time of the maximum increase in thioredoxin reductase activity for these tissues, or in liver, lung, and kidney at 98 days.

Selenium in Rat Thioredoxin Reductase

Studies were conducted to determine whether rat thioredoxin reductase contained Se. The protein was isolated by immunoprecipitation with rabbit polyclonal human thioredoxin reductase antibody or by binding to ADP-agarose from the liver, lung, and kidney of a rat fed a normal Se diet and injected with ^{75}Se. All the tissues expressed a protein that was the same size as human thioredoxin reductase and that contained ^{75}Se (Fig. 3). Liver had other ^{75}Se protein containing bands that bound to ADP-agarose and to thioredoxin reductase antibody. These may represent other forms of thioredoxin reductase, for example glycosylated, since the rat cDNA shows a putative glycosylation attachment site [33]. Kidney showed ^{75}Se incorporation into protein bound by ADP-agarose but not bound by thioredoxin reductase antibody. This may be because rat kidney contains an immunologically distinct form of thioredoxin reductase that is not recognized by the antibody we used.

Glutathione Peroxidase Activity

The activity of cytosolic glutathione peroxidase in the liver, lung, and kidney of rats on the different Se diets was measured for purposes of comparison (Fig. 4). In all three tissues, glutathione peroxidase activity showed a significant decline in animals fed a Se-deficient diet, reaching a minimum by 40 days for liver and by 98 days for kidney and lung. There was also a significant decrease in supernatant glutathione peroxidase activity of spleen and prostate after 98 days on a low Se diet (Table 1). There was no increase in the glutathione peroxidase activity of lung, liver, kidney, spleen, or prostate of animals fed a 1.0 ppm Se diet compared with rats fed the control Se diet (Fig. 4 and Table 1).

DISCUSSION

Decreased activity of a number of selenoenzymes has been observed under conditions of low dietary Se [13, 14, 16, 31, 34, 35]. A recent study has reported that thioredoxin reductase activity is decreased in the liver and kidney, but not the brain, of rats fed a Se-deficient diet [36]. In the present study, we found that a Se-deficient diet (<0.01 ppm) decreased the levels of thioredoxin reductase activity in some, but not all, rat tissues compared with the same tissues from rats fed a control (0.1 ppm Se) diet, to a lower limit of 1–2 nmol/min/mg. The time to fall to this lower limit was longer for a tissue such as kidney with a high normal thioredoxin reductase activity, than for a tissue such as lung with a lower normal activity. Thioredoxin reductase activity was not decreased significantly in spleen or prostate tissue, which, in rats fed a normal Se diet, was already at the lower limit.

TABLE 1. Thioredoxin reductase (TR) and glutathione peroxidase (GPx) activities in rat spleen and prostate on day 98*

Se diet (ppm)	Spleen		Prostate	
	TR	GPx	TR	GPx
<0.01	2.0 ± 0.4	218.7 ± 13.1†	1.7 ± 0.2	84.2 ± 3.3†
0.1	2.6 ± 0.2	541.3 ± 20.0	2.2 ± 0.9	150.0 ± 21.8
1.0	4.9 ± 0.3†	568.4 ± 28.2	3.0 ± 0.7	168.0 ± 3.6

*Values are means ± SEM of four rats and are expressed in nmol/min/mg of supernatant protein for both TR and GPx.

†$P < 0.05$ compared with the control (0.1 ppm) Se diet.

TABLE 2. Thioredoxin reductase (TR) immunoreactive protein in rat tissues*

Se diet (ppm)	Human TR (μg/mg cytosolic protein)				
	Liver		Kidney		Lung
	20 day	98 day	20 day	98 day	98 day
<0.01	1.9 ± 0.1†	0.0 ± 0.0†	0.7 ± 0.0†	0.1 ± 0.0†	1.4 ± 0.2†
0.1	3.8 ± 0.5	1.2 ± 0.8	1.3 ± 0.0	0.8 ± 0.0	3.4 ± 0.2
1.0	3.6 ± 1.0	2.0 ± 0.3	1.5 ± 0.1	0.8 ± 0.0	4.3 ± 0.8

*Values are means ± SEM of four rats.
†$P < 0.05$ compared with the control (0.1 ppm) Se diet.

Previous studies have shown that when animals are fed a diet with levels of Se of 1 ppm or more, the activity of the selenoenzymes glutathione peroxidase [13, 14, 16, 31, 35] and type 1 deiodinase [12] are not increased above the activity found in animals fed a normal Se diet. In contrast, we found that a 1 ppm Se diet increased rat thioredoxin reductase activity by 1.5-, 2.0-, and 2.1-fold in kidney, lung, and liver, respectively. However, the increase in thioredoxin reductase activity was not sustained, and activity levels began to decrease after 69 days in lung and after 20 days in kidney and liver. Liver showed the most rapid return to control values, which was complete by 40 days. Thus, thioredoxin reductase appears to differ from other selenoenzymes in showing an increase in activity in rats fed a diet containing Se above normal values, but the increase is not always sustained.

Two different mechanisms appear to account for the alteration in thioredoxin reductase activity by Se in rat tissues. A Se-deficient diet caused a decrease in the amount

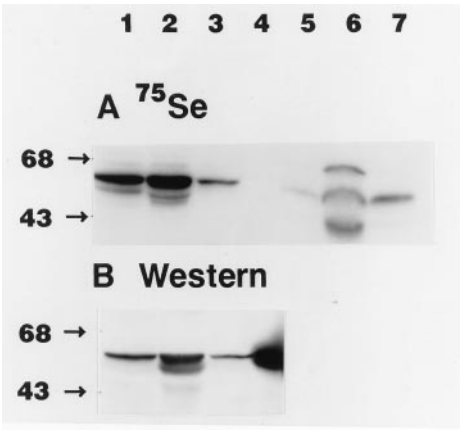

FIG. 3. Incorporation of ^{75}Se by rat tissue thioredoxin reductase. Tissue supernatant protein (0.5 mg) from a rat on a normal 0.1 ppm Se diet administered ^{75}Se for 2 days was treated with either ADP-agarose or human thioredoxin reductase antibody-protein A-agarose beads. The beads were washed, and denatured proteins were subjected to SDS-PAGE. Lanes are: protein from (1) kidney, (2) liver, and (3) lung, collected on ADP-agarose beads; (4) 0.3 μg human thioredoxin reductase standard; protein from (5) kidney, (6) liver, and (7) lung collected on thioredoxin reductase antibody-protein A-agarose beads. (A) Autoradiograph showing ^{75}Se. (B) Western blot using human thioredoxin reductase antibody detected by chemiluminescence. It was not possible to measure immunoaffinity collected thioredoxin reductase by this method because of high background caused by antibody proteins. The position of molecular weight markers in kDa is shown on the left.

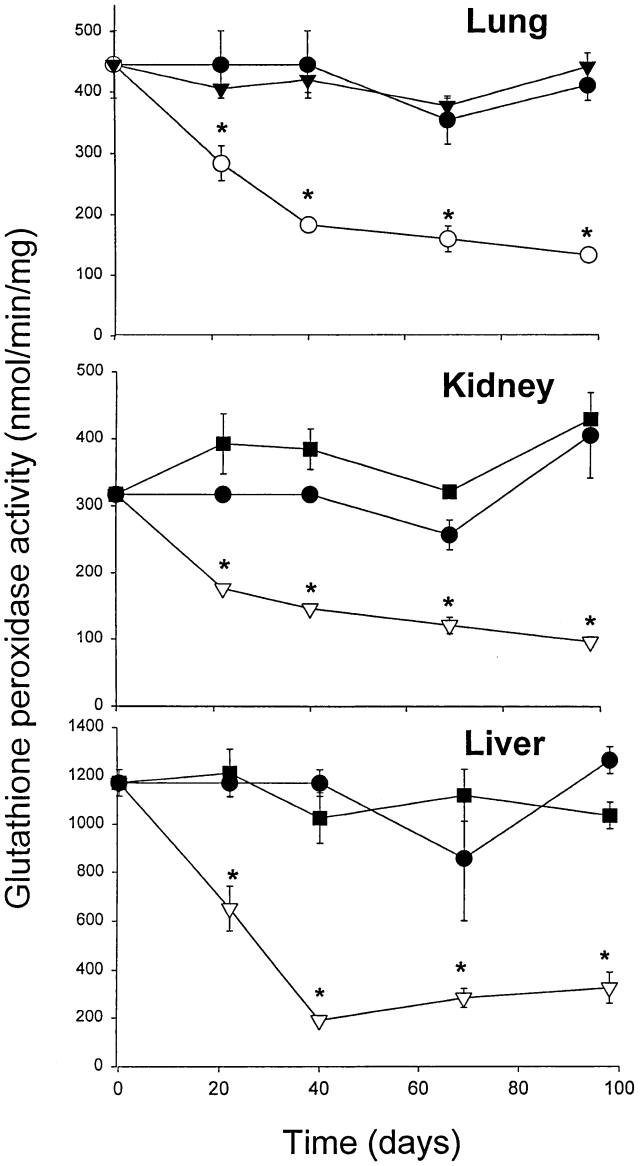

FIG. 4. Cytosolic glutathione peroxidase activity in tissues of rats fed different Se-containing diets. (○) (upper panel) and (▽) (middle and lower panels) < 0.01 ppm Se-deficient diet, (●) 0.1 ppm control Se diet, and (■) 1.0 ppm Se-supplemented diet. Each point is the mean ± SEM of four rats. Key: *$P < 0.05$ compared with control (0.1 ppm) Se diet.

of thioredoxin reductase protein by 98 days to around 10% of control values in the liver and kidney, while levels in the lung fell to 37% of control values. In none of the examined tissues of rats fed the high Se diet was there a significant increase in thioredoxin reductase protein compared with rats fed the control Se diet. In a previous study using cultured cells, we showed that the increase in thioredoxin reductase activity caused by Se was due primarily to an increase in the specific activity of the enzyme accompanied by an increase in the incorporation of SeCys into the protein, with a smaller effect of Se in increasing thioredoxin reductase protein levels [26]. The present *in vivo* results suggest that the decrease in thioredoxin reductase activity at normal and lowered levels of dietary Se may be due to a decrease in thioredoxin reductase protein and to a decrease in the specific activity of the enzyme, presumably due to decreased Se incorporation. Using ^{75}Se, we were able to show that rat thioredoxin reductase contained Se. A recent study has reported a cDNA sequence for a rat thioredoxin reductase with homology to the sequence for human thioredoxin reductase giving a putative carboxyl terminus selenocysteine residue as in the human enzyme [33]. In contrast, the increase in thioredoxin reductase activity at high dietary Se was not accompanied by an increase in thioredoxin reductase protein synthesis and was due solely to an increase in the specific activity of the enzyme. A caveat to this interpretation of the results is that some tissues, particularly kidney and liver, have higher levels of thioredoxin reductase activity than would be expected by the thioredoxin reductase protein levels, suggesting that there could be tissue differences in the regulation of Se incorporation into thioredoxin reductase or that there are other forms of thioredoxin reductase not detected by the antibody.

In summary, our studies have shown that a Se-deficient diet results in a decrease in thioredoxin reductase activity in some, but not all, rat tissues, reaching the same low value by 98 days. The decrease in thioredoxin reductase activity was due to a decreased specific activity of the enzyme and to a decreased protein synthesis. Selenium at supranutritional levels resulted in an over 2-fold increase in thioredoxin reductase activity in some, but not all, rat tissues, that was maintained better in some tissues than in others. The increase in thioredoxin reductase activity appears to be due solely to an increase in the specific activity of the enzyme, possibly due to an increased incorporation of Se.

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