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Influence of selenium status on activity of phospholipid hydroperoxide glutathione peroxidase in rat liver and testis in comparison with other selenoproteins

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Selenium-deficient rats (−Se, fed a Torula yeast-based diet containing no added selenium for 6 weeks) were injected intraperitoneally with up to 50 μg selenium per kg bodyweight (BW) and sacrificed 6 or 12 hr later. Control rats were fed a similar diet with 0.25 mg Se/kg diet added as sodium selenate. Phospholipid hydroperoxide glutathione peroxidase (phGSH-Px) and cellular glutathione peroxidase (cGSH-Px) activities were determined in liver and testis. Extracellular glutathione peroxidase (eGSH-Px) activity and selenoprotein P level were measured in plasma. Liver phGSH-Px activity in control rats was small in comparison with liver cGSH-Px activity. Much of the phGSH-Px activity measured in liver (especially under −Se conditions) was accounted for by non-specific NADPH oxidation, which was measurable in the absence of any added substrate in the reaction vial, or when a non-reactive substrate analogue was used. Gross activity of liver phGSH-Px fell only to 76% of control values in selenium deficiency and showed little response to selenium injection. Liver cGSH-Px and plasma eGSH-Px activities in −Se rats were reduced to <2% of control values under the same conditions, increasing after selenium injection only to 2 to 3% of control. Selenoprotein P level in plasma fell to 7% of control levels in −Se rats, returning to a maximum of 43% of control by 12 hr after injection of the highest selenium dose. In testis, phGSH-Px and cGSH-Px fell only to 65% and 45% of control values, respectively, and did not increase significantly in response to resupplementation of selenium under the conditions of this experiment. Based on activity levels, phGSH-Px appears to be of greater relevance in testis than liver. Activity of phGSH-Px in either tissue showed little change with selenium status. None of the peroxidases measured responded as strongly to short-term selenium repletion as did selenoprotein P. (J. Nutr. Biochem. 7:333–338, 1996.)

Keywords: selenium; phospholipid hydroperoxide glutathione peroxidase; cellular glutathione peroxidase; extracellular glutathione peroxidase; selenoprotein P; rat; liver; testis

Introduction

Phospholipid hydroperoxide glutathione peroxidase (phGSH-Px) is able to reduce esterified phospholipid hydroperoxides in addition to free hydroperoxides.[1,2] In contrast, the classical cellular glutathione peroxidase (cGSH-Px, E.C. 1.11.1.9) catalyzes the reduction of hydrogen peroxide and free lipid hydroperoxides, but reduction of phospholipid hydroperoxides by cGSH-Px is only possible after release of free lipid hydroperoxides by the action of phospholipase A_2.[3] Extracellular glutathione peroxidase (eGSH-Px) is a distinct protein from the intracellular enzyme,[4] and is able to reduce esterified phospholipid hy-

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droperoxides.[5,6] All three of these peroxidase enzymes use glutathione as a source of reducing equivalents, and catalyze the reduction of hydroperoxides by similar kinetics.[2,7]

Determination of the cDNA sequence, and peptide analysis have confirmed the presence of an in-frame TGA codon for selenocysteine in the monomeric phGSH-Px protein,[8,9,10] analogous to the presence of a single selenocysteine residue per peptide monomer in the tetrameric extra- and intracellular GSH-Pxs. The gene for human phGSH-Px has been mapped to chromosome 19,[11] whereas the human cGSH-Px gene has been mapped to chromosome 3,[12] further indicating that these proteins represent distinct gene products.

Phospholipid hydroperoxide glutathione peroxidase activity has been detected in various tissues including liver, testis, heart, lung, kidney, and retina, in numerous mammalian species including pig, rat, mouse, and in human tumour cell lines in culture.[1,13–16] Both soluble and membrane-bound forms of the enzyme from rat testes have been described.[17]

The effects of varying selenium levels on the activity of phGSH-Px have been studied in several isolated cell systems in vitro.[15,18,19] In vivo effects of selenium depletion and long-term repletion on phGSH-Px activity in various mouse organs have been described.[14] Selenium deficiency caused little change in mRNA levels for phGSH-Px in rat liver,[9] but phGSH-Px activity was not addressed in that report. Activity of phGSH-Px has been shown to decrease significantly in rat liver in response to selenium deficiency, but not in testis.[20] However, there appear to be no reports to date regarding the influence of short-term selenium dosing of selenium-deficient rats on the activity of phGSH-Px.

We investigated the effects of selenium depletion and short-term repletion on the activity of phGSH-Px in rat liver and testis. The liver is a central organ in the production of many plasma proteins, including Se-P and eGSH-Px, and contains both cGSH-Px and phGSH-Px. Testis has been shown to contain high levels of phGSH-Px activity in rats.[16] The effects of similar depletion and repletion regimens on the activities of cGSH-Px and eGSH-Px, and on the level of selenoprotein P (Se-P) in rats have been reported previously.[21,22] In the present study, these proteins were investigated to allow comparison with effects on phGSH-Px activity.

Methods and materials

Diets

A Torula yeast-based diet was formulated and supplemented with 0.3% methionine.[23] The basal diet was selenium deficient for rats, so the control diet had 0.25 mg Se/kg diet added as sodium selenate. A level of 0.1 mg Se/kg diet has previously been shown to support maximal activity of cGSH-Px.[21] Diet and tap water were available to all rats ad libitum. Rats were fed the selenium-deficient diet (−Se) or control diet (+Se) for 6 weeks before sampling. Liver and plasma GSH-Px activities have been shown to be less than 1% of control values in rats fed this selenium-deficient diet for 6 weeks.[24]

Animals

Weanling male Sprague-Dawley rats were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN, USA), and housed in an American Association for Accreditation of Laboratory Animal Care-approved animal facility, with a 12-hr light/dark photoperiod. The rats weighed between 200–330 g each at the end of the feeding period. Groups of three selenium-deficient rats were injected intraperitoneally (i.p.) with 0, 5, 10, 25, or 50 μg selenium/kg body-weight (BW) as sodium selenite dissolved in 0.15 mol/L saline, and sacrificed 6 or 12 hr later by injection of sodium pentobarbital (65 mg/kg BW, i.p.). Four control (selenium-replete) rats were used for determination of control levels or activities of selenoproteins. Blood was drawn from the aorta and transferred into tubes containing disodium EDTA (1 mg/mL), on ice. After centrifugation at 3,000 rpm, plasma was removed to clean tubes on ice, and assayed for eGSH-Px activity the same day. A portion of the plasma was frozen at −20°C for no more than 1 week before analysis for Se-P content. Liver and testes were rapidly dissected out and homogenized in 4 parts (w/v) homogenization buffer,[14] consisting of 0.1M Tris (pH 7.6), 0.3M KCl and 1% Triton X-100 (peroxide-free, Boehringer-Mannheim), or were immediately frozen in liquid nitrogen and stored at −70°C for no more than 4 weeks until analyzed.

Assays

Tissue homogenates were transferred to microcentrifuge tubes and spun at 12,500 rpm (13,000 xg) in a microcentrifuge for 15 min at 4°C. Tissue supernatants and plasma samples were assayed for glutathione peroxidase (cGSH-Px and eGSH-Px, respectively) by a coupled assay, using H_2O_2 (0.25 mmol/L) as the substrate.[25] Phospholipid hydroperoxide GSH-Px activity was assessed by a modification of this method, using 13-hydroperoxy-β-linoleoyl-γ-palmitoylphosphatidylcholine (17.5 μmol/L) as the substrate (prepared as outlined below), and incorporating 0.1% Triton X-100 (peroxide-free) into the reaction mixture.[2] To allow comparison of the relative activity in all three types of GSH-Px assay, the concentration of reduced glutathione used in all the reaction mixtures was 3 mM. Blank reactions in the absence of an enzyme source were subtracted from all measurements. In addition, a blank reaction measured with tissue supernatant, in the absence of substrate, was detected in liver but not in testis preparations during assays of phGSH-Px activity. This activity, referred to as "non-specific NADPH oxidase activity" in the discussion below, was of the same magnitude in liver as the blank activity measured, with tissue supernatant, using a reduced substrate analogue (13-hydroxy-β-linoleoyl-γ-palmitoylphosphatidylcholine, prepared as outlined below). Selenoprotein P level in rat plasma was determined by a specific radioimmunoprecipitation assay.[26,27] Protein was determined by the method of Lowry.[28]

Substrate preparation

Oxidized phosphatidylcholine compounds were synthesized using soybean lipoxygenase (Type V) with β-linoleoyl-γ-palmitoylphosphatidylcholine (Sigma), in 0.2M borate buffer (pH 9.0) containing 10 mM sodium deoxycholate, to yield the 13-hydroperoxide.[29,30] The reaction was stopped by extraction with methylene chloride: methanol. The desired hydroperoxide product was purified by reverse-phase HPLC with methanol:acetonitrile:water (90:6:4), containing 20 mM choline chloride to enhance peak definition.[31] A portion of the hydroperoxide was reduced to the corresponding hydroxide by 30-min room-temperature incubation with a slight molar excess of triphenylphosphine before HPLC purification. The purified hydroperoxy- or hydroxy-phosphatidylcholine products were recovered from the HPLC solvent by addition of water and

extraction with methylene chloride. After evaporation of the organic phase using a stream of nitrogen, the products were dissolved in methanol and quantified by UV spectroscopy using the prominent conjugated diene chromophore (λmax = 236 nm, ϵ = 23,000) and stored in methanol under argon at −70°C. The phospholipid substrates were added to enzyme assay incubations in a small volume of methanol (5–10 μL/mL reaction volume).

Statistical analyses

Activities or levels of selenoproteins from rats injected with selenium were compared with those of time-matched rats injected with saline (0 μg Se/kg bodyweight) using Student's *t*-test.

Results

Liver phGSH-Px activity in control rats was very small compared with cGSH-Px activity (*Table 1*). The response of phGSH-Px activity to Se depletion and repletion appeared to be rather variable, although the very low control activity of this enzyme meant that the absolute changes in activity were very small. The large relative magnitude of the "non-specific NADPH oxidase activity" measured in the coupled assay of phGSH-Px (as determined using the substrate analogue 13-hydroxy-β-linoleoyl-γ-palmitoylphosphatidylcholine or in the absence of substrate) made the precise measurement of the small specific activity difficult, being effectively a large background against which to try to resolve a small activity. Most of the "gross" phGSH-Px activity measured in rat livers, especially from the selenium-deficient animals, was accounted for by the non-specific reaction (*Table 1, Figure 1b, c,* phGSH-Px activities in liver are presented as "gross activity," uncorrected for the non-specific activities that are shown adjacent in each table or figure). Taking into account the non-specific reaction, no clear dose-response of liver phGSH-Px activity was seen in this study. The perceived increase in "specific" phGSH-Px activity in rat livers with increasing selenium dose, suggested by the divergence between the lines indicating "gross" phGSH-Px activity and "non-specific NADPH oxidase," was actually due to decreases in the non-specific activity (*Figure 1b, c*). In contrast, liver cGSH-Px fell to less than 1% of control values and returned only to 2.8% under the repletion conditions employed here (*Table 1*). Dose-responses in liver cGSH-Px activity were noted at both sacrifice times, attaining plateau responses above 25 μg/kg BW (*Figure 1a*).

In testis, phGSH-Px and cGSH-Px activities fell only to 65% and 45% of control values, respectively, and did not increase significantly in response to resupplementation of selenium under the conditions of this experiment (*Table 1, Figure 2*).

Plasma eGSH-Px activity fell in selenium deficient rats to 1.3% of control values, and increased under the repletion regimen employed here only to 3.4% of control (*Table 1*). A dose-response in eGSH-Px activity was observed at 12 hr post-injection (*Figure 3*). In contrast to the effects on the glutathione peroxidases, Se-P levels fell only to 7% of control, and rose to 43% in the experiment reported here. A clear dose response in plasma Se-P level was observed at both sacrifice times (*Figure 3*). These results for eGSH-Px, liver cGSH-Px and Se-P are essentially as have been reported previously.[21,22]

Discussion

The 13-hydroperoxy-β-linoleoyl-γ-palmitoylphosphatidylcholine (13-hydroperoxy-PC) may be assumed to represent a biologically relevant substrate for phGSH-Px, as it has been shown to be produced during phosphatidylcholine peroxidation mediated by activated human neutrophils.[32] Phosphatidyl choline was demonstrated to be the most susceptible phospholipid class to peroxidation in vivo.[33] The reduced form of this substrate (13-hydroxy-PC) was used in some measurements in the present study to establish that the "non-specific NADPH oxidase activity" noted in rat liver supernatants in the absence of substrate was not influenced by a lack of interaction of the PC-type structure with the enzyme.

A similar non-specific activity has been reported in phGSH-Px assays of mouse liver but not heart, lung, or kidney.[14] In the present experiment, this non-specific activity accounted for most of the measured phGSH-Px activity in rat liver. Plasma eGSH-Px has previously been shown to reduce esterified phospholipid hydroperoxides.[5,6] As the tis-

Table 1 Selenoprotein levels in control rats, and effects of selenium deficiency and short-term repletion on levels or activities of rat selenoproteins

Tissue	Selenoprotein	Control[1] level/activity	0 Se[2] level/activity (% of control)	12 hr repleted[3] level/activity (% of control)
Plasma	eGSH-Px (U/mL)*	3312 ± 716	1.3	3.4
	Se-P (μg/mL)	24.2 ± 4.6	7.0	43.0
Liver	cGSH-Px (U/mg prot)*	624 ± 14	0.8	2.8
	phGSH-Px (U/mg prot)*	3.4 ± 0.1	76.0	80.0
	nsNADPHox (U/mg prot)*	2.6 ± 0.2	97.0	88.8
Testis	cGSH-Px (U/mg prot)*	97 ± 4	45.0	40.4
	phGSH-Px (U/mg prot)*	20 ± 1	65.0	64.0

[1]Selenium-adequate rats, fed for 6 weeks on a Torula yeast-based diet containing 0.25 mg supplemental Se/kg. Values shown are mean ± standard deviation (n = 4).
[2]Selenium-deficient rats, fed for 6 weeks on a Torula yeast-based diet without supplemental Se.
[3]Selenium-deficient rats injected with 50 mg Se/kg BW (as selenite), 12 hr before sacrifice.
*Measured using 3 mM GSH.

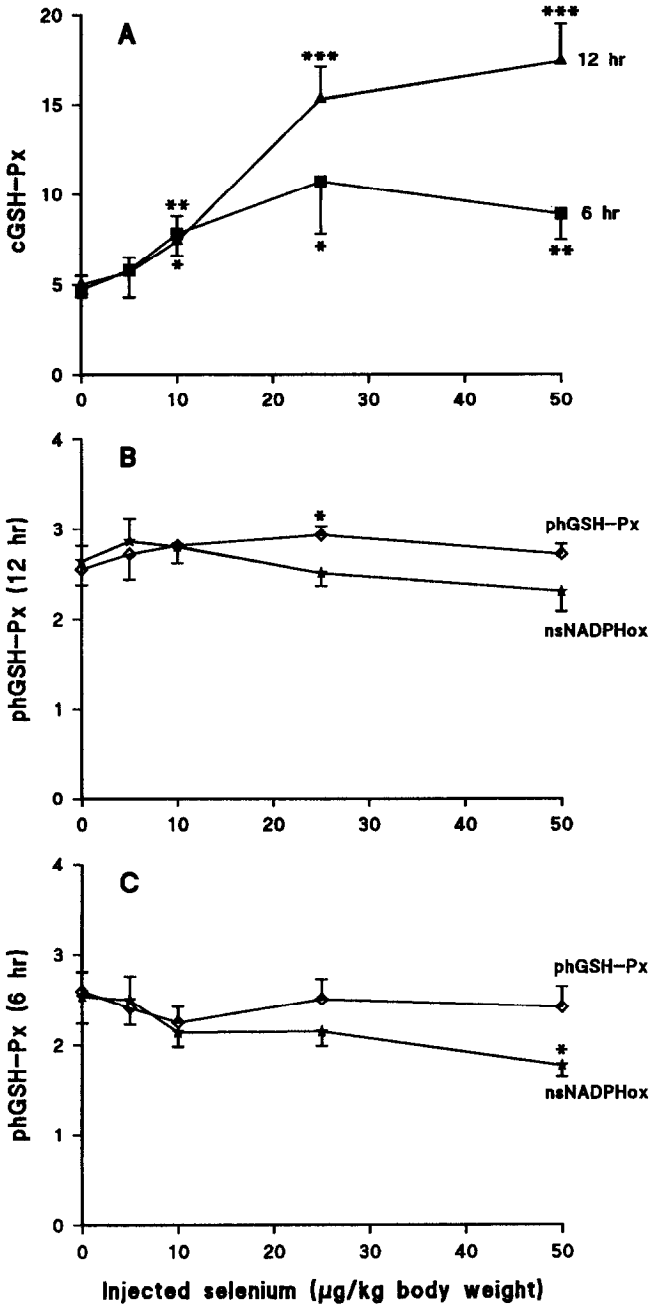

Figure 1 Phospholipid hydroperoxide glutathione peroxidase (phGSH-Px) and cellular glutathione peroxidase (cGSH-Px) activities in selenium-deficient rat liver, and responses to injection of selenium at doses up to 50 µg Se/kg bodyweight, sampled 6 or 12 hr after injection. A—liver cGSH-Px, 6 hr (solid squares) or 12 hr (solid triangles) B—liver phGSH-Px (open diamonds) or non-specific NADPH oxidase (closed stars), 12 hr C—liver phGSH-Px (open diamonds) or non-specific NADPH oxidase (closed stars), 6 hr All activities are expressed as units/mg protein. Vertical bars indicate standard deviation (n = 3). Stars indicate significant differences compared to rats dosed with 0 µg Se/kg bodyweight (two-tailed t-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

sues in the present experiment were not perfused before assay, it is possible that some portion of the remaining activity (i.e., after subtracting the non-specific activity) was due to eGSH-Px from the small amount of plasma contained

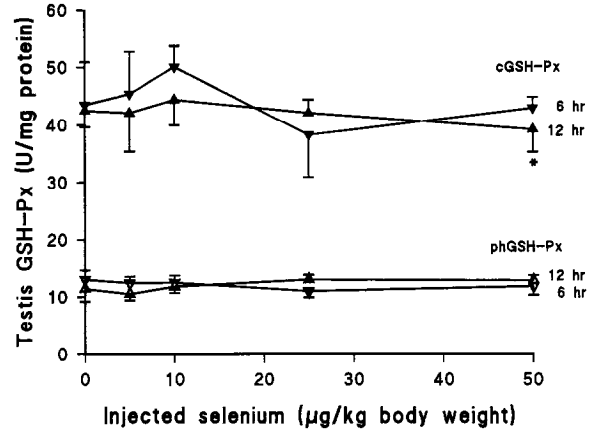

Figure 2 Phospholipid hydroperoxide glutathione peroxidase (phGSH-Px, open triangles) and cellular glutathione peroxidase (cGSH-Px, closed triangles) in selenium-deficient rat testis, and responses to injection of selenium at doses up to 50 µg Se/kg bodyweight, sampled 6 (inverted triangles) or 12 hr (upright triangles) after injection. Vertical bars indicate standard deviation (n = 3). Stars indicate significant differences compared to rats dosed with 0 µg Se/kg bodyweight (two-tailed t-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

in the tissues as sampled. Thus the activity of phGSH-Px in rat liver was very low in comparison to cGSH-Px. However, the presence of measurable, although very low, levels of mRNA for phGSH-Px in rat liver[20] suggests that the enzyme does exist in that tissue.

In previously-reported experiments with selenium-deficient mice, no response of cGSH-Px or phGSH-Px activities in liver to 10 µg Se/kg BW was observed at 114 hr after injection.[14] Our study shows a small but measurable response in rat liver cGSH-Px, but not phGSH-Px activity at earlier times (up to 12 hr) after injection of a similar dose. With a much higher dose of selenium (500 µg/kg BW),

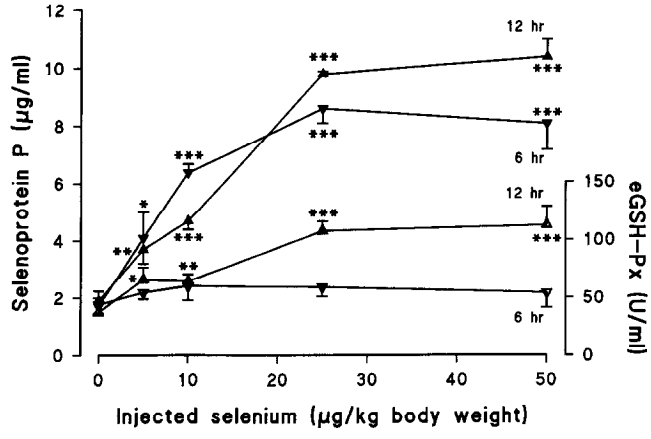

Figure 3 Plasma glutathione peroxidase (eGSH-Px, open triangles) and selenoprotein P (Se-P, closed triangles) in selenium-deficient rats, and responses to injection of selenium at doses up to 50 µg Se/kg bodyweight, sampled 6 (inverted triangles) or 12 hr (upright triangles) after injection. Vertical bars indicate standard deviation (n = 3). Stars indicate significant differences compared to rats dosed with 0 µg Se/kg bodyweight (two-tailed t-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

mouse liver cGSH-Px and phGSH-Px activities at 114 hr post-injection were significantly increased.[14] This was 10 times higher than the highest dose employed in the current study, and the lag time before measurement was almost 10 times as long (114 hr versus 12 hr in the present study). It may be argued that such a high dose of selenium is physiologically unrealistic, in which case, it would be concluded that liver phGSH-Px is unresponsive to short-term selenium repletion. There were no significant effects of selenium deficiency on levels of phGSH-Px mRNA in rat liver[9] or testis.[20] The influence of gonadal steroid hormones in regulation of phGSH-Px[10,16] appears to have been of greater magnitude than the selenium effects noted in the present study. It has been suggested that phGSH-Px may not be solely an oxidant-defense enzyme, rather also playing a role in regulation of signalling events mediated by lipid hydroperoxides.[16] The activity of phGSH-Px is purported to occur at the membrane-cytoplasm interface, and not in solution,[34] so the response of this enzyme in vivo may be different to that which has been measured with the substrate in a micellar environment in these experiments.

In conclusion, the activity of phGSH-Px was very low in rat livers assayed in this study, and showed minimal response to short-term changes in selenium supply, in comparison with selenoprotein P. In rat testes, phGSH-Px activity was at least 20 times higher than the level in rat liver. Thus, whereas phGSH-Px may play an important role in tissues such as testis or heart,[14,35] it seems to be of limited importance in rat liver. Certainly the response of rat liver phGSH-Px to short-term selenium repletion did not correlate temporally with selenium-associated protection against diquat-induced liver necrosis.[36]

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