Selective control of cytosolic glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase mRNA stability by selenium supply

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Abstract Selenium depletion of H4 hepatoma cells reduced cytosolic glutathione peroxidase (cGSH-Px) mRNA abundance but had no effect on phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px) mRNA abundance. Actinomycin D chase experiments showed that selenium depletion had no effect on the stability of PHGSH-Px mRNA but decreased the stability of cGSH-Px mRNA. In Se-replete cells puromycin decreased the stability of both cGSH-Px and PHGSH-Px mRNAs. The results suggest that when selenium supply is limiting PHGSH-Px mRNA translation is maintained more than that of cGSH-Px mRNA, and thus more cGSH-Px mRNA is released from polysomes and degraded.

Key words: Messenger RNA; Translation; Selenium; Glutathione peroxidase (rat hepatoma cell); Stability; 3 -Untranslated region

1 Introduction

The micronutrient selenium (Se) is present as selenocysteine in a number of proteins including cytosolic glutathione peroxidase (cGSH-Px) and phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px) [1]. cGSH-Px and PHGSH-Px are involved in the regulation of intracellular hydrogen peroxide and lipid hydroperoxide concentrations [2]. The ability of tissues to control synthesis of individual selenoproteins is vital for efficient utilization of limited amounts of the element for its most essential functions, and indeed, the Se content of diets modifies the activity and concentrations of selenoproteins under conditions of limited Se supply. Furthermore, the extent to which Se deficiency affects synthesis of the various selenoproteins differs between tissues and between the different enzymes [3–7]. For example, in the liver Se deficiency causes a dramatic reduction of approximately 90% in both the activity and mRNA abundance of cGSH-Px whilst the activity of PHGSH-Px is decreased by only 75% and the mRNA abundance is unchanged [5].

Although it is known that Se is incorporated into selenoproteins by recognition of the stop codon UGA as a codon for selenocysteine and that reduced availability of Se leads to termination of translation [8,9], the mechanism which underlies the difference in effects of Se deficiency on mRNA abundances of the cGSH-Px and PHGSH-Px has not been described. In rat liver the change in expression of these two genes is not brought about through changes in gene transcription [5]. In hepatoma cells in culture, Se deficiency reduces cGSH-Px mRNA expression without affecting gene transcription [10] but the effects of deficiency on the post-transcriptional control of the expression of these two peroxidase mRNAs has not been investigated. The aim of the present work was to investigate whether the differential effect of Se deficiency on cGSH-Px and PHGSH-Px expression is due to differences in stability of the two mRNAs under condition of low Se supply. In order to carry out these experiments a cell culture model for Se deficiency was developed using H4 hepatoma cells.

2. Materials and methods

2.1. Cell culture and enzyme activities

H4 rat hepatoma cells (H4-II-E-C3 from European Cell Culture Collection, Porton Down, UK) were grown to confluence in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. In order to produce Se-deficient and Se-replete cells, the cells were then grown in the same medium without serum but supplemented with either insulin (5 μg/ml), transferrin (5 μg/ml) and sodium selenite (7 ng/ml) (Se-replete medium) or with only the insulin and transferrin (Se-deficient medium) [10,11]. Medium was changed every 2 days.

cGSH-Px and PHGSH-Px enzyme activities were measured in cells which had been washed and resuspended in phosphate-buffered saline, pH 7.4 and then incubated with 0.1% peroxide-free Triton X-100, 0.3 mM NADPH, 5 mM reduced glutathione and 0.7 U/ml glutathione reductase. Oxidation of NADPH was followed spectrophotometrically at 340 nm after addition of either H_2O_2 (cGSH-Px activity) or phospholipid hydroperoxide PC-OOH (PHGSH-Px activity) to the samples [12,13].

2.2. cDNA probes and chemicals

The cGSH-Px probe [8], a gift from Dr P. Harrison, Beatson Institute, Glasgow, UK, the PHGSH-Px probe [4] from Dr R. Sunde, University of Missouri-Columbia, USA, the glyceraldehyde-3′-phosphate dehydrogenase (GAPDH) probe [14] and the 18S rRNA cDNA [15] from Dr R. Fulton, Beatson Institute, Glasgow, UK have been described previously [5]. Multiprime labelling kits, Hyperfilm-MP and [^{32}P]dCTP were purchased from Amersham International, Amersham, Bucks., UK and other chemicals were either of Analar or of Molecular biology grade.

2.3. RNA extraction and hybridisation analysis

Total RNA was extracted by the acid/guanidinium/phenol/chloroform procedure of Chomczynski and Sacchi [16] and assessed by the A_{260}/A_{280} absorbance ratio. RNA species were then separated by electrophoresis through a denaturing 2.2 M formaldehyde, 1.2% w/v agarose gel [17] and transferred to nylon membrane (Genescreen from NEN Dupont Ltd.) by capillary blotting. RNA was fixed to the membrane by exposure to UV light and the membranes were stored dry until required. Membranes were prehybridised for at least 6 h at 42°C with 0.1 mg/ml denatured salmon sperm DNA in 50% formamide, 10% dextran sulphate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrilidone, 0.2% Ficoll, 0.1% sodium pyrophosphate, 1% SDS and 50 mmol/l Tris-HCl, pH 7.5. 50–100 ng of the DNA probes were

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Abbreviations: cCGSH-Px, cytosolic glutathione peroxidase; PHGSH-Px, phospholipid hydroperoxide glutathione peroxidase

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labelled with [^{32}P]dCTP by random priming and hybridisation carried out at 42°C for 24 h as described previously [5,18]. Membranes were then washed to remove non-specifically bound probe; two washes in 2×SSC (1×SSC = 0.15 mol/l NaCl/0.015 mol/l sodium citrate) at room temperature for 5 min, followed by two washes at 65°C for 1 h in either 1×SSC, 1% SDS (cGSH-Px, PHGSH-Px, and GAPDH) or 0.2×SSC, 1% SDS (18S rRNA), and a final wash in 0.1×SSC at room temperature. Specific hybridisation was then detected both by direct imaging using a Canberra Packard Instantimager and by autoradiography using Hyperfilm-MP (Amersham International, UK) at −70°C. After analysis membranes were washed in 0.1% SDS for 5–7 min at 95°C before rehybridisation to other probes.

Quantification of the bound probe was carried out using the Instantimager and results for each probe expressed per unit of hybridisation achieved with the 18S rRNA probe; this allowed correction for any variation between loading of RNA on the gel or transfer to the nylon membrane. RNA abundances were estimated in this manner from cells analysed in duplicate for each time point and all hybridisations were carried out on duplicate filters. Estimates of mRNA half-life were calculated by combining the data from both filters.

3. Results

During culture of cells in Se-deficient medium there was a dramatic decrease in cGSH-Px activity. The major change in cGSH-Px enzyme activity occurred during the first 2–4 days in Se-deficient medium, with a reduction of 80% by 2–4 days (see Table 1). Culture in Se-deficient medium for longer periods produced a further gradual decrease in cGSH-Px activity, with a reduction of 92% after 8 days compared to activity in cells grown in Se-replete medium. Subsequent replacement of Se (7 ng/ml sodium selenite) in the medium led to an increase of cGSH-Px activity such that after 8 days the cells showed activities comparable to the control levels [19]. The extent and timing of these changes in cGSH-Px during Se depletion and repletion are similar to those found in Hep3B cells [10]. Measurement of PHGSH-Px activity showed an approximately 50% reduction in cells cultured in Se-deficient medium for 3 days (Table 1), a considerably smaller decrease than with cGSH-Px activity. Northern hybridisation analysis of RNA extracted from cells grown for 3 days in either Se-deficient or Se-replete medium showed that there was a large reduction in the abundance of cGSH-Px mRNA but not of PHGSH-Px mRNA (Fig. 1). Quantification of the amount of specific probes bound to the RNA and expression of the data per unit of 18S rRNA showed no difference in the abundance of PHGSH-Px mRNA between Se-replete and Se-depleted cells, but the abundance of cGSH-Px mRNA was reduced by 66% in cells grown in Se-deficient medium (Table 1).

The stability of the cGSH-Px and PHGSH-Px mRNAs was assessed by measuring their abundances over a 12 h period following inhibition of transcription. Cells were grown in either Se-replete or Se-deficient medium for 3 days and then transcription inhibited by addition of actinomycin D (5 μg/ml). RNA was extracted at 0, 4, 8 and 12 h after addition of the inhibitor and the levels of cGSH-Px and PHGSH-Px mRNAs measured by Northern hybridisation. Following inhibition of transcription there was a reduction in cGSH-Px and PHGSH-Px mRNA abundances, indicating degradation of the mRNAs (Fig. 2), as well as a very rapid reduction in

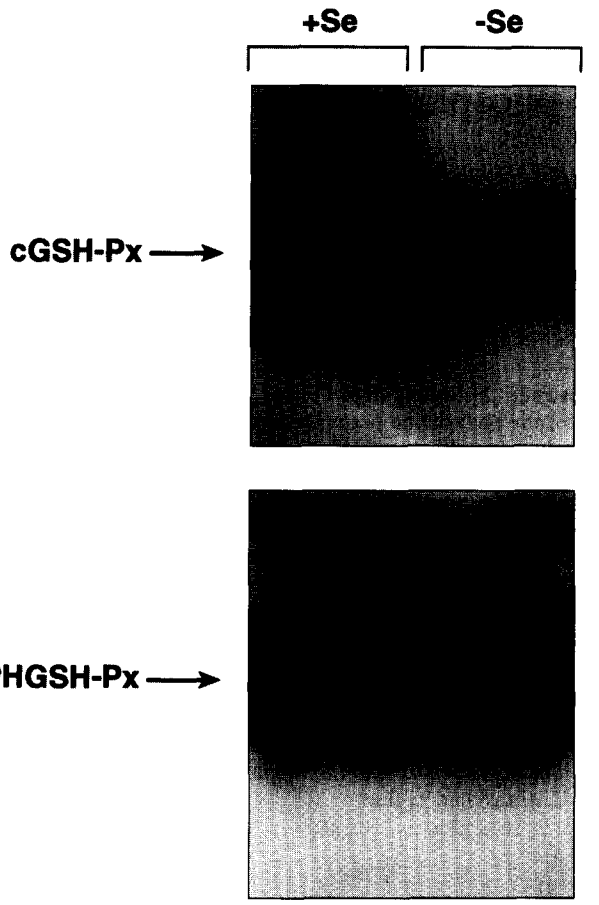

Fig. 1. Northern hybridization of total H4 cell RNA showing the effect of selenium deficiency on cGSH-Px and PHGSH-Px mRNA abundance. All lanes were loaded with 20 μg of total RNA and filters were hybridised successively with cGSH-Px and PHGSH-Px. Results show the levels of the two corresponding RNAs for duplicate samples detected by autoradiography. For a given probe samples from Se-replete (+Se) and Se-depleted (−Se) cells were hybridised under identical conditions. Note the reduction in cGSH-Px mRNA abundance in Se-deficient condition and the lack of effect on PHGSH-Px abundance.

Table 1
The effect of Se deficiency on cGSH-Px and PHGSH-Px enzyme activity and mRNA abundance in H4 cells

	Se-replete	Se-deficient
Enzyme activity (mU/mg protein)		
cGSH-Px	34.9 ± 6.2 (5)	10.0 ± 0.7 (5)**
PHGSH-Px	4.5 ± 0.6 (3)	2.1 ± 0.3 (3)*
mRNA abundance (arbitrary units)		
cGSH-Px	14.8 ± 3.7 (5)	5.0 ± 1.3 (5)*
PHGSH-Px	41.1 ± 16.8 (5)	36.1 ± 13.8 (5)

Values shown are means ± S.E.M. with number of experiments in parentheses. Groups were compared using a two-tailed paired *t*-test; *$P < 0.05$, **$P < 0.02$ compared with the Se-replete group.

c-*myc* mRNA abundance (half-life 30 min, results not shown), consistent with the unstable nature of this mRNA [20]. Under Se-replete conditions the half-life of cGSH-Px mRNA was 10–13 h and that of PHGSH-Px was 9–10 h. In cells grown in Se-deficient medium the rate of degradation of PHGSH-Px mRNA after addition of actinomycin D was unchanged compared with cells grown in Se-replete medium. In contrast there was more rapid degradation of cGSH-Px mRNA (Fig. 2).

Quantification of the data from five experiments showed that the half-life of the PHGSH-Px mRNA was similar in cells grown in either Se-replete (10 ± 2 h) or Se-deficient medium (10 ± 3 h) but that the half-life of cGSH-Px mRNA was significantly reduced from 13 ± 2 h in the Se-replete controls to 8 ± 1 h ($P < 0.05$, using a two-tailed paired *t*-test) in the cells grown in Se-deficient medium. The stability of the mRNA for the non-selenoprotein glyceraldehyde-3-phosphate dehydrogenase was unchanged by Se deficiency: the half-life of the mRNA was 10 ± 1 h in the Se-replete cells and 10 ± 2 h in the cells grown in Se-depleted medium. When 25 μg/ml 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole [21] was used instead of actinomycin D to block mRNA synthesis in a further three experiments a differential effect of Se deficiency on cGSH-Px and PHGSH-Px mRNA stability was again observed: the half-life of the PHGSH-Px mRNA was similar in cells grown in either Se-deficient (8 ± 0.3 h) or Se-replete (9 ± 0.7 h) medium but the half-life of cGSH-Px mRNA was reduced by 28% in the cells grown in Se-deficient medium (3 ± 1 h) compared to that in the cells grown in Se-replete medium (11 ± 2 h).

In order to investigate whether the stability of either cGSH-Px or PHGSH-Px mRNA is affected by the extent to which they are translated and retained in polyribosome complexes, the effects of the protein synthesis inhibitor puromycin on mRNA stability was investigated. As shown in Table 2, puromycin caused a decrease in the half-life of both cGSH-Px and PHGSH-Px mRNAs in H4 cells grown in Se-replete medium. The extent of the decrease in half-life was similar for the two mRNAs (cGSH-Px, 40%; PHGSH-Px 44%) and was of a similar order to the observed effect of Se deficiency on cGSH-Px mRNA.

4. Discussion

In animals and man Se deficiency is characterised by a large decrease in hepatic cGSH-Px activity [1,3]. Using cGSH-Px activity as a measure of Se status, the present results show that with H4 hepatoma cells, as with H3B and HL-60 myeloid cells [10,11], culture in medium without a supplement of sodium selenite led to the cells becoming Se-depleted. After 3 days in such medium the H4 cells had lost 80% of their cGSH-Px activity. This loss of cGSH-Px activity was accompanied by a reduction in cGSH-Px mRNA and a smaller reduction in PHGSH-Px activity, but there was no change in PHGSH-Px mRNA abundance. Thus, in the Se-deficient H4 cells, as in rat liver during Se deficiency [5], there was a differential effect of Se-depletion on the activity and mRNA abundance of the cGSH-Px and PHGSH-Px mRNAs. The regulation of these enzymes during Se depletion of H4 cells appears to be similar to that in Se-deficient rat liver and therefore presents a suitable model to study the control of these genes by Se supply.

Table 2
The effect of puromycin on stability of cGSH-Px and PHGSH-Px mRNAs in H4 cells grown under Se-replete conditions

	mRNA half-life (h)	
	no puromycin	+puromycin
cGSH-Px	10 ± 2	6 ± 1*
PHGSH-Px	9 ± 1	5 ± 0.3*

Values shown are means ± S.E.M. from three separate experiments. mRNA half-lives were measured as described in the text. Groups were compared using a two-tailed paired *t*-test; *$P < 0.05$ compared with the control groups without puromycin.

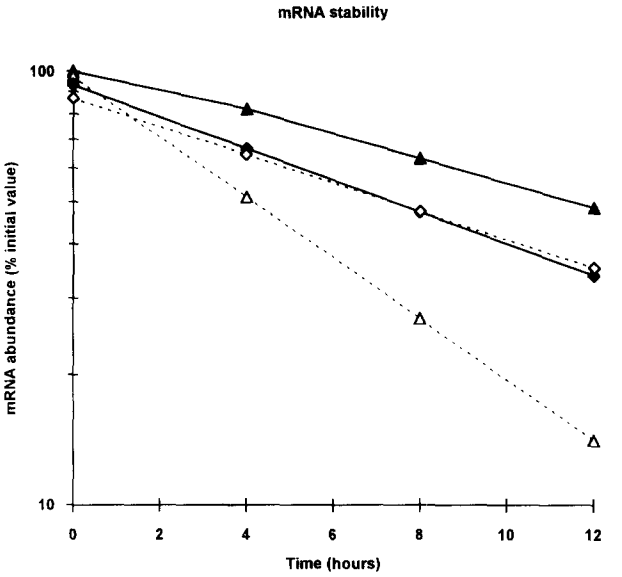

Fig. 2. Stability of cGSH-Px and PHGSH-Px mRNAs in H4 cells grown in Se-deficient and Se-replete medium. mRNA stability was assessed by measurement of transcript levels by Northern hybridisation of total RNA isolated from cells at 4, 8 and 12 h after treatment with 5 μg/ml actinomycin D. Hybridisation of the cGSH-Px and PHGSH-Px probes was quantified by electronic autoradiography and abundances calculated per unit of 18S rRNA: the level of expression was then calculated relative to the control levels before addition of actinomycin D (100%). Abundances of cGSH-Px mRNA in Se-replete (▲) and Se-deficient (△) cells are shown together with those of PHGSH-Px mRNA in Se-replete (◆) and Se-deficient (◇) cells. Note the faster rate of reduction in cGSH-Px mRNA in the Se-deficient cells, indicating a decrease in mRNA stability. Results are mean values from 2–3 separate RNA preparations.

As shown in Fig. 2, Se depletion had a selective effect on the stability of cGSH-Px and PHGSH-Px mRNAs: as judged by their half-lives estimated after inhibition of mRNA synthesis, Se depletion had no effect on the half-life, and therefore stability, of the PHGSH-Px mRNA but decreased the half-life, and therefore the stability, of the cGSH-Px mRNA. This difference in the effect of Se deficiency on stability of cGSH-Px and PHGSH-Px mRNAs can explain the observations that, both in cultured cells (Fig. 2, Table 1) and in rat liver [5], Se deficiency causes a reduction in the abundance of cGSH-Px mRNA but has no effect on that of PHGSH-Px mRNA. The decrease in cGSH-Px mRNA stability in Se deficiency also accounts for the reduced abundance of this mRNA in liver or hepatoma cells in the absence of any change in transcription [5,10].

Inhibition of translation by puromycin causes dissociation of the polyribosome complex and release of the nascent polypeptide chains and mRNA [22]. In Se-replete culture condi-

tions the inhibition of translation with puromycin caused a decrease in stability of both cGSH-Px and PHGSH-Px mRNAs (Table 2). The decrease in stability was similar for the two mRNAs and the data show that both mRNAs become more unstable when not being translated.

The results in this paper show that cGSH-Px mRNA stability is decreased by either release from polysomes in Se-replete conditions or by Se-depletion. Since incorporation of Se into selenoproteins such as cGSH-Px and PHGSH-Px involves use of the UGA stop codon for incorporation of Se-cysteine [8,9] and there is a premature termination of the translation of selenoprotein mRNAs in the absence of selenium [9,23], the most likely explanation for the decreased stability of cGSH-Px mRNA in Se deficiency is that the effect is secondary to decreased translation. We propose therefore that the selective effect of Se deficiency on cGSH-Px and PHGSH-Px mRNA stability and abundance is due to a differential effect of Se deficiency on translation of the two mRNAs: when Se is limiting in liver or hepatoma cells translation of PHGSH-Px mRNA is maintained more than that of cGSH-Px and thus more cGSH-Px than PHGSH-Px mRNA is released from polysomes, so resulting in more degradation of cGSH-Px mRNA. This hypothesis is compatible with two observations; firstly, PHGSH-Px activity is affected less than that of cGSH-Px in hepatic cells (see Table 1 and [5]); and secondly, in Se deficiency a greater proportion of PHGSH-Px mRNA than cGSH-Px mRNA is retained in polysomes in the liver [5].

The incorporation of Se-cysteine at specific UGA codons in the selenoenzyme mRNAs requires stem-loop structures in the 3′-untranslated regions (3′-UTRs) of these mRNAs [9,23,24]. Furthermore, the efficiency of translation is affected by the 3′-UTR, as indicated by the observations that in transfected cells the activity of the selenoenzymes type I and type III deiodinases are altered by exchanging their native 3′-UTRs for those of other selenoenzymes [9,25]. It is likely therefore that the differential effect of Se depletion on stability and translation of cGSH-Px and PHGSH-Px mRNAs in hepatic cells is due to the PHGSH-Px 3′-UTR being more efficient than the cGSH-Px 3′-UTR at maintaining translation under conditions of limiting Se supply. Different or additional controls may exist in other cell types such as the thyroid [5].

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