Glutathione Peroxidase Protein

Absence in Selenium Deficiency States and Correlation with Enzymatic Activity

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Abstract

Glutathione peroxidase (GSHPx) activity is an indicator of selenium status in selenium-deficient individuals. Utilizing polyclonal monospecific antibodies to purified erythrocyte GSHPx, we were able to determine the relationship between enzymatic activity and protein content. In erythrocytes from a selenium-deficient individual who was treated with selenium, and in HL-60 cells grown in the absence of selenium and then returned to selenium-containing medium, there was a direct relationship between enzymatic activity and protein content. Thus, selenium deficiency results not only in a decrease of GSHPx activity, but also in a decrease of GSHPx protein.

Introduction

It has been known for some time that selenium in the form of selenocysteine is part of the catalytic site of mammalian glutathione peroxidase (GSHPx)[1] (1–2), an enzyme involved in detoxification of hydrogen peroxide. Experimental selenium deficiency in animals results in a decrease in cellular and plasma GSHPx activity (3–4). Some patients with gastrointestinal disorders who have been treated for prolonged periods of time with total parenteral nutrition become markedly selenium-deficient as well as deficient in erythrocyte, granylocyte, and platelet GSHPx activity (5–7). Supplementation of both animals and patients with selenium results in an increase in cellular levels of GSHPx activity (3–4, 8–11). We have recently shown that when patients deficient in selenium and GSHPx are supplemented with intravenous selenous acid, there is a time-dependent recovery in their erythrocyte GSHPx activity occurring over 3–4 mo and that this recovery occurs only in cells made in the presence of selenium (12). Since all of the above studies were performed using an enzymatic assay for GSHPx activity, it was not possible to determine whether the GSHPx protein was synthesized in the absence of selenium. In order to answer this question, a radioimmunoassay for GSHPx was established.

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1. *Abbreviation used in this paper:* GSHPx, glutathione peroxidase.

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Methods

GSHPx activity. GSHPx activity using *tert*-butyl hydroperoxide as a substrate was assayed by an adaptation (10) of the method of Beutler (13). Both sample and reference cuvettes contained 0.1 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 0.2 mM NADPH, 1 U glutathione reductase from Baker's yeast (Sigma Chemical Co., St. Louis, MO), 2 mM reduced glutathione, and the appropriate amount of enzyme in 1.0 ml. The oxidation of NADPH by *tert*-butyl hydroperoxide (70 μM), added to the sample cuvette only, was followed spectrophotometrically at 340 nm at 37°C. 1 U of enzyme activity is defined as the oxidation of 1 μmol of NADPH per minute.

Immunization of GSHPx and isolation of antibody. GSHPx was purified from human erythrocytes according to the methods of Awasthi et al. (14). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15) of the purified GSHPx showed a single stained band with a molecular weight of 22,000, similar to what has been previously described (14). 150 μg of purified GSHPx was mixed with Freund's complete adjuvant and injected intramuscularly in four sites on the back of a New Zealand white rabbit. This procedure was repeated weekly for 4 wk. Serum was collected 5 wk after the first immunization. Since rabbit serum itself contains GSHPx activity, the IgG fraction free from GSHPx activity was isolated using ammonium sulfate precipitation followed by DEAE cellulose DE-52 chromatography.

Erythrocytes and HL-60 cells. Samples of erythrocytes were obtained from a selenium-deficient individual during the course of selenium repletion as previously described (12). Selenium-deficient HL-60 were grown as previously described (16). After 14 d in selenium-deficient medium, the cells were placed in the same medium containing sodium selenate (5 ng/ml) at 0 time. At the times noted, the cells were washed twice in cold phosphate-buffered saline, pH 7.5, centrifuged at 500 *g* for 5 min at 4°C and the pellet was frozen at −80°C. The frozen cells were resuspended in 0.34 M sucrose containing 0.02 M Tris-HCl, pH 7.4, at a concentration of 1×10^8 cells/ml and placed in a sonicator bath for 6–8 s. The lysates were centrifuged at 70 *g* for 5 min at 4°C. The resulting supernatant was centrifuged at 12,000 *g* for 20 min at 4°C. The cytosolic solutions were assayed for GSHPx activity and protein.

Radioimmunoassay of GSHPx. Radioimmunoassay was performed according to the method of Roberts and Parker (17) with a slight modification. Purified erythrocyte GSHPx was radioiodinated using the Bolton-Hunter reagent (New England Nuclear, Boston, MA) (18). All samples were incubated with the same amount of anti-GSHPx IgG for 15 min at 25°C followed by a 60-min incubation at 25°C with an aliquot of ^{125}I-labeled purified GSHPx. The reaction mixture contained 50 mM Tri-HCl, pH 8.0, 2.5 mM EDTA, 0.02% NaN_3 and 0.2% bovine serum albumin in a total volume of 160 μl. After the 60-min incubation, 40 μl of goat anti-rabbit IgG antibody (Calbiochem-Behring Corp., La Jolla, CA) was added at equivalence and incubated for 15 min at 25°C. The mixtures were then centrifuged at 10,000 *g* for 15 min at 4°C and the radioactivity of the resulting immune precipitates was determined. The amount of GSHPx in samples was calculated from a standard curve obtained with purified GSHPx (1–100 ng). The protein concentration of the pure enzyme was measured by the method of Lowry et al. (19),

using bovine serum albumin as a reference protein. Hemoglobin concentration was determined with Drabkin's reagent (13).

Results

Characterization of antibody against GSHPx. Our objective was to have monospecific polyclonal antibodies that would bind to and precipitate GSHPx from erythrocytes, but would not inhibit its enzymatic activity. Rabbit anti-GSHPx antibody produced single lines of precipitation in an Ouchterlony double diffusion analysis when the ammonium sulfate–precipitated fraction of the erythrocyte hemolysates and the purified GSHPx were examined. A zone of identity was present between the lines formed with the crude fraction and the purified enzyme. Table I shows that the rabbit IgG fraction did not inhibit human erythrocyte GSHPx activity. However, as also indicated in Table I, incubation of the erythrocyte hemolysate with the antibody, followed by centrifugation, resulted in a supernatant fraction devoid of GSHPx activity. The same procedure performed with rabbit preimmune IgG did not precipitate GSHPx activity. We therefore had polyclonal, monospecific, precipitating antibodies to erythrocyte GSHPx directed against sites other than the active site of the enzyme.

Erythrocyte GSHPx activity and protein. We examined the hemolysates from a selenium-deficient individual during supplemental with selenium. The recovery of GSHPx activity in these hemolysates has previously been reported (12). As shown in Fig. 1, the protein content of GSHPx parallels the enzyme activity found in these hemolysates. The coefficient of correlation was 0.97.

HL-60 GSHPx activity and protein. We examined GSHPx activity and protein content in the human promyelocytic cell line HL-60, which had been cultured in defined medium in the presence and absence of sodium selenate. Cells grown in the absence of selenium had 4.6% of the control GSHPx activity and 4.2% of the control GSHPx protein content. In addition, as shown in Fig. 2, there is a direct relationship between the recovery of GSHPx protein and activity in selenium-deficient HL-60 cells transferred to medium containing sodium selenate. The coefficient of correlation between activity and protein content was 0.97.

Discussion

Erythrocytes from selenium-deficient individuals not only lack GSHPx activity but also lack the GSHPx protein as assessed by radioimmunoassay. Since erythrocytes are not capable of synthesizing protein, it is possible that an inactive GSHPx protein is made in erythroid precursors, but because of its abnormality it is rapidly degraded and therefore not present in circulating erythrocytes. Alternatively, selenium may affect the rate of synthesis of the GSHPx protein. In order to attempt to answer this question, we examined GSHPx activity and protein in HL-60 cells cultured in the presence and absence of selenium. There appears to be a direct correspondence between the levels of GSHPx activity and protein content even in cells capable of undergoing protein synthesis, including the synthesis of this specific enzyme (16). These results are consistent with the ability of selenium to control the synthesis of the GSHPx protein. We cannot, however, eliminate the possibility of very rapid degradation of an incomplete or abnormal protein.

These results must be explained in light of the recent observations by Sunde and Evenson (20) that serine is the carbon source for selenocysteine in rat liver GSHPx. Their observations suggest that selenium is incorporated into GSHPx either cotranslationally or posttranslationally. The observations reported here could be explained by a co-translational modification of the protein with selenium, such that in the absence of selenium, protein synthesis cannot proceed. Yoshida et al. (21) did not find any evidence for an immunochemically recognizable protein in selenium-deficient rat liver. In addition, recent studies utilizing ^{75}Se in rat liver suggest that there exists a specific selenocysteine transfer RNA (22). If the same is true in human cells, then deficiency of selenium would lead to a decrease in the amount of

Table I. Precipitation of Erythrocyte Hemolysate GSHPx by Rabbit Anti-GSHPx IgG

	GSHPx activity ($\times 10^{-3}$ U)	
Preparation	Before centrifugation	After centrifugation
Hemolysate	7.0	7.2
+ Rabbit anti-GSHPx	6.9	<0.1
+ Rabbit pre-immune IgG	6.7	6.5

Erythrocyte hemolysates were incubated at 25°C for 60 min either alone or with rabbit anti-GSHPx IgG or control rabbit IgG. The mixtures were then either directly assayed for GSHPx activity or centrifuged at 100,000 *g* for 30 min at 4°C and the resulting supernatants were assayed for GSHPx activity.

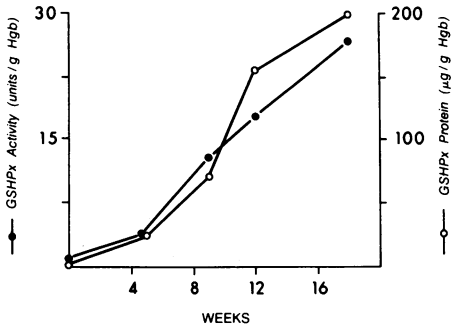

Figure 1. Time course for the recovery of erythrocyte GSHPx activity and protein after selenium repletion. All samples were obtained from a selenium-deficient individual during the course of selenium repletion as previously described (12). The hemolysates were assayed for hemoglobin content, GSHPx activity, and protein as described in Methods.

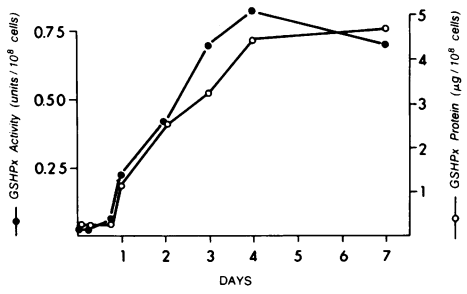

Figure 2. Time course for the recovery of HL-60 GSHPx activity and protein with selenium repletion. The cytosolic solutions of HL-60 were assayed for GSHPx activity and protein as described in Methods.

the selenocysteine-transfer RNA complex. In the absence of this complex, translation and synthesis of GSHPx could not proceed to completion.

The results obtained in this study also explain the previous observations that only erythrocytes synthesized in the presence of selenium contain GSHPx activity (12). Since there is no apoenzyme present in the erythrocytes of selenium-deficient individuals, and since erythrocytes do not synthesize protein, only new cells made after the introduction of selenium into the diet contain GSHPx.

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