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Phospholipid Hydroperoxide Glutathione Peroxidase of Rat Testis

GONADOTROPIN DEPENDENCE AND IMMUNOCYTOCHEMICAL IDENTIFICATION*

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Antonella Roveri‡, Andrea Casasco§, Matilde Maiorino‡, Paolo Dalan‡, Alberto Calligaro§, and Fulvio Ursini‡¶

From the ‡Department of Biological Chemistry, University of Padova, §Institute of Histology and Embriology, University of Pavia, and ¶Institute of Chemistry, University of Udine, Italy

A high glutathione peroxidase activity toward phospholipid hydroperoxides is present in rat testis. The attribution of this activity to the selenoenzyme phospholipid hydroperoxide glutathione peroxidase (PHGPX) was supported by cross-reactivity with antibodies raised against pig heart PHGPX which had been purified and characterized. Rat testis PHGPX is partially cytosolic and partially linked to nuclei and mitochondria. The soluble and organelle-bound enzymes appear identical by Western blot analysis.

PHGPX, but neither selenium-dependent nor non-selenium-dependent glutathione peroxidase activity, is expressed in testes only after puberty, disappears after hypophysectomy, and is partially restored by gonadotropin treatment.

Specific immunostaining of testes by antiserum against PHGPX appears as a fine granular brown pattern localized throughout the cytoplasm in more immature cells but is confined to the peripheral part of the cytoplasm, the nuclear membrane, and mitochondria in maturating spermatogenic cells. As expected, immunostaining of spermatogenic cells in hypophysectomized animals was negative, but gonadotropin treatment only marginally increased the immunoreactivity.

The expression of PHGPX in testes is consistent with the previously described specific requirement for selenium for synthesis of a 15–20-kDa selenoprotein which is related to the production of functional spermatozoa.

The role of selenium as a micronutrient has been clearly established for several years (1), and different selenoproteins have been identified through the use of radiolabeled selenium (2, 3). Among these, only four have been characterized in mammalian tissues as enzymes containing a selenocysteine which is involved in the catalytic cycle and which is coded by an inframe TGA codon: glutathione peroxidase (GPX)[1] (4), glycosylated extracellular glutathione peroxidase (5), phospholipid hydroperoxide glutathione peroxidase (PHGPX) (6), and type I iodothyronine deiodinase (7–9). Mammalian cells, therefore, contain at least two molecularly characterized Se-dependent glutathione peroxidases; these being GPX (10) which is a tetrameric enzyme that reduces hydrogen peroxide as well as small molecular weight water-soluble hydroperoxides, and PHGPX (11), which is a monomeric enzyme that reduces hydroperoxide derivatives of lipids inserted in membranes. Despite showing a common ping-pong kinetic mechanism these two enzymes are different with respect to substrate specificity (12) and structure (6). The overall identity between PHGPX and GPX is below 40%, while the identical amino acids are centered in the clusters that constitute the active site of these selenoenzymes, suggesting a common ancestor and an early evolutionary divergence of PHGPX (6).

While PHGPX has been formerly characterized by its activity on phospholipid hydroperoxides (12), it has also been recently shown to reduce cholesterol hydroperoxides (13). This peroxidase activity toward all membrane lipid hydroperoxides, apparently, accounts for the observed inhibition of lipid peroxidation (14). Furthermore, by reducing the hydroperoxides generated by interaction of lipid hydroperoxyl radicals with vitamin E, PHGPX enhances the antioxidant effect of the latter and accounts for the synergistic antiperoxidant effect of selenium and vitamin E (15).

The physiological role of PHGPX, thus, appears to cope with antioxidant protection, since lipid hydroperoxides both perturb membrane structure and function and may generate free radicals by their decomposition. Nevertheless, another possible physiological function of this enzyme could be related to the suggested physiological roles of lipid hydroperoxides. PHGPX would serve as a switch-off signaling mechanism of a biological event mediated by lipid hydroperoxides (16).

Selenium is specifically required for normal spermatogenesis (17), and its concentration in testis is regulated by a homeostatic mechanism which assures a priority in the supply of selenium to male gonads over other tissues (18). Furthermore, the concentration of selenium in testis is depressed by hypophysectomy and restored by gonadotropins (18), suggesting a hormonal control of selenium uptake and utilization. In this report we present enzymological and immunohistochemical evidence for a high expression of PHGPX in rat testes which appears to be gonadotropin-dependent and suggests a specific role for this enzyme in the overall process of spermatogenesis.

MATERIALS AND METHODS

Enzymatic Activity and Cellular Fractionation—PHGPX, GPX, and non-Se-GPX were measured as previously described (11, 19). Rat testis and liver subcellular fractions were prepared by the usual procedure (20, 21) using a differential centrifugation of a 1:3 tissue homogenate in 50 mM Tris-HCl, 250 mM sucrose, pH 7.4. The pellet obtained after centrifugation at 500 × *g* for 10 min contained unbroken cells and cell debris and was discarded, the pellet at 2000 × *g* for 15 min contained the nuclear fraction, the pellet at 20,000 × *g* for 30 min contained the mitochondrial fraction, the pellet at 105,000 × *g* for 1 h contained the microsomal fraction, and the final supernatant

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[1] The abbreviations used are: GPX, selenium-dependent glutathione peroxidase; GSH, reduced glutathione; non-Se-GPX, non-selenium-dependent glutathione peroxidase; PBS, phosphate buffer-saline; PHGPX, phospholipid hydroperoxide glutathione peroxidase;

was considered as soluble fraction. Homogeneity of particulate fractions was tested by electron microscopy and by cytochrome c oxidase activity which was partitioned as follows: 70% in mitochondrial fraction, 20% in nuclear fraction, and 10% in microsomal fraction. Microsomal fraction was not washed, to avoid the loss of possibly loosely bound activities and so has to be considered as partially contaminated with cytosolic activities.

Preparation of Antiserum and Western Blot Analysis—Phospholipid hydroperoxide glutathione peroxidase was purified to homogeneity from pig heart as described (11). Furthermore, to minimize the possibility of raising antibodies against possible minor contaminants, slab gel electrophoresis was carried out on the final preparation of PHGPX and, after a mild staining with Coomassie Blue, the band containing the enzyme was isolated. The strip of gel, containing approximately 100 μg of protein was chopped, equilibated overnight in PBS, and homogenized in PBS using a Potter homogenizer. To 1.2 ml of this homogenate 1.2 ml of complete Freund adjuvant (Difco) and 3.5 mg of dried heat-killed *Mycobacteria tubercolisis* (Difco) were added. The emulsion was made using two syringes connected by a double needle and used to immunize a New Zealand rabbit. The immunization procedure was according to Vaitukaitis (22). Serum was prepared at different times during all the immunization period and checked by Western blot analysis on crude preparations of PHGPX. The antiserum that generated a clear spot at a dilution of 1:1000 by interacting with 5 ng of pig heart PHGPX was used for the immunohistochemical studies.

Western blot analysis was carried out as follows: proteins or extracts were run on 0.1% sodium dodecyl sulfate, 12% polyacrilamide gel and transferred on nitrocellulose sheets using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blotting was according to Towbin *et al.* (23) as modified by Szewczyk and Kozloff (24). The detection of the antigen was made by using alkaline phosphatase conjugate according to Blake *et al.* (25).

Immunoprecipitation and Immunoinhibition—Rat testis cytosol (0.15 ml) was incubated for 1 h at room temperature under continous agitation with one volume of appropriate dilutions of immune serum and pre-immune serum, as a negative control, in PBS. After this incubation, two volumes of Staphylococcus A suspension (Sigma) in PBS were added. After 1 h of gentle shaking the suspension was centrifugated at 3,000 × *g* for 5 min. Remaining activity was measured in the supernatants.

Total membrane fraction of rat testis homogenate was resuspended in five volumes of PBS. The suspension (0.15 ml) was then incubated with the immune serum at different dilutions for 1 h before measurement of activity. Preimmune serum was used as control.

Hypophysectomy—Hypophysectomized rats were supplied by Nossan (Italy)-HSD-Indianapolis (Indianapolis, IN). The surgical approach used by the supplying company was parapharyngeal, and animals were anaesthetized with ketamine-xylazine. Animals were hypophysectomized either at the 25th or at the 56th day after birth. The efficiency of the hypophysectomy was evaluated by the macroscopically lower dimension of testes in comparison with controls or sham-operated animals (18). Animals from the same colony were used as controls.

Gonadotropin Treatment—Human chorionic gonadotropin (Serono, Italy) was injected intraperitoneally at a dosage of 500 units per day per rat for 30 days. The treatment started at the 55th day after birth for the animals hypophysectomized at the 25th day and at the 86th day after birth for the animals hypophysectomized at the 56th day. The efficiency of the treatment was evaluated by the macroscopic increase of testis weight (18).

Immunocytochemistry—Testes were obtained from five 60-day-old rats and from two 63-day-old rats, 28 days after hypophysectomy. Samples were fixed in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, for 6 h, dehydrated through graded concentrations of alcohol, and embedded in paraffin. Sections were cut at 10 μm and processed for the immunocytochemical demonstration of PHGPX using the streptavidin-biotin immunoperoxidase method (26). Briefly, sections were incubated serially with the following solutions: 1) 0.3% hydrogen peroxide for 30 min, to remove endogenous peroxidase activity; 2) normal goat serum (diluted 1:20) for 30 min, to minimize nonspecific background staining; 3) antiserum to PHGPX (diluted 1:1000) overnight at 4 °C; 4) biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame CA) (diluted 1:100) for 1 h at room temperature; 5) streptavidin-biotinylated peroxidase complex (Amersham International, Amersham, United Kingdom) (diluted 1:200) for 1 h at room temperature; 6) 0.03% (w/v) 3-3′-diaminobenzidine tetrahydrochloride, to which 0,02% hydrogen peroxide was added just before use, for 5 min at room temperature. Each solution was prepared in 0.05 M Tris-HCl, 0.1 M NaCl, pH 7.4. The same buffer was used for washing the sections after each step of immunostaining procedure. Some sections were lightly counterstained with haematoxylin. Sections were finally dehydrated and observed under a light microscope.

Pertinent specificity tests were performed, including absorption of antiserum to purified PHGPX, omission of the first step, and substitution of an inappropriate or a preimmune serum for the specific antiserum. The immunoprecipitation was abolished by using the antiserum preabsorbed with PHGPX (10 μM in antiserum diluted 1:1000 in PBS).

RESULTS

Rat testis contains a GSH peroxidase activity toward phospholipid hydroperoxides (PHGPX activity) that is much higher than in any other tissue far analyzed. Notably, in testis homogenate this activity is 20-fold higher than in liver and accounts for the majority of peroxidase activity of this tissue (Table I). As purified PHGPX (12), this activity in testis is completely inhibited by iodoacetate but only in the presence of a millimolar concentration of thiols (data not shown). This strongly suggests the presence of a selenium involved in the catalytic cycle (11 and references quoted therein).

Data reported in Table I also show that PHGPX specific activity in rat testis is higher in nuclear and mitochondrial fractions, while the specific activity in the liver is similar throughout different subcellular fractions, being only higher in cytosol.

When the polyclonal antiserum prepared using pig heart soluble PHGPX as antigen was used in Western blot analysis of cytosolic and membrane proteins from rat testis, a single protein with the same molecular mass (18 kDa) as pure PHGPX of pig heart cytosol was observed (Fig. 1).

PHGPX activity of rat testis cytosol was inhibited (not shown) and could be immunoprecipitated (Fig. 2*A*) by this antiserum. On the other hand, the immunoprecipitation of the membrane-bound enzyme was not possible due to aggregation and nonspecific precipitation of membranes in the presence of Staphylococcus A. Nevertheless, also the membrane-bound activity was inhibited by this antiserum (Fig. 2*B*).

These findings support the conclusion that rat testis contains a protein that corresponds to the pig heart cytosolic PHGPX, both in the cytosol and organelles. Furthermore, the absence of any significant cross-reactivity of the antiserum against other rat testis proteins permitted the immunocytochemical study of this enzyme.

PHGPX activity in testis was not detectable during the first 3 weeks after birth. Thereafter enzyme activity appeared and increased for 7–8 weeks (Fig. 3, *A–D*). It is notable that the delayed expression is specific for PHGPX, but not GPX and non-Se-GPX (a side activity of some glutathione *S*-transferases). The time course of the appearance of PHGPX activity was similar in the different subcellular compartments, with the exception of microsomes, where the activity was very low and much more delayed.

The pattern of the expression of PHGPX suggested that it could be related to the onset of puberty. This was confirmed by the observation that PHGPX is absent in testis (Fig. 4) but not liver (not shown) of animals hypophysectomized before puberty. Treatment of these animals with corionic gonadotropins led to the expression of PHGPX activity, although at a lower level than in control animals, at least during the 30-day treatment (Fig. 4).

The gonadotropin dependency of PHGPX activity expression was confirmed also in adult animals where hypophysectomy caused a rapid decline of the activity (Fig. 5). In these

TABLE I

Subcellular distribution of glutathione peroxidases in rat liver and testis

Specific activities were measured as described under "Materials and Methods."

Fraction	Liver			Testis		
	PHGPX	GPX	Non-Se-P	PHGPX	GPX	Non-Se-P
	mmoles/min/mg prot.					
Homogenate	4.2 ± 0.4	363 ± 25	871 ± 85	95 ± 5	62 ± 7.8	42.2 ± 5.8
2,000 × *g*	5.9 ± 0.6	321 ± 24	781 ± 66	102 ± 9	62 ± 5.7	50.2 ± 4.0
20,000 × *g*	2.3 ± 0.2	131 ± 12	313 ± 27	94 ± 9	58 ± 6.7	44.5 ± 5.1
105,000 × *g*	2.5 ± 0.3	183 ± 17	447 ± 52	2 ± 0.3	4.2 ± 0.3	5.1 ± 0.7
Supernatant	8.9 ± 0.6	873 ± 78	1265 ± 135	18 ± 3	40 ± 4.1	28.4 ± 3.5

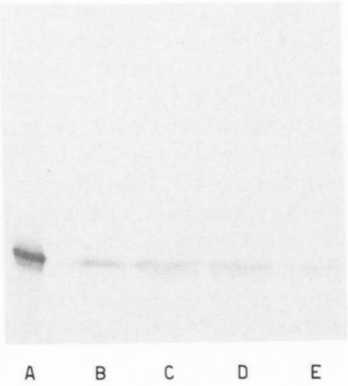

FIG. 1. **Cross-reactivity of an antiserum specific for pig heart PHGPX with PHGPX in rat testis subcellular fractions.** Western blot analysis was carried out as described under "Materials and Methods." *a*, PHGPX from pig heart cytosol (0.4 μg of protein); *b*, rat testis homogenate (100 μg of protein); *c*, rat testis nuclei (50 μg of protein); *d*, rat testis mitochondria (50 μg of protein); *e*, rat testis cytosol (100 μg of protein).

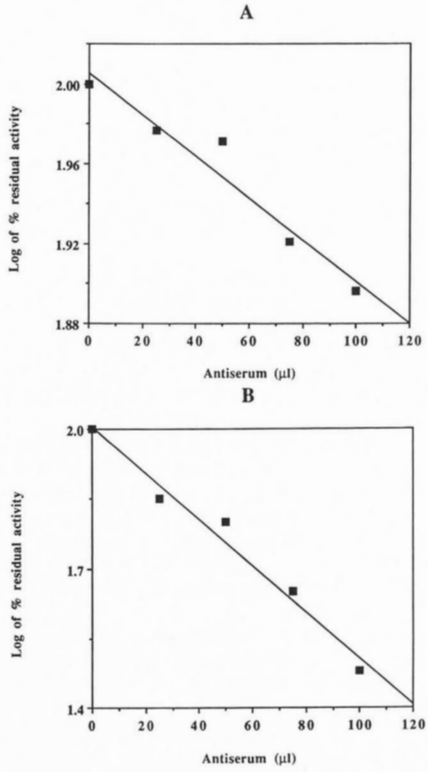

FIG. 2. **Immunoprecipitation of PHGPX from rat testis cytosol (*A*) and immunoinhibition of PHGPX activity in rat testis membranes (*B*).** Incubations and enzyme activity measurements were carried out as described under "Materials and Methods."

animals the activity was restored, although partially, by gonadotropin treatment.

The effect of gonadotropins on the expression of PHGPX was rather specific since neither GPX, nor non-Se-GPX activity was affected by hypophysectomy or gonadotropin treatment (Table II).

Specific immunolabeling of testis by antiserum against PHGPX appeared microscopically as a finely granular brown end-product localized throughout the cytoplasm in more immature cells, namely type B (clear) spermatogonia, but confined to the peripheral regions of the cytoplasm, the nuclear membrane, and mitochondria of maturating primary spermatocytes of normal testis. However, not all primary spermatocytes and spermatogonia displayed this immunoreactivity. Spermatids and spermatozoa, as well as Sertoli cells, were not immunostained.

In hypophysectomized animals immunostaining was completely negative, with the exception of rare immunoreactive cells in the suprabasal layer of the epithelium of tubules (Fig. 6*b*).

Although more cells become immunostained following gonadotropin treatment, this did not completely restore the immunohistochemical pattern observed in control animals (not shown).

DISCUSSION

PHGPX is a major peroxidase of rat testis. This is supported by: (i) the specific activity on specific substrates (*e.g.* phospholipid hydroperoxides); (ii) the inhibition of the activity by iodoacetate, which, only in the presence of thiols, alkylates the selenol moiety (12); (iii) the reactivity of a specific antiserum raised against homogeneous PHGPX, which inhibits and precipitates the activity and immunostains a 18-kDa protein.

Although PHGPX was first discovered in liver cytosol (14) and routinely purified from heart cytosol (11), a variable amount of the activity is strongly associated with cellular membranes (10–30% in different organs).[2] The specific activity measured in nuclear and mitochondrial fractions of testis is by far the highest so far observed. The soluble and membrane-bound enzymes have the same molecular weight, they are both inihibited by iodoacetate in the presence of GSH, and are both cross-reactive with the same antiserum. They can be considered, therefore, very similar, until the membrane-bound enzyme will be purified in an amount that permits molecular characterization. Tentatively, we submit that the difference between the soluble and the membrane-bound form of the enzyme could be due to a posttranslational modification, leading to increased lipophilicity or binding capacity of the membrane bound enzyme.

[2] A. Roveri, A. Casasco, M. Maiorino, P. Dalan, A. Calligaro, and F. Ursini, unpublished data.

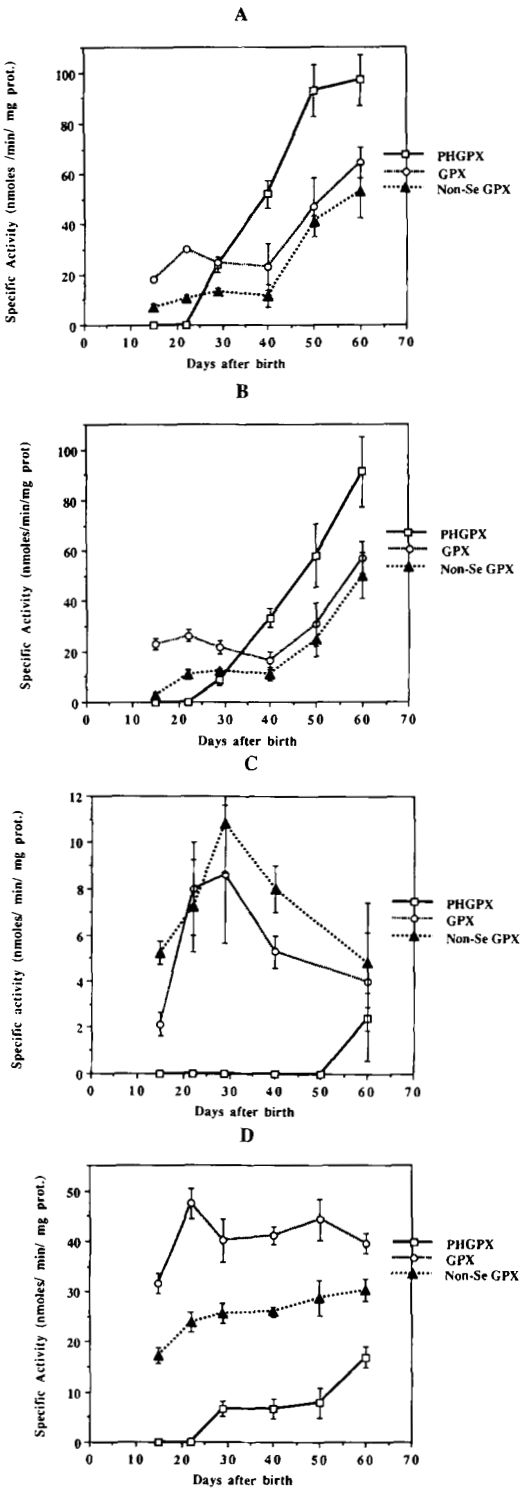

FIG. 3. **Time course of GSH peroxidases expression in rat testis.** The specific activity of different peroxidases was measured as described under "Materials and Methods" in nuclei (*A*), mitochondria (*B*), microsomes (*C*), and cytosol (*D*) or rat testis. Results are the mean ± S.D. of five independent measurements.

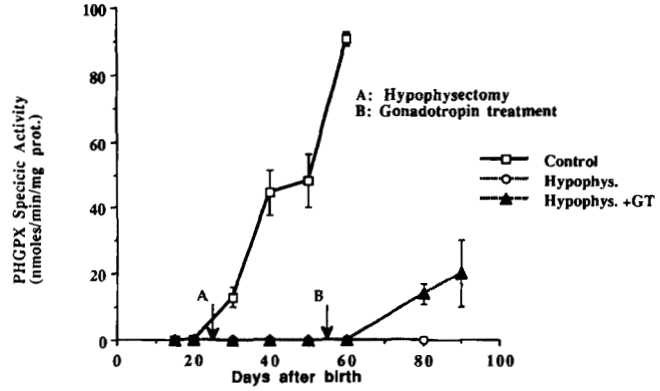

FIG. 4. **Effect of hypophysectomy before puberty and gonadotropin treatment on PHGPX activity in rat testis.** Hypophysectomy was carried out at the time *A* on the rats of group *Hypophys.* (□) and the group *Hypophys + GT* (▲). Gonadotropin treatment on the rats of the group *Hypophys + GT* started at time *B*. Results are the mean ± S.D. of five independent measurements (see "Materials and Methods" for details).

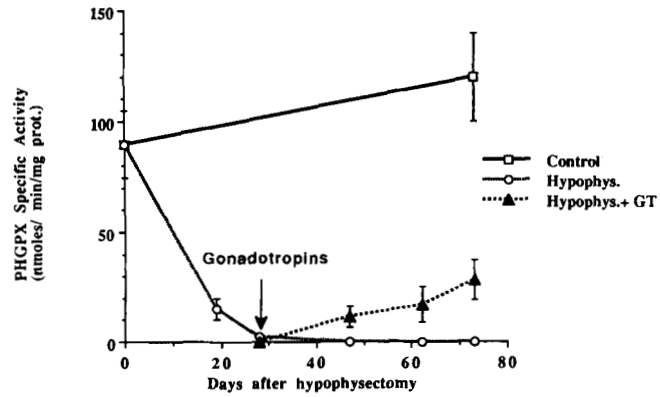

FIG. 5. **Effect of hypophysectomy after puberty and gonadotropin treatment on PHGPX activity in rat testis.** Gonadotropin treatment on the group *Hypophys + GT* (▲) started when indicated. Results are the mean ± S.D. of five independent measurements (see "Materials and Methods" for details).

TABLE II

Se-dependent (GPX) and non-Se-dependent (non-Se-GPX) glutathione peroxidase activities in rat testis after hypophysectomy and gonadotropin treatment

Rats were hypophysectomized (H) at the 25th day after birth and treated with gonadotropins (G) at the 56th day after birth, as described under "Materials and Methods."

Age	Treatment	GPX	Non-SE-GPX
days		*nmoles/min/mg prot.*	
80	None	65.2 ± 5.3	42.6 ± 4.2
80	H	65.8 ± 6.2	40.2 ± 5.3
80	H + G	57.5 ± 2.8	36.8 ± 8.5
90	None	68.7 ± 4.8	45.1 ± 3.8
90	H	61.7 ± 6.9	37.6 ± 8.2
90	H + G	57.3 ± 8.3	30.9 ± 7.5

The identification of PHGPX as a major selenoprotein in testis appears particulary relevant in the light of previous observations that selenium is required for spermatogenesis and supplied to the testes with an apparent priority over other tissues, allowing the synthesis of a selenoprotein different from GPX (18).

Our results, in agreement with data on selenium metabolism, suggest that the latter is specifically required for the expression of PHGPX, which, in turn, is necessary for the proper differentiation of germinal cells. Furthermore, the appearance of PHGPX activity after puberty, and the observed dependency on gonadotropins, supports the view that PHGPX is involved in the process of spermatogenesis.

The morphological data demonstrate that PHGPX is localized within maturating spermatogenic cells of the seminiferous tubules, whereas Sertoli and Leydig cells are not stained

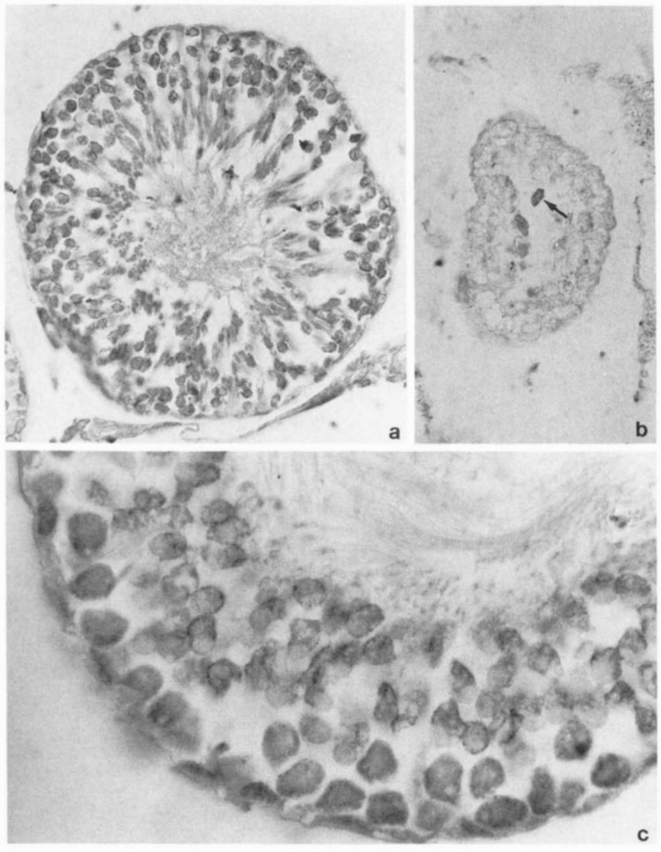

FIG. 6. *A-C*, immunocytochemical demonstration of PHGPX in testis from normal (*a*, *c*) and hypophysectomized (*b*) rats. Only rare positive cells (*arrow*) are visible after hypophysectomy. Magnification 430 X (*a*, *b*) and 1070 (*c*).

by the immunocytochemical reaction. The pattern of immunolocalization appears to change after the early stages of differentiation, when a diffuse immunoreactivity is apparent, while in the later phases the immunoreactivity is more concentrated in the mitochondria and nuclear envelop, in agreement with enzymological data. It is notable that the immunoreactivity completely disappears in spermatozoa. Thus, it may be concluded that the expression of PHGPX declines in completely differentiated cells. However, it is also possible that in spermatozoa the enzyme could be embedded in a complex matrix hampering the interaction with antibodies. Our recent unpublished observation that PHGPX can be identified by Western blot analysis among spermatozoa proteins supports the second possibility.

Tracer incorporation experiments showed that radioactive selenium is incorporated into a 15–20-kDa protein localized in the mid-piece of spermatozoa (27), accounting for the abnormality of spermatozoa in selenium-deficient rats. This protein has been previously identified with a major cysteine-rich structural protein in sperm mitochondria. The identity of PHGPX with this protein, although suggestive, may be ruled out by comparing the deduced amino acid PHGPX sequence with the recently reported amino acid sequence of the cysteine-rich protein deduced from cDNA sequence (28). Furthermore, neither selenocysteine nor the TGA codon have been identified in cysteine-rich protein and cDNA sequence (22). In conclusion, the similar molecular weights of the PHGPX and the cystein-rich protein accounts for a possible overlapping of the two proteins by gel permeation and electrophoretic analysis and the incorrect attribution of selenium to the cysteine-rich protein rather than to PHGPX. On the other hand, a different, possibly nonspecific, incorporation of selenium in the cysteine-rich protein (*e.g.* in the form of an -S-Se-S- adduct) is not excluded. Nevertheless, the two proteins are unquestionably different and only the PHGPX has been documented as a selenoprotein.

A plausible role for PHGPX in testes may be the protection of the genetic material from harmful lipid hydroperoxides, particularly when the genetic material is prone to damage during periods of rapid proliferation and gametogenesis. On the other hand, it is also possible that the control of peroxide tone in membranes by PHGPX might have a specific role in cellular regulation, particularly in cells undergoing proliferation and differentiation.

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