Expression of Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) mRNA in Rat Testes

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We investigated the expression of reactive oxygen species (ROS)-related enzyme mRNA to clarify the role of ROS in reproduction. In the present study, we investigated the expression of phospholipid hydroperoxide glutathione peroxidase (PHGPx) mRNA in rat testes by *in situ* hybridization. To prepare digoxigenin-labeled probes, we obtained 5'-phosphorylated oligonucleotides containing sense and antisense sequences (93-mer) with EcoR I or Hind III restriction sites as protruding cohesive ends (total 99-mer). Then, both oligonucleotides were annealed and cloned into a pGEM4Z vector. The resultant plasmid DNA was linearized with EcoR I or Hind III and used as a template for the synthesis of labeled sense or antisense riboprobes.

PHGPx mRNA was expressed stage-specifically during spermatogenesis in adult rats. It was not expressed during spermatogonia, but its expression first appeared in stage VII pachytene spermatocytes, became evident in stage VIII pachytene spermatocytes and increased gradually until becoming diplotene spermatocytes. It decreased slightly during the metaphase of meiosis, and then gradually increased as the spermatids differentiated. The expression was marked in spermatids between steps 7 and 12, and the maximum expression was observed in step 9–10 spermatids. Expression was diminished completely in step 18 spermatids. These results suggest that PHGPx plays an essential role in spermatogenesis.

Key words: Phospholipid hydroperoxide glutathione peroxidase, *In situ* hybridization, Testes, Rat

I. Introduction

Reactive oxygen species (ROS) are known to be involved in pathological conditions, while controlled concentrations of ROS are related to redox regulation under physiological conditions. In rat female reproductive organs, manganese superoxide dismutase (Mn-SOD) mRNA expression is observed in the theca interna, granulosa and lutein cells in ovulated follicles, and the epithelial cells of uteri and vaginas sequentially during the estrous cycle [14]. Localization of the transcripts in the ovary is consistent with the sites of progesterone synthesis and we concluded that hydrogen peroxide is involved in the process from pregnenolone to progesterone. High expression of Mn-SOD mRNA in the zona fasciculata and zona reticularis in the adrenal glands also supports this hypothesis [15].

In male reproductive organs, ROS is involved in sperm function [6, 8], while the deleterious effects of reactive oxygen species on spermatozoa are induced by membrane lipid peroxidation because of the high concentration of polyunsaturated fatty acids in the cell membrane [1]. Therefore, the role of antioxidant enzymes in the testes has been investigated. Among ROS-related enzymes, phospholipid hydroperoxide glutathione peroxidase (PHGPx) is specifically expressed in the testes of many mammals and is a major peroxidase in rat testes [2].

Selenium deficiency has been known to cause spermatozoa abnormalities [2]. PHGPx, one of the selenoproteins in the testis, is a slow responder to selenium deficiency, and selenium is rapidly stored in PHGPx after resupplementation [2], suggesting that PHGPx plays an important physiological role in spermatogenesis [2, 17]. Selenium is incorporated into selenocysteine of the enzyme, forming an active enzyme site with tryptophan and glutamine and is encoded by an in-frame UGA codon [5]. Thus, in the present study, we synthesized antisense

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Fig. 1. Probe preparation. Oligonucleotides (Table I) containing sense and antisense sequences for PHGPx were annealed and cloned into a pGEM4Z vector. The resultant plasmid DNA was finally linearized with *EcoR* I or *Hind* III and used as a template for the synthesis of antisense or sense riboprobes which were labeled with non-radioactive (digoxigenin) nucleotides.

and sense probes for PHGPx using a modified multi-labeling method [19] with nucleotides 231–323 including T²⁷⁷G²⁷⁸A²⁷⁹ for selenocysteine in a rat cDNA sequence [16] and investigated the detailed expression pattern of PHGPx mRNA in rat testes via *in situ* hybridization.

II. Materials and Methods

Tissue preparation

Adult male Wistar rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Then, testes were perfused with Bouin's fixative and immersed in the same fixative for 24 hr, dehydrated in ethanol, substituted with benzene, and embedded in paraffin. Paraffin sections (5 μ m thick) were cut on a microtome and mounted on silane-coated slides.

Preparation of probes

Probes for PHGPx were designed using a modified multi-labeling method (Fig. 1) [19]. 5'-phosphorylated oligonucleotides (Table I) containing sense and antisense sequences (nucleotides 231–323, 93-mer) [16] with *EcoR* I or *Hind* III restriction sites as protruding cohesive ends (total 99-mer) were obtained as purified and lyophilized products (Takara Biochemicals, Japan).

Both oligonucleotides were mixed and incubated together at 70°C for 10 min. Then, the solution was slowly cooled to room temperature and allowed to stand for a further 30 min to complete annealing. The annealed double-stranded DNA was then cloned into the pGEM4Z vector. The resultant plasmid DNA was linearized with *EcoR* I or *Hind* III. Digoxigenin (DIG)-labeled riboprobes for PHGPx were synthesized with an *in vitro* transcription kit in the presence of T7 or SP6 RNA polymerase according to the instructions provided by the

Table 1. Sequences of two 5'-phosphorylated oligodeoxynucleotides obtained from a commercial source for the preparation of PHGPx sense and antisense probes. Sense and antisense sequences (b) are flanked by *EcoR* I (a) or *Hind* III (c) restriction sites as protruding cohesive ends. T²⁷⁷G²⁷⁸A²⁷⁹ which codes for selenocysteine is underlined.

(a)	(b)
sense sequence 5'-pAATTC-TAAGTACAGGGGTTGCGTGTGCATCGTCACCAACGTGGCC	
antisense sequence 3'-G-ATTCATGTCCCCAACGCACACGTAGCAGTGGTTGCACCGG	
(b)	(c)
TCGCAATGAGGCAAAACCGACGTAACTACACTCAGCTAGTCGATCTGCATGC-A-3'	
AGCGTTACTCCGTTTTGGCTGCATTTGATGTGAGTCGATCAGCTAGACGTACG-TTCGAp-5'	

manufacturer (Roche Diagnostics Co., Germany). After DNase treatment, the product was ethanol-precipitated and the pellet was resuspended in the hybridization mixture. Limited hydrolysis after DNase treatment was omitted as the probes were less than 100-mers. Sequential homology of the probes' PHGPx element with classical GPx was under 60%.

Hybridization

Sections were dewaxed with toluene and ethanol, then sequentially treated with 0.2 N HCl, 1 µg/ml proteinase K in 2 mM of CaCl₂ and 20 mM of Tris-HCl buffer (pH 7.5), 2 mg/ml glycine in Dulbecco's phosphate buffered saline and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) to prevent nonspecific binding of the probes. The slides were washed with 2×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate) and then prehybridized in a solution containing 50% formamide and 2×SSC at 45°C for more than 1 hr. Hybridization was performed on slides using 20 µl of a hybridization mixture containing 50% formamide, 2×SSC, 1 mg/ml tRNA, 0.5 mg/ml sonicated salmon sperm DNA, 2 mg/ml bovine serum albumin, 10% dextran sulfate and the riboprobe (about 2 µg/ml) at 45°C for 16 hr in a moist chamber. The slides were then rinsed three times with 2×SSC and 50% formamide at 45°C for 20 min each. After further rinsing of the slides with 0.1×SSC, anti-digoxigenin alkaline phosphatase conjugate (1 : 2500) was placed on the sections at room temperature for 2 hr. After rinsing the slides, the enzyme-catalyzed color reaction was continued overnight with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt according to the instructions provided with the kit. The sections were stained with methyl green and observed with a light microscope.

When stage classification of spermatogenesis in rat testes was investigated, the same serial sections were stained with periodic acid Schiff-hematoxylin [9, 18].

III. Results

The expression of PHGPx mRNA was marked and stage-specific during spermatogenesis in adult rats. Figure 2A-M show the expression patterns of PHGPx mRNA at each stage in the seminiferous epithelium observed

typically in one section.

PHGPx mRNA was not expressed in spermatogonia, early spermatocytes or Sertoli cells constituting the basal layer of seminiferous tubule (Fig. 2P, periodic acid-Schiff-Hematoxylin stained section), but its expression became evident in stage VIII pachytene spermatocytes (Fig. 2G, arrowhead) and increased gradually until becoming diplotene spermatocytes (Fig. 2H-L). It decreased slightly during the meiosis metaphase (Fig. 2M), and then gradually increased as the spermatids differentiated. The expression was very marked in spermatids between steps 7 and 12 (Fig. 2F-K). The maximum expression was observed in step 9-10 spermatids (Fig. 2H-I).

In stages I-IV (Fig. 2A-C), PHGPx mRNA was expressed weakly in step 1-4 spermatids, and very weakly in step 15-17 spermatids. In stages V-VI (Fig. 2D-E), its expression began to increase in step 5-6 spermatids, whereas it was diminished in step 18 spermatids (stage VI, Fig. 2E). In stages VII-VIII (Fig. 2F-G), the expression became strong in step 7-8 spermatids. In these stages, it first clearly appeared in stage VIII pachytene spermatocytes (Fig. 2G, arrowhead). In stages IX-X (Fig. 2H-I), the expression in spermatids (step 9-10) reached the maximum, and in stages XI-XIII (Fig. 2J-L), it decreased. The expression in spermatocytes continued to increase in stages IX-XIII (Fig. 2H-L). The expression became weak in the meiotic division metaphase (stage XIV, Fig. 2M).

When sense probes were used as a control, few signals were observed (Fig. 2N).

PHGPx mRNA expression was not found in Leydig cells with *in situ* hybridization using the present probes.

IV. Discussion

In the present study, PHGPx mRNA was expressed in rat testes stage-specifically during spermatogenesis, but was not observed in spermatogonia, early primary spermatocytes or spermatozoa. Data showing PHGPx mRNA expression in spermatids has been consistently reported previously [11] and is compatible with findings on Northern analysis [24] and the data that PHGPx was expressed in the testes only after puberty [17]. The fact that the expression started in the pachytene spermatocytes of rat testes was consistent with results obtained in mouse testes [12].

Fig. 2. Adult rat seminiferous tubules hybridized with DIG-labeled antisense (A-M) or sense (N) probes for PHGPx. Arabic numerals represent steps of spermatids differentiation. A) stage I, B) stages II-III, C) stage IV, D) stage V, E) stage VI, F) stage VII, G) stage VIII, H) stage IX, I) stage X, J) stage XI, K) stage XII, L) stage XIII, M) stage XIV, N) seminiferous tubules hybridized with a DIG-labeled sense probe. P) section of stage IX seminiferous tubule stained with periodic acid Schiff-Hematoxylin. P, pachytene spermatocytes; L, leptotene spermatocytes; S, Sertoli cell. PHGPx mRNA expression first became apparent in stage VIII pachytene spermatocytes (G, arrowhead). $\times 240$.

Before PHGPx was identified [22], selenoproteins in testis had been studied, as selenium deficiency caused severe spermatozoa abnormalities. Concerning localiza-

tion of selenoprotein in rat spermatozoa, Calvin *et al.* reported that radioactive selenium is incorporated into a 15-20 kDa protein localized in the midpiece of spermato-

zoa [3]. They also reported that incorporation of ^{75}Se into mitochondrial capsule protein (MCP) begins in late primary spermatocytes, increases in step 1–6 spermatids, is highest in step 7–12 spermatids, and declines to the levels of spermatozoa in step 15 spermatids [4]. ^{75}Se incorporation into MCP occurred at a timing similar to the expression of PHGPx mRNA, becoming marked in stage VIII pachytene spermatocytes (Fig. 2G, arrowhead) and highest in step 7–12 spermatids (Fig. 2F–K) and the labeling was more intense in the residue fraction than in the cytosol [4]. These results coincided with findings later obtained for PHGPx [17, 24].

Recently, Ursini *et al.* clarified that this protein, the mitochondrial capsule protein in spermatozoa, was PHGPx, which is an enzymatically inactive, oxidatively crosslinked, insoluble structural protein [23]. This explains perfectly the damage to the mitochondrial midpiece of spermatozoa in Se deficiency [23].

Although the role of PHGPx as a structural protein became apparent in mature spermatozoa, PHGPx exists as an enzymatically active, soluble protein in spermatids [23] and its role in spermatids is not clear. Among ROSs, the dual action of hydrogen peroxide on spermatozoa has also been emphasized. When found at low concentrations, it acts as a mediator of normal sperm functions such as capacitation, but when it is produced in excess, it is highly toxic to the cell [6, 8]. However, PHGPx reduces phospholipid hydroperoxide [21] and cholesterol hydroperoxides [20] much more effectively than it can reduce hydrogen peroxide.

The role of PHGPx in spermatogenesis is not clear, but it is studied using cultured cells. Nomura *et al.* reported that human cells that overexpress mitochondrial PHGPx prevent the release of cytochrome c, activation of caspase-3 and apoptosis induced by the exposure of 2-deoxyglucose [13]. They suggested that hydroperoxide generated in mitochondria is a major factor in apoptosis, and that mitochondrial PHGPx plays a critical role as an anti-apoptotic agent in mitochondrial pathways. In rat testes, PHGPx specific activity in the mitochondrial fraction is 5-fold higher than that in the cytosol [17], and PHGPx isolated from rat testis mitochondria is a membrane-bound enzyme, largely localized at contact sites between the two membranes [7]. Therefore, almost all signals in the figures may represent the presence of mitochondrial PHGPx mRNA. PHGPx may play a role as an anti-apoptotic agent, as well as in spermatid differentiation through the regulation of hydroperoxide levels.

In our previous studies, we reported that Mn-SOD mRNA was expressed cyclically in the epithelium of rat ovaries, uteri and vagina during the estrous cycle, and that hydrogen peroxide may play important roles in ovarian steroid metabolism [14]. In addition, ROSs, including hydrogen peroxide, may be involved in the triggering of apoptosis in the tadpole tail [10]. The present results support the important roles of hydroperoxide in the male,

as well as female, reproductive organs.

PHGPx is synthesized as a long form containing a leader sequence that is required for transport to mitochondria and as a short form which lacks the sequence [16]. In the present study, both the mitochondrial L-form and the non-mitochondrial S-form can be detected by the probes for PHGPx mRNA which is synthesized by the multiple-labeling method [19]. The probes prepared by this method are riboprobes and can be labeled with multiple reporter molecules such as digoxigenin or ^{35}S , which form highly stable hybrids with mRNA. If information regarding the target cDNA sequence is available, synthetic oligonucleotides can be obtained from commercial sources at low cost. So this method may be a useful tool for further *in situ* hybridization studies after screening for gene expression alterations using cDNA microarray or cDNA chips analysis. Precise studies showing the expression patterns of various ROS-related enzymes may be used to study the toxic effects of various agents on the reproductive organs.

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VI. References

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