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Phospholipid hydroperoxide glutathione peroxidase is involved in the maintenance of male fertility under cryptorchidism in mice

Ki Youn Jung[a,1], Jung-Min Yon[a,1], Chunmei Lin[a], A Young Jung[a], Jong Geol Lee[a], In-Jeoung Baek[b], Beom Jun Lee[a], Young Won Yun[a], Sang-Yoon Nam[a,*]

[a] College of Veterinary Medicine and Research Institute of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea
[b] Asan Institute for Life Sciences, Asan Medical Center and University of Ulsan, Seoul 138-736, Republic of Korea

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ABSTRACT

Severe oxidative stress by cryptorchidism leads to infertility. To assess the functional significance of phospholipid hydroperoxidase glutathione peroxidase (PHGPx) under cryptorchidism, PHGPx expression was spatiotemporally analyzed in testes and epididymis excised at 1, 4, 7, 14, 21, and 28 days after experimental bilateral cryptorchidism in adult mice. In testes, while apoptosis-related caspase 3 and Bcl-xL mRNAs were significantly changed after 14 days, 3 beta-hydroxysteroid dehydrogenase mRNA was greatly reduced immediately after cryptorchidism. Under cryptorchidism, PHGPx was significantly decreased in both organs after 21 days, while its mRNA was greatly reduced in testes after 14 days and in epididymis after 4 days. However, PHGPx was upregulated in degenerative spermatids, multinucleated giant cells, and Leydig cells in testes and desquamous spermatids in epididymis until 21 days, but was weakly detected in the spermatids at 28 days. These findings suggest that PHGPx is necessary for maintenance of male fertility under cryptorchidism in testes.

1. Introduction

Oxidative stress in male reproductive systems has been implicated in the pathogenesis of developmental defects and diseases such as cryptorchidism, testicular torsion, varicocele, infection, and hormone imbalance [1]. Scrotal testes in most mammals are 2–8 °C lower than the core body temperature, and any disruption of this testicular temperature homeostasis may cause large-scale loss of testicular germ cells [2]. Cryptorchidism is a congenital defect of testis in many species of mammals, which leads to infertility by over-production of reactive oxygen species [3,4]. Although it has been reported that endogenous antioxidant systems are essential to decreasing oxidative damage to sperm lipids and DNA [5,6], the critical antioxidant enzyme related to dynamic spermatogenic disorders induced by cryptorchidism is still unclear.

Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant that belongs to the GPx superfamily and can reduce hydrogen peroxide, alkyl peroxides, and fatty acid hydroperoxides like other GPx, as well as hydroperoxides in lipoproteins and complex lipids [7]. PHGPx is associated with regulation of mitochondrial apoptosis and protection of mitochondrial ATP generation against oxidative stress and eicosanoid production in somatic cells [8,9]. PHGPx is expressed in the mitochondria, cytosol and nuclei of all tissues, and is found in especially high levels in testis and preferentially expressed after puberty [10,11]. Transgenic mice that do not have PHGPx protein were found to be viable, but exhibited structural abnormalities in the sperm [12,13]. Although previous studies proposed that PHGPx may be involved in sperm function in male fertility and could be considered a prognostic parameter for fertilization capacity [14,15], further studies are necessary to understand the functional significance of PHGPx on spermatogenesis, especially under cryptorchidism.

In the present study, the expression profiles of PHGPx were spatiotemporally investigated in the testes and epididymis of cryptorchid mice to better understand the function of PHGPx on spermatogenesis under cryptorchidism.

2. Materials and methods

2.1. Experimental cryptorchidism

Male ICR mice (8 weeks old; Koatech Co., Gyeonggi, Korea) were housed in polycarbonate cages in a well-ventilated room maintained at 21 ± 2 °C and 55 ± 10% relative humidity under a 12 h

* Corresponding author at: Laboratory of Veterinary Anatomy, College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Republic of Korea. Tel.: +82 43 261 2596; fax: +82 43 271 3246.
E-mail address: synam@cbu.ac.kr (S.-Y. Nam).
[1] These authors contributed equally to this work.

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light/dark cycle. Mice were fed a standard mouse chow (Samyang Ltd., Incheon, Korea) and provided with tap water ad libitum.

Bilateral cryptorchidism was experimentally induced in ICR mice under pentobarbital anesthesia as previously described [16]. Briefly, both inguinoscrotal incisions were made, and the gubernaculums were separated from the abdominal wall. After the testes were displaced from the scrotum to the abdomen, the external inguinal ring was sutured to prevent testis descent. At 1, 4, 7, 14, 21, and 28 days after operation, the animals were sacrificed under pentobarbital anesthesia and the testes and epididymides were removed ($n = 15$ per group). The left testes and epididymides were fixed with Boun's solution for immunohistochemistry, while the right organs were immediately frozen at −70 °C for RNA or protein extraction. Sham-operated animals (8 weeks old) served as a control and were sacrificed according to the cryptorchidism schedule. All procedures were conducted in compliance with the Guide for Care and Use of Animals (NIH # 86-23) and approved by Chungbuk National University Animal Care Committee (CBNUA-588-13-01).

2.2. Quantitative real-time PCR

Total RNA was isolated from mouse testes and epididymides using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA concentrations were determined based on the UV absorbance. Next, 2 μg of total RNA were reverse-transcribed using random primers and high capacity cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). Quantitative PCR reactions were performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Amplifications of PHGPx (a mitochondrial isoform), apoptosis-related genes, and 3 beta-hydroxysteroid dehydrogenase type VI (3β-HSD) were performed using a Model 7500 Real-Time PCR System (Applied Biosystems) and primers designed by TIB Mol-Bio Synthesis (Berlin, Germany). Primer sequences used in this study were as follows: PHGPx (NM_008162) forward 5′-TAA GAA CGG CTG CGT GGT-3′ and reverse 5′-GTA GGG GCA CAC ACT TGT AGG-3′; Caspase-3 (NM_009810) forward 5′-AAA GCC GAA ACT CTT CAT CAT-3′ and reverse 5′-GTC CCA CTG TCT TCA-3′; Bcl-xL forward 5′-TGA CCA CCT AGA GCC TTG GA-3′ and reverse 5′-TGT TCC CGT AGA GAT CCA CAA-3′; 3β-HSD (NM_153193) forward 5′-GAC TGC TGA CAC ACC ACA CC-3′ and reverse 5′-GGG AGT GAG GTT AAC TTA ATG TAC G-3′; β-actin (NM_007393) forward 5′-TTT CCA GCC TTC CTT CTT GGG TAT G-3′ and reverse 5′-CAC TGT GTT GGC ATA GAG GTC TTT AC-3′. Each PCR program was started with UNG (uracil-N-glycosylase) incubation at 50 °C for 2 min, followed by incubation at 95 °C for 10 min. This was followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Data were acquired and analyzed with the 7500 system SDS software (version 1.3.1.21, Applied Biosystems). Amplification kinetics was recorded in real-time mode as sigmoid process curves for which fluorescence was plotted against the number of amplification cycles. Beta-actin was used as an internal standard to normalize expression of the target transcripts. Data from five independent runs were analyzed using a comparative Ct method as previously described [17].

2.3. Protein extraction and Western blotting analysis

Total protein was extracted from mouse testes and epididymides. To quantify protein concentrations in tissues, Western blotting was conducted as previously described [18]. Anti-PHGPx polyclonal antibody, anti-cytoplasmic GPx (GPx1) polyclonal antibody, and anti-GAPDH monoclonal antibody were purchased from Epitomics, Inc. (Burlingame, CA, USA), abcam (Cambridge, MA, USA), and Cell Signaling Technology (Beverly, MA, USA), respectively. Secondary horseradish peroxidase-linked anti-rabbit IgG antibody was purchased from Ab Frontier (Seoul, Korea). Membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:7000; Ab Frontier) for 1 h at room temperature, after which the blot was developed using the WEST-one™ Western Blot Detection System (iNtRON, Gyeonggi, Korea). Membranes were subsequently exposed to X-ray film from Agfa HealthCare (Mortsel, Belgium) for 5 and 20 s. After digitalizing the film, Image J software from the National Institutes of Health (Bethesda, MD, USA) was used to quantify band intensities. Experiments were performed in each of five testes and epididymides and data were presented as the means ± SE.

2.4. Immunohistochemistry

Tissue sections were deparaffinized with xylene and rehydrated through ethanol gradients. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol for 15 min, after which sections were washed in PBS for 20 min 4 times. Sections were then blocked from nonspecific binding by 20 min of incubation in diluted normal serum and then incubated with the PHGPx antibody for 1 h at 37 °C. Next, sections were incubated for 30 min at room temperature with a biotinylated secondary antibody (Vectastain ABC Kit; Vector Lab., Burlingame, CA, USA) followed by 40 min at room temperature with the peroxidase-conjugated biotin-avidin complex (Vector Lab.). Finally, the bound peroxidase was revealed by immersing the sections in diaminobenzidine (Vector Lab.). Sections were rehydrated for 15 min in deionized water to remove any precipitated Tris and dehydrated in a series of ethanol and xylene. Negative control experiments for the antibodies were performed as described above with omission of the primary antibody.

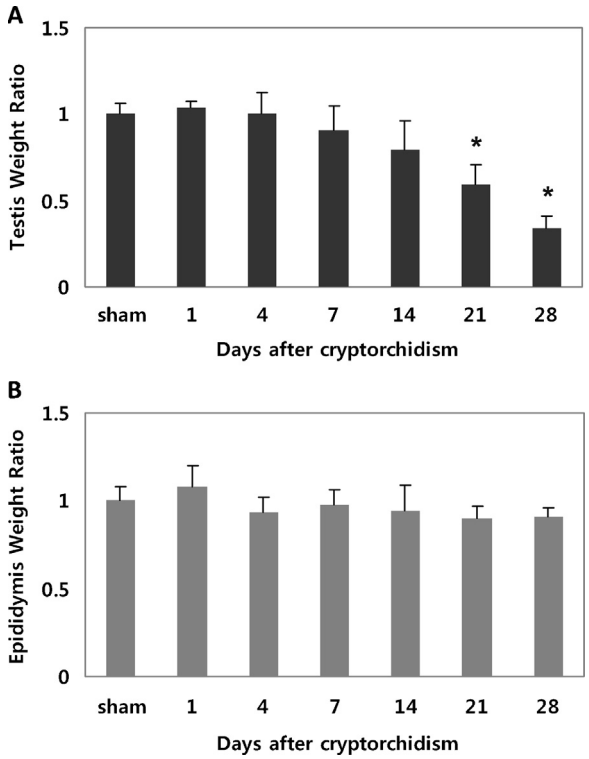

Fig. 1. Time course changes in testes and epididymides weights after cryptorchidism. Bilateral cryptorchidism was surgically performed in 8-week-old mice. The testes (A) and epididymides and (B) weights relative to body weights were measured on days 1, 4, 7, 14, 21 and 28 after surgery. Data represent the means ± SE ($n = 5$). *Significantly different from the sham control at $P < 0.05$.