Bactericidal/permeability-increasing protein in the reproductive system of male mice may be involved in the sperm–oocyte fusion

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Abstract

Bactericidal/permeability-increasing protein (BPI) is a 455-residue (~55 kDa) protein found mainly in the primary (azurophilic) granules of human neutrophils. BPI is an endogenous antibiotic protein that belongs to the family of mammalian lipopolysaccharide (LPS)-binding and lipid transport proteins. Its major function is to kill Gram-negative bacteria, thereby protecting the host from infection. In addition, BPI can inhibit angiogenesis, suppress LPS-mediated platelet activation, increase DNA synthesis, and activate ERK/Akt signaling. In this study, we found that *Bpi* was expressed in the testis and epididymis but not in the seminal vesicles, prostate, and solidification glands. BPI expression in the epididymis increased upon upregulation of testosterone, caused by injection of GnRH. In orchidectomized mice, BPI expression was significantly reduced, but its expression was restored to 30% of control levels in orchidectomized mice that received supplementary testosterone. The number of sperm fused per egg significantly decreased after incubation with anti-BPI antiserum. These results suggest that BPI may take part in the process of sperm–oocyte fusion and play a unique and significant role in reproduction.

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Introduction

Bactericidal/permeability-increasing protein (BPI) was first described by Weiss *et al.* (1978). BPI is a 455-residue (~55 kDa) protein that was originally identified in rabbit and human primary azurophilic granules of neutrophil granulocytes (Weersink *et al.* 1993). It can also be found on the surface of neutrophils and monocytes and in exposed epithelial areas of the nose, mouth (Canny *et al.* 2002, Canny & Levy 2008), pharynx, lungs (Holweg *et al.* 2011), digestive tract, and organs of sensation (Gerald *et al.* 2007).

BPI belongs to a family of mammalian lipopolysaccharide (LPS)-binding and lipid transport proteins. This family includes four well-characterized members, namely, BPI, LPS-binding protein (LBP), cholesteryl ester transfer protein, and phospholipid transfer protein. These proteins are 45–65% homologous at the mRNA level and are structurally and functionally related to each other (Qiu *et al.* 2007). For example, the genes for *Bpi* and *Lbp* are highly homologous and are encoded in the same region of chromosome 20. BPI and LBP are two key members of the innate immune response to Gram-negative bacteria. LBP catalyzes the extraction and transfer of individual LPS molecules to CD14, forming

a monomeric LPS–CD14 complex that is a key intermediate in the delivery of LPS to MD-2/toll-like receptor 4 (TLR4) and in TLR4-dependent cell activation. In contrast, BPI, which has a higher affinity for LPS, represses inflammation by preventing LBP from delivering LPS to CD14 (Eckert *et al.* 2006, Palmer *et al.* 2011).

The BPI molecule has a highly elongated, ‘boomerang’ shape. The N- and C-terminal domains of this protein share little sequence identity but are very similar in global architecture (Beamer 2003). The N-terminal domain is rich in cations and can specifically bind to the lipid A portion of LPS (Gazzano-Santoro *et al.* 1992), thereby killing Gram-negative bacteria by changing the permeability of the bacterial cell wall. LPS-neutralizing activity is also localized to the N-terminal region of the protein. A recombinant amino-terminal fragment of BPI (rBPI21) can protect animals by inhibiting the effects of Gram-negative bacteria and endotoxin (Elsbach & Weiss 1998, Guinan *et al.* 2011). The function of the C-terminal domain of BPI has been less reported; all that is known is that this domain may protect BPI from degradation by intracellular enzymes and that it enhances the opsonic activity of the molecule (Iovine *et al.* 1997).

BPI has been conserved over evolution, as orthologs have been found in a number of non-mammalian

vertebrates and invertebrates. During the process of evolution, BPI in low-grade organisms may function as a surface molecule to identify environmental microbes and then kill or neutralize them (Krasity *et al.* 2011).

In addition to its well-documented anti-infective properties, BPI has been shown to possess additional bioactivities; for example, it has been demonstrated to act as an endogenous inhibitor of angiogenesis (der Schaft *et al.* 2000, 2002) via induction of endothelial cell apoptosis. In addition, BPI activates ERK and serine/threonine protein kinase (Akt) and increases DNA synthesis in retinal pigment epithelial cells and pericytes, meanwhile reducing ischemia-induced retinal neovascularization and diabetes-induced retinal permeability (Yamagata *et al.* 2006). PLUNC (palate, lung and nasal epithelium clone) is a small, secreted protein that is expressed in the oropharynx and upper airways of humans, mice, and rats (Leclair 2003) and is structurally related to BPI. PLUNC inhibits *Pseudomonas aeruginosa* biofilm formation without acting directly as a bactericide (Bartlett *et al.* 2011). Auto-antibodies against BPI (BPI-ANCA) are associated with poor prognosis in cystic fibrosis, as they induce less inflammatory response than the non-ANCA-associated strains (Carlsson *et al.* 2011). BPI inhibits LPS-mediated platelet activation (Luo *et al.* 2012) and mitigates radiation-induced bone marrow aplasia and death (Guinan *et al.* 2011). Patients with chronic obstructive pulmonary disease present with decreased plasma levels of BPI (Chen *et al.* 2012).

The testis and epididymis are two important organs of the male reproductive system. The main functions of the testis are sperm production and androgen secretion. The epididymis is one of the reproductive accessory glands; its epithelium functions in absorption, secretion, and enrichment, which is consequently responsible for sperm surface remodeling, promotion of sperm maturation, sperm storage, and protection (Bedford 1967, Orgebin-Crist 1967). All these functions of the testis and epididymis are regulated by testosterone.

In this study, BPI was found in the testis and epididymis of C57BL/6J mice but was undetectable in the seminal vesicles, prostate, and solidification glands. In the epididymis, BPI expression was regulated by testosterone levels. Immunofluorescence analysis showed BPI localization in the seminiferous tubule, caput epididymis epithelium, and sperm. Although it has been reported that BPI protein is associated with the acrosome region of mice epididymal spermatozoa (Yano *et al.* 2010), BPI expression was not seen in the epithelia of the corpus and cauda epididymis. Results from sperm-oocyte fusion assays indicated that anti-BPI antiserum may influence the fusion of sperm and oocytes, although the mechanism by which this occurs is still unclear and needs further investigation. Together, our data indicate that in addition to its other biological functions, BPI may play an important role in male reproduction.

Materials and methods

Animals

The Institutional Animal Care and Use Committee (IACUC) of School of Medicine, Shanghai Jiao Tong University, approved this study (SYXK (Hu) 2008 0050). All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. C57BL/6J mice (male: aged 8 weeks, body weight 22–24 g; female: aged 7–9 weeks, body weight 18–20 g) were purchased from Shanghai Laboratory Animal Commission (Shanghai, China) and were accommodated in the animal facility for at least 1 week before experiments.

Chemicals

Unless otherwise stated, all reagents were purchased from Sigma–Aldrich Corporation.

Reverse transcriptase-PCR

Total RNA from the seminal vesicles, prostate, solidification glands, testes, and epididymis of mice was prepared using TRIzol Reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. Then, the cDNA from the mouse testis and epididymis was reverse transcribed with RT Master Mix (Takara, Otsu, Japan). *Bpi* cDNA was amplified with the forward primer, 5'-AGCAGGGAGTGGTTGAGT-3' and reverse primer, 5'-GATGGTGGTGATGTGGC-3'. β -Actin was amplified with the forward primer, 5'-CCCATCTACGAGGGCTAT-3' and the reverse primer, 5'-TGTCACGCACGATTTC-3'.

Immunofluorescence analysis

To determine the localization of BPI in the mouse testis and epididymis, frozen sections of 8 μ m thickness were prepared and then fixed in 4% paraformaldehyde for 20 min at 4 °C. All subsequent incubations were performed in a humid chamber. The slides were blocked in 10% goat serum (Invitrogen) for 30 min at room temperature followed by a subsequent overnight incubation at 4 °C with rabbit anti-BPI antibodies (1:200 dilution) and rabbit normal IgG as a negative control. After washing, the samples were incubated with FITC-labeled goat anti-rabbit IgG (Invitrogen; 1:200 dilution) for 1 h at 37 °C followed by incubation with 5 μ g/ml propidium iodide (Wako, Osaka, Japan) for 5 min to counterstain the nucleus. Finally, the fluorescence-stained sections were viewed under a laser scanning confocal microscope (Carl Zeiss LSM-510, Jena, Germany). Immunofluorescence experiments were repeated at least three times.

Gonadotrophin releasing hormone (GNRH) treatment

Male mice were divided into two groups: ten animals received GNRH by i.p. injection (10 μ g/day for 5 days) and ten animals received injections of saline as a negative control. Serum testosterone concentrations and BPI expression in the testis and epididymis were analyzed.

Surgical castration

Male mice were divided into three groups consisting of three mice each: castration, sham-operated (negative control), and no operation (normal control) groups. Mice in the castration group were killed 1–8 days after the operation.

Testosterone supplement

Testes were removed aseptically, and damage to the epididymis was avoided. Male mice were divided into three groups consisting of three mice each: the treatment group that underwent i.p. injection of 200 µl testosterone at a concentration of 2×10^{-7} mol/l, the control group that underwent i.p. injection of physiological saline, and the blank control group that had no treatment. The mice were killed the day after testosterone treatment. Serum testosterone concentrations and BPI expression in the epididymis were analyzed in three independent experiments.

Serum testosterone detection

Blood (200 µl) from each mouse was collected from the inner canthus. Blood samples were allowed to clot for 30 min before centrifugation for 15 min at 1000 g. The serum was removed, followed by quantitative analysis of testosterone levels with a testosterone ELISA Kit (R&D, Minneapolis, MN, USA) according to the manufacturer's protocol.

Quantitative real-time PCR

SYBR green-based quantitative real-time PCR (qPCR) assays for the quantitative detection of BPI were performed to analyze *Bpi* gene expression in the testes and epididymis. The cDNA was obtained as previously described. *Bpi* primers were as follows: forward primer, 5'-CCCGCCAGTAATGAGGTT-3'; reverse primer, 5'-CAGGGTCATCTTCAAGGTT-3'. Primers for the β -actin gene, which served as an internal control, were as follows: forward primer, 5'-AGGTGACAGCATTGCTTCTG-3'; reverse primer, 5'-GCTGCCTCAACACCTCAAC-3'. QPCR was performed using the Applied Biosystems 7500 real-time PCR

System, according to the manufacturer's protocol. The $2^{-\Delta\Delta C_t}$ method was used to analyze the data.

Preparation of protein specimens

Mice testis and epididymis were homogenized using T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Ameresco, Solon, OH, USA). Protein extracts were incubated in a rotating ice bath for at least 30 min to allow proteins to dissolve completely, followed by centrifugation at 12 000 g for 10 min. The supernatant was saved, and protein concentration was determined using the Pierce BCA Protein Assay Kit.

Western blot analysis

Protein samples (30 µg) were separated by SDS–PAGE and then transferred to PVDF membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer apparatus (Bio-Rad). Membranes were blocked in 5% BSA. Immunoblotting was performed with rabbit anti-BPI-C polyclonal antibodies at a suitable dilution followed by incubation with secondary antibody conjugated to HRP (Abgent, San Diego, CA, USA) at a 1:5000 dilution. Signals were detected by ECL (Millipore) according to the manufacturer's protocol. β -Actin (1:5000; Abcam, Cambridge, MA, USA) served as the internal reference.

Gamete preparation

Female C57BL/6J mice were superovulated by abdominal injection of 10 IU of pregnant mare's serum gonadotropin (PROSPEC, Rehovot, Israel) followed by injection of 10 IU human chorionic gonadotropin (hCG; Li Zhu drug plant, Zhuhai, China) after 46–48 h. Cumulus-enclosed egg complexes were collected from the oviducts 15–16 h post-hCG injection and treated with 0.1% hyaluronidase in Medium 16 (M16) to disperse the cumulus cells. The zona pellucida was removed with 0.6% proteinase K, after which the eggs were incubated in M16 containing 3% BSA at 37 °C, 5% CO₂ for 30–60 min.

Figure 1 Analysis of *Bpi* mRNA expression in the male mouse reproductive tract by RT-PCR. (A) Upper section shows amplification of the *Bpi* gene from total RNA of adult mouse (8 weeks) testes, epididymis, prostate, seminal vesicles, and coagulation gland respectively. The lower section shows amplification of the β -actin gene that corresponds with the genes in the upper lanes. (B) Upper section shows amplification of the *Bpi* gene from total RNA of adult mouse (8 weeks) caput epididymis, corpus epididymis, and cauda epididymis respectively. The lower section shows amplification of the β -actin gene that corresponds to genes in the upper lanes.

Mature spermatozoa were collected from the epididymal cauda of C57BL/6J male mice by rough cutting in 1.5 ml of M16 containing 3% BSA. Motile spermatozoa were allowed to swim out for 10–15 min, after which the tissue was removed from the medium. One part of the spermatozoa was cultured for 2 h in M16 (containing 3 mg/ml BSA and 5 μ mol/l calcium ionophore A23187) at 37 °C, 5% CO₂. The rest of the spermatozoa were cultured for 90 min in M16 containing 3 mg/ml BSA at 37 °C, 5% CO₂, which allowed them to capacitate and undergo spontaneous acrosome reaction. Next, spermatozoa (adjusted to 10⁷ sperm/ml) were incubated for 40 min in the presence or absence of the same dilutions of purified rabbit anti-mouse BPI-C antiserum (inactivated complement), purified rabbit anti-mouse BPI-N antiserum (inactivated complement), pre-immunized rabbit serum, or wash buffer (control), followed by a 1:40 dilution with M16 containing 3% BSA for use in the sperm–oocyte fusion assay. The final sperm concentration was 2.5 $\times$ 10⁵ sperm/ml.

Sperm–oocyte fusion assay

For every sperm–oocyte fusion assay, all oocytes and spermatozoa used in an experiment were combined from several female and male mice and then randomly divided into each group. Zona pellucida-free oocytes were directly added to the sperm suspensions that were prepared as described earlier, and the gametes were co-incubated for 3 h at 37 °C, 5% CO₂. Then, loosely bound spermatozoa were removed from the oocytes by gentle pipetting. Next, the oocytes were fixed in 4% paraformaldehyde for 15 min and then treated with Hoechst 33342 for 10 min to stain the chromatin. Finally, images were collected using a laser scanning confocal microscope and results were obtained as the average number of penetrating sperm per oocyte. Each assay was repeated at least three times.

Statistical analysis

All values are presented as mean $\pm$ S.E.M., and statistical analysis of the data was carried out using SAS 8.0. Data was assessed for normality using the Shapiro–Wilk test. Statistically significant differences in the serum testosterone in gonadotropin-releasing hormone (GnRH) treatment, surgical castration and testosterone supplement after surgical castration, mRNA values, and the numbers of sperm fused to oocytes were analyzed using ANOVA. The Student–Newman–Keuls test was used to account for variance. Pearson correlations were performed to test the correlation between serum testosterone concentration and BPI mRNA expression in the epididymis. The false discovery rate was utilized to adjust the level of significance to account for multiple comparisons. Statistical significant difference was considered to be $P < 0.05$.

Results

Bpi mRNA expression in the reproductive system of male mice

In order to analyze *Bpi* mRNA expression in the reproductive system of adult male mice, qPCR was

Figure 2 Localization of BPI protein in the adult mouse testis and epididymis. BPI-positive signals had a green fluorescence, and the nucleus, which was counterstained with propidium iodide, had a red fluorescence. (a, b, c, d and e) These images represent the adult mouse (8 weeks) testis, efferent duct, caput epididymis, corpus epididymis, and cauda epididymis respectively. Enlargement of the seminiferous tubule in (A) is indicated with a yellow box, enlargement of the interstitial tissue is indicated with a light blue box, and enlargement of the sperm from the testis is indicated with a white box. (f, g, h, i and j) The negative controls (rabbit normal IgG) corresponding to images (a, b, c, d and e) respectively.

performed. *Bpi* mRNA was remarkably expressed in the testis and epididymis but not in the other organs examined, including the seminal vesicles, prostate, and solidification glands (Fig. 1A). With regard to the epididymis, which is divided into three main regions: the caput, corpus, and cauda, *Bpi* expression was only detected in the caput epididymis epithelium but not in the epithelia of the corpus and cauda epididymis (Fig. 1B).

BPI localization in the testis and epididymis

Immunofluorescence analysis with an anti-BPI-C terminal polyclonal antibody (Supplementary Figure 1, see section on supplementary data given at the end of this article) was performed to determine the localization of BPI in the mouse testis and epididymis. A positive BPI signal was seen in spermatocytes and spermatids (Fig. 2a, Supplementary Figure 2), as well in mature spermatozoa (Fig. 2e), indicating that BPI was expressed throughout the processes of spermatogenesis in the testis and sperm maturation in the epididymis. BPI was also expressed in Leydig cells. Consistent with the qPCR data, BPI expression in the epididymis was confined to the epithelia of the efferent duct and caput epididymis. BPI expression in the efferent duct was higher than that in the caput epididymis (Fig. 2b, c, d and e).

Testosterone-influenced *Bpi* expression

To determine the effect of increased testosterone levels on *Bpi* expression, GNRH was used to enhance testosterone synthesis and secretion in male mice. After 5 days of i.p. injection with GNRH, serum testosterone levels increased more than 30-fold compared with that in control mice that were only treated with saline (Fig. 3A, $P < 0.01$). In addition to increased testosterone levels, there was an approximate doubling of *Bpi* mRNA expression in the epididymis (Fig. 3B, $P < 0.05$). Nevertheless, *Bpi* expression in the testis did not increase upon GNRH treatment (Fig. 3C). These results indicated that *Bpi* expression in the epididymis was more sensitive to the testosterone level.

In order to assess the effect of decreased testosterone on BPI expression, surgical castration was carried out to decrease testosterone synthesis. After castration, serum testosterone concentrations began to decrease on the first day and were maintained at stable low levels over the following days (Fig. 4A, $P < 0.01$). Interestingly, similar to changes in serum testosterone levels after castration, *Bpi* mRNA expression levels in the epididymis also decreased to about 20% of those in the control group, the day after surgical castration. After 3 days, *Bpi* expression decreased to levels of 10% or less than that in the control mice (Fig. 4B, $P < 0.01$, Supplementary Figure 3). Accordingly, serum testosterone

Figure 3 Serum testosterone concentrations and *Bpi* mRNA levels after stimulation with GNRH. (A) After the mouse was stimulated with GNRH for 5 days, ELISA was performed to determine the serum testosterone concentration. The control group was treated with physiological saline instead of GNRH. Data are presented as mean $\pm$ s.d. and showed statistical significance (** $P < 0.01$) when comparing the GNRH and control groups. (B and C) After the mouse was stimulated with GNRH for 5 days, qPCR was carried out to analyze *Bpi* mRNA expression in the epididymis and testis. The control group received physiological saline instead of GNRH. Data are presented as mean $\pm$ s.d. and showed statistical significance (* $P < 0.05$) when comparing the GNRH and control groups.

Figure 4 Serum testosterone concentrations and *Bpi* mRNA levels after surgical castration. (A) At 1 to 8 days after surgical castration, an ELISA was performed to determine serum testosterone concentrations. Sham operation was used as day 0, and the group that did not undergo an operation was the blank. The ELISA was performed three times. Data are presented as mean $\pm$ s.d., and showed statistical significance (** $P<0.01$) when comparing the castration group to the control and blank groups. (B) At 1 to 8 days after surgical castration, qPCR analysis was performed to analyze *Bpi* mRNA expression in the epididymis. Sham operation was used as day 0, and the group that did not undergo an operation was the blank. Experiments were performed three times. Data are presented as mean $\pm$ s.d. and showed statistical significance (** $P<0.01$) when comparing the castration group to the control and blank groups. (C) Correlation between serum testosterone concentration and *Bpi* mRNA expression in the epididymis.

concentrations in male mice positively correlated with *Bpi* mRNA expression levels in the epididymis (Fig. 4C, $r=0.91$, $P<0.001$). The mice were given testosterone in order to slow down the decrease of testosterone. Daily i.p. injection of testosterone at a concentration of 2×10^{-7} mol/l led to retention of serum testosterone concentrations at over half of physiological concentrations compared with only 25% in the control group (Fig. 5A, $P<0.05$). Although *Bpi* expression decreased to about 30% of that in the blank group (Fig. 5B, $P<0.01$), the expression of *Bpi* was almost 150% compared with the control group (Fig. 5B, $P<0.05$). These results indicate that *Bpi* expression in the epididymis is influenced by testosterone levels.

BPI protein expression in response to the different treatments was determined by western blot analysis. As shown in Fig. 6, protein expression upon GNRH treatment and surgical castration was in accordance with *Bpi* mRNA levels. In other words, *Bpi* expression in the testis seemed unrelated to testosterone levels; however, there was a correlation in the epididymis.

BPI plays a role in the sperm–oocyte fusion process

As shown in Figs 7, 8 and Supplementary Figure 4 the number of sperm fused to oocytes significantly decreased after incubation with either anti-BPI-N or anti-BPI-C antiserum compared with those incubated with pre-immunized rabbit serum as a control ($P<0.01$). The results indicated that both the N- and C-terminal domains of the BPI protein might play a significant role in the sperm–oocyte fusion process.

Discussion

The male reproductive system is an open duct from the body to the external environment. *Bpi* is expressed in the male reproductive system (Lennartsson *et al.* 2005) and has been reported to have anti-Gram-negative bacteria activity. Although our results confirmed that *Bpi* is expressed in the male reproductive tract, it was unexpected to find that *Bpi* gene expression was localized in the testis and epididymis, and not in the seminal vesicles, prostate, and solidification glands, which are the organs closer to the external environment. This suggests that in the male reproductive system, BPI may not function as just an anti-microbial protein, as previously described in other organs.

In the testis, a BPI-positive signal was found in Leydig cells, spermatocytes, spermatids, and sperm in the seminiferous tubule. BPI protein expression in the epididymal epithelial cells showed significant regional differences, which indicates that it may have different functions. The expression of BPI in the epithelia of the efferent duct and caput epididymis suggests that it may play a critical role in the early stage of epididymal

Figure 5 Serum testosterone concentrations and the *Bpi* mRNA levels after castrated mice were stimulated with testosterone. (A) After castration, mice were stimulated with 10^{-7} mol/l testosterone for 1 day followed by ELISA to determine the serum testosterone concentration. The control group received saline instead of testosterone, while the blank group did not undergo an operation. Data are presented as mean $\pm$ s.d. and showed statistical significance (*with shorter bar, $P < 0.05$) when comparing the testosterone treatment group with the control group. Data also showed statistical significance (*with longer bar, $P < 0.05$) when comparing the testosterone treatment group and the control group with the blank group. (B) qPCR analysis was used to analyze *Bpi* mRNA expression in the epididymis in aforementioned three groups. Data are presented as mean $\pm$ s.d. and showed statistical significance (*with shorter bar, $P < 0.05$) when comparing the testosterone treatment and control groups. Data also showed statistical significance (**with longer bar, $P < 0.01$) when comparing the testosterone treatment and control groups with the blank group.

maturation. As our results show, in the seminiferous tubules, BPI was expressed in spermatocyte cells, spermatid cells, and spermatozoa that passed through the epididymis. BPI secreted by the efferent duct and caput epididymis may be continuously absorbed onto

spermatozoa. In other words, BPI is expressed throughout the processes of spermatogenesis in the testis, and sperm maturation in the epididymis. It is noteworthy that BPI-positive signals were strong in Leydig cells. BPI belongs to a family of mammalian LPS-binding and lipid transport proteins. Two members of this family have been identified that are related to lipid transport. One of the important functions of Leydig cells is producing androgens that are synthesized using lipid as raw material. We need to do more research to confirm whether BPI has a function of lipid transport in Leydig cells. In addition, it has been reported that BPI is localized in the matrix of the acrosome and attaches to the spermatozoa plasma membrane surface in the acrosomal region (Yano *et al.* 2010), suggesting that BPI may play a role in fertilization in addition to its anti-microbial function.

Testosterone is a steroid hormone that is primarily produced by testicular Leydig cells (Haider 2004). In addition to its traditional role in spermatogenesis, and development and maintenance of primary and secondary sex characteristics, testosterone has several other biological functions. Preliminary experiments have indicated that BPI expression peaks at sexual maturity and is lower in older or younger men, which made us speculate that there may be some relationship between BPI expression and androgen. Here, we found that *Bpi* expression in the epididymis was altered according to either increases or decreases in serum testosterone concentrations. In particular, after castration, the expression of *Bpi* in the epididymis was extremely low, and the expression of *Bpi* was restored to 30% of control

Figure 6 BPI expressed as determined by western blot analysis. The signal at 50 kDa represents the BPI protein. The signal at 43 kDa represents β -actin. (A) Protein expression of BPI and β -actin in the epididymis after stimulation with GnRH. (B) Protein expression of BPI and β -actin in the testis after stimulation with GnRH. (C) Protein expression of BPI and β -actin in the epididymis after surgical castration at each time point.

Figure 7 Images of sperm–oocyte fusion. The head of the sperm had blue fluorescence. And the blue fluorescence picture of each operation was placed in the left array. (a, b, c and d) These images represent incubation with anti-BPI-C antiserum, anti-BPI-N antiserum, negative control, and blank control respectively. (e, f, g and h) These images represent the white photos from the images shown in (a, b, c and d).

levels by administration of testosterone. These results corroborate our speculation.

It is reported that the synthesis and secretion of epididymal proteins are regulated by temperature (Belleannee *et al.* 2012), as well as by endocrine (Primiani *et al.* 2007, Sipila *et al.* 2011), paracrine (Turner *et al.* 2007a, 2007b, Belleannee *et al.* 2012) and lumicrine factors (Turner & Bomgardner 2002, Sipila *et al.* 2011,

Turner 2011), which are from the testis or from upstream regions of efferent ducts. Epididymal BPI may be regulated by these factors in addition to testosterone, which may explain the fact that *Bpi* expression was restored to 30% of its normal levels in orchidectomized mice treated with testosterone. However, BPI expression in the testis was less sensitive to increases in testosterone compared with BPI expression in the epididymis upon GNRH treatment. This may be due to the insensitivity of germ cells to androgens. Together, these results suggest that a certain amount of testosterone is essential for BPI expression, and high levels of testosterone may increase BPI expression in the epididymis. In fact, it has been reported that a number of proteins in the testis and epididymis involved in sperm maturation and fertilization, such as glutathione peroxidase (Klapcinska *et al.* 2008) and γ -glutamyl transferase (Haring *et al.* 2012), are regulated by testosterone and play a role in reproductive activities. Therefore, the regulation of BPI expression by testosterone suggests that BPI may play a crucial role in fertilization.

The mechanism underlying the process of sperm–oocyte fusion remains unclear. It is generally thought that the mammalian acrosome reaction is induced by the zona pellucida. There are a number of proteins, such as acrosin, sperm adhesion molecule 1 (Hardy *et al.* 2004, sperm–oocyte binding antigen 2 (Lefevre *et al.* 1997), SKP2 (FLB1; Boue *et al.* 1995), fertilization antigen (Naz 2002), and cystatin-related epididymal spermatogenic protein (Wang *et al.* 2012)), that take part in the acrosome reaction and sperm–oocyte fusion process. For instance, it has been demonstrated that cystatin-related epididymal spermatogenic protein has both anti-microbial functions and a role in the sperm–oocyte fusion process. BPI is located in the matrix of the acrosome and attaches to the spermatozoa plasma

Figure 8 Statistical analysis of sperm–oocyte fusion between each operation. Data are presented as mean $\pm$ s.d. Data showed statistical significance (** $P < 0.01$) when comparing anti-BPI-C antiserum and anti-BPI-N antiserum operation groups with control and blank groups.

membrane surface of the acrosomal region (Yano *et al.* 2010). Data from our sperm–oocyte fusion assay suggest that both the N- and C-terminal domains of BPI may be directly involved in the sperm–oocyte fusion process. When sperm fuse with an oocyte, the microenvironment around the gamete is not sterile. BPI may play an indirect role in sperm–oocyte fusion by the interaction between the protein and microorganism. There are plenty of microorganisms in our body, especially in the intestinal tract, that are in mutualism with us. They are parts of us. Interestingly, it was recently reported that BPI may have a role in the mutualism between the Hawaiian bobtail squid (*Euprymna scolopes*) and the bioluminescent bacterium (*Vibrio fischeri*) (Krasity *et al.* 2011). Thus, we speculate that BPI may also be involved in sperm–oocyte fusion by its function in mutualism. For example, BPI on sperm may not directly interact with the oocyte but may serve as an intermediary between the oocyte and microorganisms, thereby playing the role of a bridge molecule. In other words, BPI may interact with some bacterium, and the anti-microbial role of BPI may be involved in the normal process of sperm–oocyte fusion.

Conclusions

Bpi expression was only found in the testis and caput epididymis and not in other reproductive accessory glands. Testosterone levels regulated BPI expression. In addition, anti-BPI antiserum incubated with sperm suppressed sperm–oocyte fusion. These results suggest that BPI may not only function as an anti-microbial protein but may also play a role in mammalian fertilization.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0127>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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