G protein-coupled estrogen receptor (GPER) in adult boar testes, epididymis and spermatozoa during epididymal maturation

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ABSTRACT

The G protein-coupled estrogen receptor (GPER) is a transmembrane receptor considered as a mediator of rapid non-genomic responses. GPER has been found in the male reproductive tract of many mammalian species. However, in adult boars, GPER has been reported only in ejaculated spermatozoa. Therefore, we focused on GPER detection in testicular and epididymal tissues and sperm cells in adult boars. We found GPER in Leydig cells and seminiferous tubules of boar testes and in the secretory epithelium of epididymis. A weaker signal was visible in smooth muscle cells and spermatozoa in the epididymal tubule. In spermatozoa isolated from epididymal parts, GPER was found to localize mainly in the sperm acrosome and flagellum. We immunodetected several protein bands in the extracts of the tissues and epididymal spermatozoa. A significantly higher amount of GPER mRNA was detected in the spermatozoa from caput epididymis, whereas the mRNA expression was lower in tissues of testes and caput epididymal. Our results showed the first evidence of GPER in boar epididymal spermatozoa. Moreover, the GPER localization in adult boar testes, epididymis, and mature spermatozoa suggests the involvement of estrogens via transmembrane receptor and rapid non-genomic signaling in both the sperm development and post-testicular maturation.

1. Introduction

Estrogens play an important role in spermatogenesis, sperm maturation, capacitation, acrosome reaction, and fertilization [1]. The effect of estrogens is determined by the presence of estrogen receptors (ERs) in the cells of targeted tissues to which they are able to bind, thus initiating the cellular responses [2–4]. Besides classical nuclear/cytoplasmic ERs, ESR1 (ERα) and ESR2 (ERβ) [2–4], the G protein-coupled estrogen receptor GPER (GPR30) has been described [5–7]. This transmembrane receptor is considered to be a mediator of rapid non-genomic estrogen responses in various types of cells [8,9]. It is structurally unrelated to classical estrogen receptors ESR1 and ESR2 [10]. Some investigators have claimed that it is strictly a cytosolic or endoplasmic reticulum-associated receptor, while others have maintained that it is a plasma membrane-associated one [8,11]. The biological function of GPER could be related to the cell type and localization [5,7]. It has been confirmed that the GPER signaling cascade also includes the release of intracellular calcium ions [12], which is closely related to the sperm

capacitation and acrosomal reaction [13] and indicates the GPER presence in spermatozoa. The proof of this is the detection of GPER in spermatozoa of humans and pigs [14]. GPER has been studied in the testes and epididymides of several mammals, such as the mouse [15,16], rat [17,18], sheep [19] and human [20,21]. In the pig, the GPER expression has been reported only in the epididymal tissue of postnatal boars [22]. In adult boar reproductive tissues, GPER detection has not been described yet. The available literature suggests that the detection and localization of GPER in the male reproductive tissue and spermatozoa has not yet been verified and fully documented. Additionally, the distribution of ERs has not been monitored across a single stage of sperm development by a uniform methodology, and particularly in the case of GPER, the findings can be described as very incomplete. In our present study, we focused on the detection of membrane estrogen receptor GPER in the testicular and epididymal tissues in adult boars as well as their localization in sperm cells during their development and maturation.

2. Materials and methods

The study was carried out according to Directive 2010/63/EU and guidelines of the Czech legislation (Directive 208/2004 Sb.).

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E-mail address: pavlam@ibt.cas.cz (P. Maňásková-Postlerová).

2.1. Collection of spermatozoa and tissues from boar reproductive organs

Epididymides and testes of adult boars (4 animals of Large white boars, slaughter weight of 110–115 kg, 9–12 months of age; 2 miniature pigs, slaughter weight of 70–100 kg, 7–12 months of age) were collected immediately post-mortem from the breeding station at the Institute of Animal Physiology and Genetics (Liběchov, Czech Republic) and at slaughterhouse of the Institute of Animal Science (Praha-Uhříněves, Czech Republic) and transported on ice to the laboratory. Spermatozoa from the main parts of the epididymis (caput, corpus and cauda) were obtained by swimming up from the epididymal organ into phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO; 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4) after incubation for 30 min at 37 °C. Tissue pieces were removed by centrifugation for 5 min at 50g. Then spermatozoa were separated from the buffer by centrifugation (20 min at 300g) and used for protein extraction, mRNA isolation and immunofluorescence.

Tissues of the boar reproductive tract (testes, caput, corpus, and cauda epididymis) were cut to small pieces and kept at −70 °C for the isolation of mRNA and preparation of protein extracts. Pieces of the testes and main parts of epididymis were frozen in blocks with tissue-freezing medium (Jung, Nussloch, Germany) in liquid nitrogen and stored at −70 °C. Cryosections of 5-μm thickness were prepared.

2.2. Sperm and tissue extract preparation

Proteins from spermatozoa isolated from different parts of the epididymis (caput, corpus, and cauda) were extracted using sodium dodecyl sulphate (SDS) sample buffer (2% SDS in 0.5 M Tris-HCl, pH 6.8) for 30 min on ice and vortexed every 5 min. For sperm samples, 5×10^7 sperm cells counted in Makler counting chamber were dissolved in 150 μl of SDS sample buffer. Testicular and epididymal tissues from caput, corpus and cauda (100 mg) were homogenized in 500 μl of 1% Triton X-100 in 50 mM Tris-HCl (pH 7.8) with 50 mM NaCl using homogenizer IKA T10 basic (IKA Werke, Staufen, Germany). Detergent was removed from the tissue extracts using 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol. Extracts were dissolved in reducing sample buffer (2% SDS in 0.5 M Tris-HCl, pH 6.8) for sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

2.3. SDS-electrophoresis with immunoblotting

SDS-electrophoresis and the Western blot technique were used for GPER detection and were carried out using protocols based on standard methods [23,24]. Samples containing protein equivalent to 5×10^6 sperm cells and 25 μg per lane were run in 4% stacking and 12% running SDS polyacrylamide gel using Precision Plus Protein™ Dual Color Standards (Bio-Rad, Hercules, CA) as molecular weight markers. After transferring proteins onto a nitrocellulose membrane, nonspecific sites were blocked with 5% Blotting-Grade Blocker (Thermo Fisher Scientific, Waltham, MA) in PBS at 4 °C overnight. GPER was detected by primary polyclonal rabbit antibody against human GPR30 (K-19-R, Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:500 in PBS, followed by peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad). The antibody reaction was visualized by chemiluminescent substrate SuperSignal™ West Pico PLUS (Thermo Scientific) and the blot was screened with Azure c600 Gel Imaging System (Sierra Ct, Dublin, CA) to visualize the corresponding interaction bands. As a negative control, blots were incubated with immunoglobulins from rabbit serum (Sigma-Aldrich) in the same concentration as the primary antibody.

2.4. Indirect immunofluorescence technique – localization of GPER in the tissues and spermatozoa

Indirect immunofluorescence was used to detect the presence of GPER in the main parts of boar epididymis (caput, corpus and cauda) and testes, and in spermatozoa isolated from the boar adult epididymis by specific polyclonal antibody. Cryosections (5-μm thick) and spermatozoa of the epididymis were fixed in frozen (−20 °C) acetone and methanol (1:1) for 10 and 5 min, respectively. After blocking with SuperBlock (PBS) Blocking Buffer (Thermo Fisher Scientific, Waltham, MA), samples were incubated with rabbit polyclonal antibody against human GPR30 (K-19-R,Santa Cruz) diluted 1:100 in PBS overnight at 4 °C followed by incubation with goat anti-rabbit immunoglobulins conjugated with FITC (Sigma-Aldrich, St. Louis, MO). For acrosome integrity evaluation, spermatozoa were incubated with PNA-Rhodamine (Vector Laboratories, Burlingame, CA). Samples covered by VectaShield-DAPI (Vector Laboratories) were viewed and evaluated with a Nikon Eclipse E600 fluorescent microscope with Nikon Plan Fluor lenses and a Nikon Digital Sight DS-Fi 1 camera (Nikon, Japan) with the aid of NIS Elements imaging software (Laboratory imaging, a.s., Prague, Czech Republic). As controls, tissue sections and spermatozoa were incubated with the secondary antibody only.

2.5. RNA extraction and reverse transcription-PCR analysis

2.5.1. Optimization of PCR

Total RNA was isolated using Hybrid R™ (GeneAll Biotechnology, Co., Ltd., Seoul, Korea), from exactly the amount of spermatozoa and tissues (tissues and spermatozoa from caput, corpus and cauda epididymis, and testicular tissue), according to the manufacturer's instructions. Total RNA was quantified fluorometrically (Qubit 3.0, Invitrogen, Thermofisher). First-strand cDNAs were synthesized from individual RNA using HyperScriptTM (GeneAll Biotechnology). The cDNA amplification was performed by using specific forward and reverse primers (GPER1 [Sus scrofa] forward: 5′CTGAGCTTCAAGGCCGATGT3′ and reverse: 5′CCCTTAAGTAGGCGCAGAGC3′; GenBank accession: NC_010445.4) derived from the porcine GPER sequence. Amplification was performed with initial heating at 95 °C for 5 min followed by 35 cycles of 94 °C for 1 min, annealing temperature at 70 °C for 45 s and 72 °C for 1 min, and terminal amplification at 72 °C for 5 min in the PCR Thermal Cycler (Bio-Rad). PCR products were electrophoresed in 1.5% agarose gel and visualized by staining with ethidium bromide.

2.5.2. Quantitative PCR (qRT-PCR)

Total RNA isolation and cDNA synthesis was described in the previous section. The same primer pair used in PCR was also used in qRT-PCR. Five-fold serial dilution of the PCR product was prepared and used as a template for generation of the standard curve. To ensure repeatability of the experiments, each sample was run in eight replicates. The qRT-PCR was set up using 2 μl first-strand cDNA template, 7.75 μl deionized H$_2$O, 0.25 M forward and reverse primer, and 10 μl 1× SYBR Green Supermix (Bio-Rad). The thermal cycling conditions were 2 min at 50 °C, 10 min at 95 °C followed by 45 cycles of 30 s at 95 °C, 30 s at 70 °C and 45 s at 72 °C, and at the end, melting curve from 65 °C to 95 °C by 0.5 °C increments for 5 s. The experiments were performed using the CFX ConnectTM, Real-Time System (Bio-Rad). An amplification-based threshold and adaptive baseline were selected as algorithms. The GAPDH housekeeping gene (forward: 5′ACCCAGAAGACTGTGGATGG 3′ and reverse: 5′ACGCCTGCTTCACCACCTTC 3′) derived from the porcine sequence (GenBank accession No. AF017079; [25]) was used for data normalization. Final results were reported as the relative abundance level after normalizing with the mRNA expression level of the housekeeping gene.

2.6. Data analysis

Our data are from at least four independent experiments. Data obtained from RT-qPCR were analyzed by STATISTICA 12 software (StatSoft, Czech Republic) with one-way ANOVA with post-hoc Tukey test. Quantification of Western blot immunodetection was subjected to densitometric analysis in the program Image Studio Digits Ver 4.0 (LI-COR, Lincoln, NE). The antibody signal was related to the total protein concentration in the samples.

3. Results

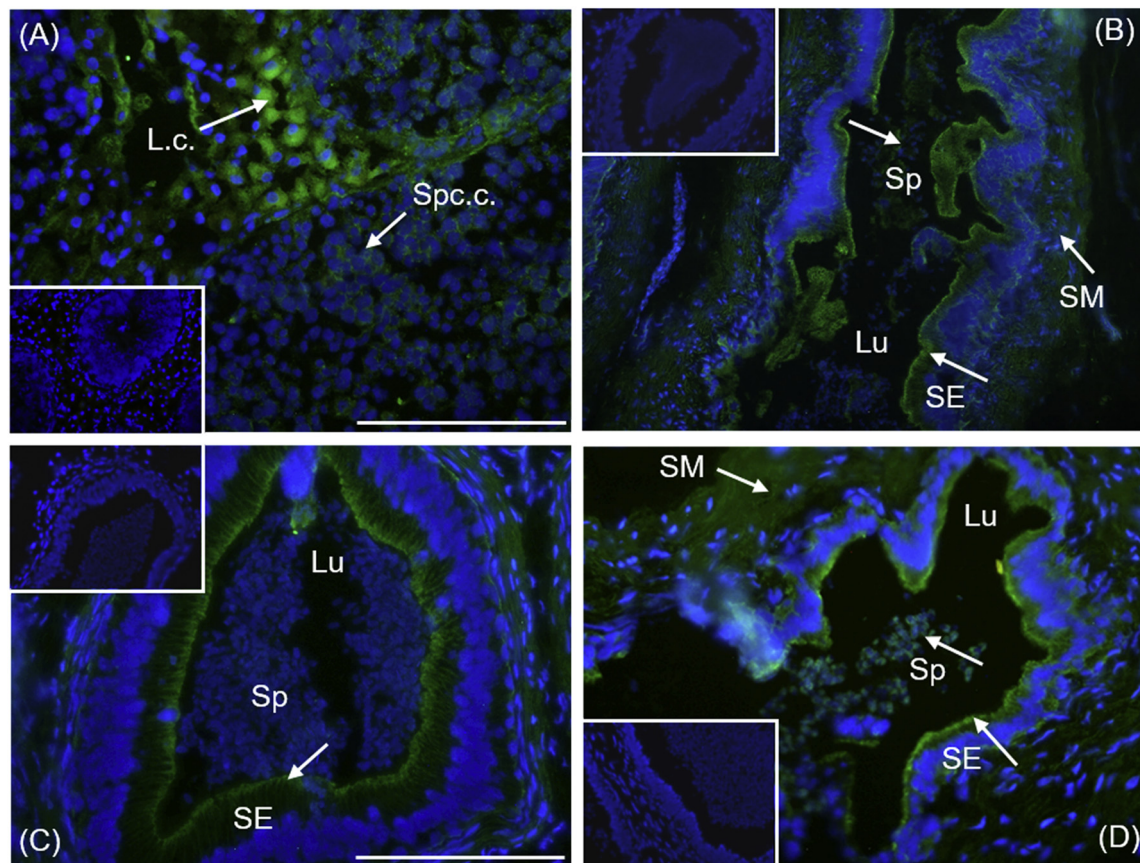### 3.1. Localization of GPER in the boar testes and epididymis

GPER localization was determined in cryosections of adult boar testes and epididymal parts with a specific polyclonal antibody. In the testicular tissue (Fig. 1), the GPER antibody stained the Leydig cells (L.c.) and spermatic cells (Scp.c.). GPER was localized in all parts of the epididymal tissue sections (caput, corpus and cauda). In caput epididymis, the antibody showed GPER in the membrane of secretory epithelium (SE) and in spermatozoa (Sp) within the tubule (Fig. 1B). A weaker signal was also observed in smooth muscle (SM). In the corpus part of boar epididymis, strong antibody staining was found in the membrane of secretory epithelium (SE) and a weak signal was visible in the smooth muscle (SM) tissue. Spermatozoa (Sp) inside the tubule were not clearly stained (Fig. 1C). The GPER signal was also observed in crysections of cauda epididymis, namely in the secretory epithelial membrane (SE), smooth muscle (SM), and spermatozoa (Sp) within the epididymal tubule, likely in the acrosomal region (Fig. 1D).

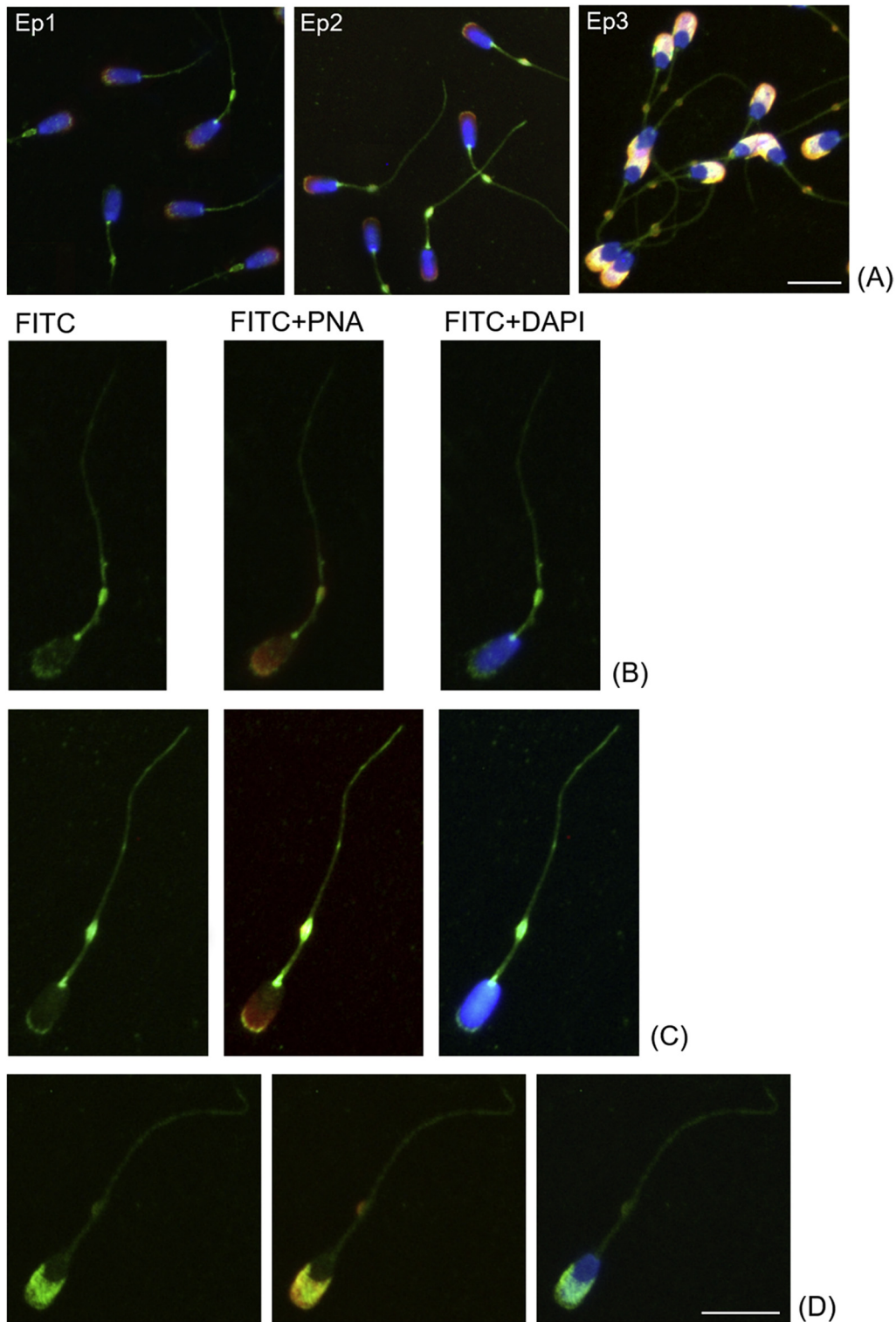### 3.2. Localization of GPER in boar epididymal spermatozoa

We found a weak antibody staining corresponding to the GPER protein in the flagellum of epididymal spermatozoa isolated from all investigated parts (caput, corpus and cauda) of the boar epididymis (Fig. 2A–D). Antibody staining with higher intensity was also observed in the neck and cytoplasmic droplets in the midpiece of some epididymal spermatozoa. In spermatozoa isolated from caput epididymis, the antibody also showed a weak diffuse staining in the sperm acrosome (Fig. 2A-Ep1 and 2B). Immunofluorescent labeling in spermatozoa from the corpus epididymal part was concentrated in the apical area of the sperm head (Fig. 2A-Ep2 and 2B). In contrast, spermatozoa isolated from cauda epididymis were clearly stained with the GPER antibody in the whole acrosome (Fig. 2A-Ep3 and 2D). In figures of epididymal spermatozoa, the acrosome integrity was proved by PNA-lectin staining of the outer acrosomal membrane (Fig. 2A–D, red).

3.3. Detection of GPER in protein extracts from boar epididymal sperm and reproductive tissues

We detected the GPER protein in the extracts from boar testicular and epididymal tissues with specific polyclonal antibody (Fig. 3A; black arrows). The antibody clearly recognized the protein band of 42 kDa in the caput and corpus epididymal extracts. In the cauda epididymal tissue extract, a band of 38 kDa was observed. In the testicular extract, the antibody detected two protein bands with molecular masses of 38 and 48 kDa. Additionally, a strong band was visible in molecular mass of approximately 60 kDa in the testicular tissue homogenate.


Fig. 1. Localization of GPER in adult boar testes (A), and parts of epididymis: caput (B), corpus (C) and cauda (D) with specific anti-GPER (K-19-R) polyclonal antibody using indirect immunofluorescent microscopy. L.c. – Leydig cells, Spc.c. – spermatic cells, SE – secretory epithelium, Lu – lumen; Sp – spermatozoa, SM – smooth muscle tissue; green color (FITC) – immunofluorescence presenting the reaction of antibody with antigen; blue color (DAPI) – staining of the cell nucleus; arrows show antibody staining; magnification 200×, scale bar = 100 μm. Negative controls are inserted in corners. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Localization of GPER in boar spermatozoa isolated from epididymal tubule: caput (Ep1), corpus (Ep2) and cauda (Ep3). (A) Panel shows overlaid images with antibody staining of GPER, PNA-lectin staining of sperm acrosome and DAPI staining of sperm nucleus; (B) caput epididymal spermatozoon, (C) corpus epididymal spermatozoon, (D) cauda epididymal spermatozoon. Green color (FITC) – immunofluorescence presenting the reaction of polyclonal antibody (K-19-R) with antigen; blue color (DAPI) – staining of the cell nucleus; red color (Rhodamin) – PNA-lectin staining of sperm acrosome; magnification 400× and 600×, scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The same band with less intensity was also registered in all epididymal extracts. In addition, we detected a weak band with molecular mass of 48 kDa in the testicular tissue. A diffuse band of approximately 54 kDa appeared in all tissue extracts, suggesting non-specific reaction with the polyclonal rabbit antiserum (Fig. 3A,B; grey arrow).

Specific polyclonal antibody to GPER recognized a protein band with molecular mass of 42 kDa in the extracts of spermatozoa isolated from the caput and corpus epididymal parts. In the cauda epididymal sperm extract, two bands with molecular masses of 38 and 48 kDa and similar density were determined, and one clear protein band approximately of
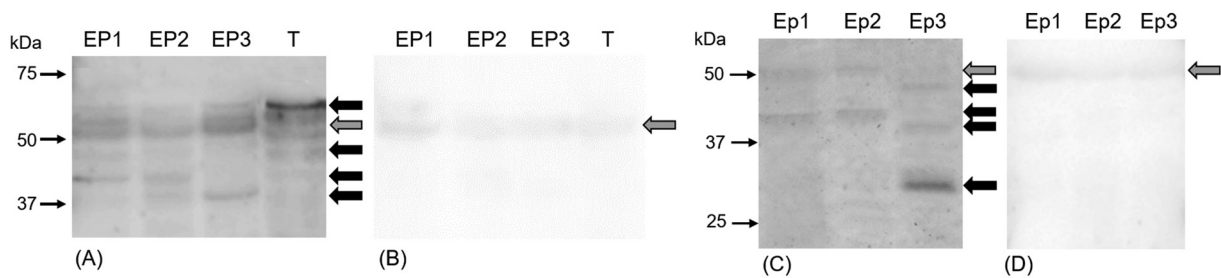
Fig. 3. Western blot analysis of the GPER detection in protein extracts from boar reproductive tissues: EP1 – caput epididymis, EP2 – corpus epididymis, EP3 – cauda epididymis, T – testes; (A) reaction with polyclonal rabbit antibody (K-19-R), (B) control with rabbit immunoglobulins. Western blot analysis of the GPER detection in protein extracts from spermatozoa isolated from boar epididymis: Ep1 – caput epididymis, Ep2 – corpus epididymis, Ep3 – cauda epididymis, T – testes; (C) reaction with polyclonal rabbit antibody (K-19-R), (D) control with rabbit immunoglobulins.

30 kDa was detected (Fig. 3C; black arrows). A diffuse band of approximately 50 kDa was discovered in all tissue extracts, suggesting non-specific reaction with the polyclonal rabbit antiserum (Fig. 3C,D; grey arrow).

3.4. mRNA expression in boar epididymal spermatozoa and testicular and epididymal tissues

Using mRNA isolated from the tissues of boar testes and three epididymal parts (caput, corpus and cauda) and spermatozoa from the same epididymal regions, we carried out qRT-PCR with primers for the porcine *GPER1* gene and the housekeeping *GAPDH* gene as a control. Higher expression of mRNA ($P < 0.05$) was found in the caput (EP1) and cauda (EP3) epididymal tissues in comparison with testicular (T) and corpus (EP3) epididymal samples (Fig. 4A). Nevertheless, a higher amount ($P < 0.01$) of mRNA was found in the spermatozoa isolated from caput epididymis (Ep1) in comparison with the boar spermatozoa from subsequent epididymal parts, corpus (Ep2) and cauda (Ep3) (Fig. 4B).

4. Discussion

Both endogenous estrogens and estrogens from the environment affect the organism via estrogen receptors (ERs). ERs were found not only in the cells of the female but also of the male reproductive tract. The effect of estrogens on the male reproductive tissues and development of gametes is essential for the reproductive success. Localization of ERs in the male reproductive tract and spermatozoa is therefore a prerequisite for understanding the estrogen effects on fertilization.

G protein-coupled estrogen receptor (GPER) is a membrane receptor considered as a mediator of the non-genomic responses of estrogens. In our study, we focused on the detection and localization of GPER in the testes and epididymis and in spermatozoa during their epididymal maturation. In a previous study, it has already been postulated that the expression of GPER in the corpus and cauda epididymis suggests a role for non-classical estrogen signaling in the sperm maturation in the corpus, and sperm protection/storage in the cauda [18].

Literature data show the presence of both GPER membrane estrogen receptor and mRNA expression in the germ cells and testicular and epididymal tissues of several mammals. GPER has mostly been described in germ cells and reproductive organs of rats, where it was studied in spermatocytes and round spermatids [26,27], in Sertoli cells associated with normal testes development and function [17,28], and in Leydig cells [29]. In the rat epididymis, the highest GPER expression was found in the corpus and cauda, and lower expression was observed in the apical membrane and cytoplasm of epithelial cells in the caput [18]. Contrary to rats, the findings on GPER expression in humans have been inconsistent. Rago et al. [30] did not find GPER in germ cells but only in Leydig and Sertoli cells. In contrast, Oliveira et al. [31] reported expression of this estrogen receptor not only in Sertoli cells, but in the human germ cells, whereas Sandner et al. [32] identified GPER in interstitial and peritubular cells only. However, there is very little information on GPER detection in the reproductive tissues of adult boars. Katleba et al. [22] are the only authors to have detected GPER in prepubertal pigs (age 6.5 weeks) in the corpus epididymis. They immunolocalized GPER in epithelial cells, smooth muscle and stromal cells labeled with varying intensity [22]. We investigated GPER localization in all parts of the boar adult epididymis. We proved the presence of this receptor in the membrane of secretory cells, and a weak signal was visible in smooth muscle cells and spermatozoa within the tubule. In boar adult testes, our results showed the presence of GPER in Leydig cells and in cells within the seminiferous tubule; however, it is not evident which developmental stage of sperm cells was stained or whether GPER localized into Sertoli cells.

In addition to immunofluorescence studies, GPER has also been detected at the protein level in the extracts from germ cells or testicular and epididymal tissues of different mammals (rats, humans and sheep), where the GPER protein was reported with molecular mass of 42 kDa [19,26,27,30,32,33]. In another study of rats and humans, Western blot analysis showed a strong protein band corresponding to GPER between 50 and 60 kDa, and weak antibody reaction was visible in a
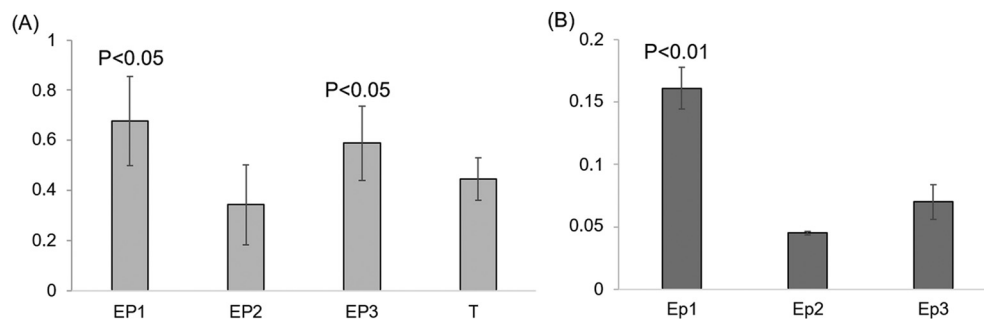
Fig. 4. Relative expression of GPER mRNA in tissues (A) of adult boar epididymis (EP1 – caput, EP2 – corpus, and EP3 – cauda) and testes (T), and in epididymal spermatozoa (B) isolated from epididymal parts (Ep1 – caput, Ep2 – corpus, and Ep3 – cauda).

protein band of 40 kDa in the extract from the testes [29]. Lu et al. [19] detected GPER in spermatocytes, spermatogonia, Sertoli and Leydig cells, and testicular spermatozoa of 6–12 month old rams, and in the main and interstitial cells of the corpus and cauda epididymis, where the amount of GPER protein and mRNA has been found the highest.

In Western blot analysis, the highest abundance of GPER was found in the testes represented by a strong antibody reaction with a protein band of ~60 kDa. The antibody showed a lower amount of GPER in the epididymal parts. We found a clear protein band of ~42 kDa in the extract from caput and corpus epididymis and the same band with weak intensity in the testes. This band corresponded to the GPER band of ~41 kDa in the corpus epididymis of prepubertal boars described by Katleba et al. [22]. Additionally, we detected other GPER-positive bands of 38 and 48 kDa in protein extracts of both tissue homogenates. Similarly, the protein band with molecular mass of 38 kDa has been found in germ cells and Sertoli cells in the cellular fractions of human testes [31]. In tissue extracts of the rat epididymis, two proteins have been shown to demonstrate the presence of GPER, namely 43 kDa and a weak band of 55 kDa [18]. Our protein bands detected with different molecular masses corresponding to GPER may suggest various glycosylated and non-glycosylated isoforms occurring in the spermatozoa and male reproductive tissues, similarly as described in MCF-7 breast cancer cells and myometrium [34].

Hess et al. [20] reported a positive finding of GPER mRNA in the epididymis of adult rats with high expression in human corpus epididymis. We found no significant differences in the amount of mRNA in all boar epididymal parts and testicular tissues. Nevertheless, we registered higher expression in the caput and cauda epididymis than in the testes and corpus epididymis. Fietz et al. [21] demonstrated significant expression of GPER mRNA in Leydig cells of the human testes.

In mature sperm cells, GPER has only been reported in ejaculated spermatozoa in the pig and human [14] and in stallion [35,36]. In stallion, this receptor was detected in the connecting piece (neck) of the sperm [35], and using electron microscopy, GPER was found at subcellular localization in the midpiece, neck, flagellum, and head [36]. In boar ejaculated sperm, the GPER receptor was localized in the acrosomal part and equatorial segment of the head and in the mitochondrial part of the flagellum [14]. In our study, we demonstrated the presence of GPER in spermatozoa isolated from distinct parts of the epididymal tubule with different patterns and intensity. Labeling of GPER in epididymal spermatozoa from the caput and corpus part was weaker and diffuse in the sperm acrosome and stronger in the flagellum with marked cytoplasmic droplets. In epididymal sperm from the cauda, the GPER localization was obvious predominantly in the acrosome. Previous GPER localization in ejaculated sperm [14] is similar to our findings on spermatozoa isolated from the cauda epididymis. This may suggest that during the epididymal maturation, GPER is present in the flagella without significant change up to their mature stage, whereas in the sperm acrosome its change is substantial. The intensity of antibody staining may suggest the exposure of epitopes for specific GPER antibody in the acrosomal part of spermatozoa during their passage throughout the epididymis. It is well-known that during sperm epididymal maturation, spermatozoa undergo a number of changes that lead to their fertilizing ability. These changes include the binding of proteins secreted by the epididymis to the sperm surface or post-translational processing of sperm proteins [37]. Proteins expressed mainly in the transition between the caput and corpus epididymis [38] are likely to bind to the sperm plasma membrane, and may thus cover the GPER epitopes recognized by the used antibody. This results in weak and diffuse GPER antibody staining of the acrosome in spermatozoa from the caput and corpus epididymis. Subsequently, in the cauda epididymis, some of these proteins leave the sperm surface [39], and thus GPER epitopes for the binding of antibodies can be revealed.

The GPER protein with a molecular weight of 42 kDa was detected in the extract from ejaculated sperm of the pig and human by Rago et al. [14]. We used the same polyclonal anti-GPER antibody as these authors (Santa Cruz, K-19-R) and detected not only the protein band of 42 kDa in the caput and corpus epididymal sperm extracts, but also bands of 30, 38 and 48 kDa in the cauda epididymal spermatozoa. The protein band corresponding to GPER with a molecular mass of 38 kDa has also been reported in the extract from equine ejaculated spermatozoa [35].

The study by Rago et al. also described the presence of GPER mRNA in human and boar sperm. This is probably the first report on the positive finding of GPER in mature mammalian sperm [14]. We detected GPER mRNA in epididymal spermatozoa with differing relative expression. The highest amount of mRNA was found in spermatozoa from the caput epididymal part compared to spermatozoa from the two following parts. The lower amount of detected mRNA may indicate its active translation into the protein during epididymal maturation of spermatozoa or its possible degradation. The translation of mRNA into the protein in the sperm is questionable; however, it was described during the final maturation steps before fertilization by Gur and Brietbart [40].

The available literature suggests that the detection and localization of ERs in the male reproductive tissue and spermatozoa has not yet been verified and fully documented. The distribution of ERs has not been monitored across a single stage of sperm development by a uniform methodology, and particularly in the case of GPER, the findings can be described as very incomplete. Our results were the first to confirm the presence of GPER in epididymal spermatozoa not only of boars, but also of mammals. The protein was also found in adult boars in testicular and epididymal tissues at the protein and mRNA levels. Its localization in the epididymal epithelium and spermatozoa suggests potential involvement of estrogens not only in the sperm development, but also in post-testicular maturation in the epididymis.

Authors' contribution

R.K. was responsible for sample preparation, immunofluorescent methods, and manuscript preparation. M.M. carried out the RT-qPCR method. V.S. performed immunofluorescent microscopy and participated in the manuscript preparation. E.L. performed WB analysis. R.R. accomplished data analysis and participated in the manuscript preparation. P.P. designed the experiments, analyzed data and prepared the results for the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

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