Steroid regulation of early postnatal development in the corpus epididymidis of pigs

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Abstract

Development of the epididymis including blood–epididymal barrier formation is not required until sperm reach the epididymis peripuberally. Regulation of this development in the early postnatal period is largely unknown. The current objectives were to evaluate potential roles of endogenous estrogen and androgen signaling during early development of the corpus epididymidis and to determine the timing of formation of the blood–epididymal barrier in the pig. Effects of endogenous steroids were evaluated using littermates treated with vehicle, an aromatase inhibitor (letrozole) to reduce endogenous estrogens, an estrogen receptor antagonist (fulvestrant) or an androgen receptor antagonist (flutamide). Phosphorylated histone 3 immunohistochemistry was used to identify proliferating epithelial cells. Lanthanum nitrate and electron microscopy were used to analyze formation of the blood barrier in the corpus epididymidis. Reducing endogenous estrogens increased the number of proliferating corpus epithelial cells at 6 and 6.5 weeks of age compared with vehicle-treated boars (P < 0.01 and P < 0.001 respectively). Blocking androgen receptors did not alter proliferation rate at 6.5 weeks of age. Although barrier formation was similar between 6 and 6.5 weeks of age in vehicle-treated animals, intercellular barriers increased in letrozole-treated littermates at 6.5 weeks of age. Fulvestrant treatment, which should mimic aromatase inhibition for regulation through ESR1 and ESR2 signaling but potentially stimulate endogenous estrogen signaling through the G protein-coupled estrogen receptor (GPER), had the opposite effect on aromatase inhibition. These responses in conjunction with the presence of GPER in the corpus epididymidis suggest early corpus epididymal development is regulated partially by GPER.

Introduction

The epididymis forms prenatally and continues to grow and develop postnataally such that when spermatozoa reach the epididymis at puberty, they can develop motility and gain fertilizing ability during their transit through the organ. The blood–epididymal barrier isolates these maturing sperm from the immune system and permits the existence of specific microenvironments along the course of the epididymis for sperm maturation. Tight junctions between epithelial principal cells are a critical component of the blood–epididymal barrier (Cyt et al., 2002). Most of what is understood about the blood–epididymal barrier has been learned from studies in rodents. Formation of
anatomical tight junctions in the epididymis appears to be regulated during two time intervals, first during embryonic development when tight junction formation is initiated (Suzuki & Nagano 1978a) and subsequently when the number of junctional strands increase to form the mature anatomical barrier (Cyr et al. 2007). The fully formed anatomical tight junction is associated with a large number of cytoplasmic proteins including dual function nuclear transcription factors and junctional components. Development of tight junctions may reduce epithelial cell proliferation due to sequestering of such dual location proteins to the cytoskeletal complexes, although regulation appears to be complex (Balda & Matter 2009, Farkas et al. 2012, Spadaro et al. 2012, Hase et al. 2013).

The exact mechanisms by which early postnatal epididymal development is regulated are unknown, but the importance of testicular factors has been recognized for many years. Orchidectomy, which removes androgens, estrogens, and other testicular factors, may affect the formation and the maintenance of the anatomical barriers in rabbits and rodents, as well as reducing epididymal growth (Suzuki & Nagano 1976, Orgebin-Crist et al. 1983, Cyr et al. 1992). Both androgens and estrogen regulate the expression of genes in the mouse epididymis (Chauvin & Griswold 2004, Pereira et al. 2014). Expression and activation of androgen receptors are critical for murine epididymal development (O’Hara et al. 2011, Sipila et al. 2011). The temporal associations between expression of tight junction proteins, occludin, claudin 1 and e-cadherin (Forster 2008) in mouse or rat epididymides and peak androgen levels, and restoration of expression of these proteins in orchidectomized animals by androgen administration, support a role for androgens in regulating tight junction formation (Cyr et al. 1992, 1995, 1999, Gregory et al. 2001). Although assumed to be the primary regulators of epididymal development and function, androgens are aromatized to estrogens by the widespread aromatase enzyme (Bondesson et al. 2015). The discovery of the crucial role of estrogen-mediated fluid resorption in efferent ducts of mice, albeit not in all species, generated awareness of estrogen-mediated events in male reproduction (Lubahn et al. 1993, Hess et al. 2000, 2001, Pearl et al. 2007). Species differences, including diurnally opposite responses to estrogens in different species, as appears to be the case for Sertoli cell proliferation, indicate the value of expanding studies to species in multiple orders of mammals (Berger et al. 2008, 2013, Cappon et al. 2011).

The pig is an ideal species to examine effects of estrogens on epididymal maturation as reducing endogenous estrogens does not detectably alter androgens, pituitary gonadotropins, inhibin or prolactin (At-Taras et al. 2006, Berger et al. 2013). Testicular estrogen secretion is relatively high in neonatal boars compared with estrogen levels measured in males of other species but temporarily decreases to a low level by 6.5 weeks of age (Ford 1983, Wagner & Claus 2008, Berger et al. 2013). In addition, the porcine epididymis expresses both ESR1 and ESR2 receptors in all regions of the epididymis (Pearl et al. 2007). However, only limited investigation of epididymal development in the pig has been published compared with development in rodents. Therefore, the present studies were conducted to begin to investigate the relative importance of androgens and estrogens on epididymal maturation in pigs. Since the corpus appeared to be the most sensitive to perturbation of the epididymal regions in rodents (Cyr & Robaire 1992, Levy & Robaire 1999), the corpus was the focus of these studies. Rather than using pharmacological levels of exogenous estrogens, we have taken advantage of an aromatase inhibitor, letrozole, to reduce production of endogenous estrogens, and the use of fulvestrant to inhibit ESR1 and ESR2-mediated signaling. Efferent ducts were not sensitive to reduced endogenous estrogens in the pig (Pearl et al. 2007); hence, they were not further studied in these experiments.

Materials and methods

Animals

Initial female breeding stock and semen (Lines 210 and GR1065) were provided by PIC (Hendersonville, TN, USA, currently a subsidiary of genus, PLC) and maintained at the UC Davis swine facility. This study was conducted in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and approved by the Animal Care and Use Advisory committee at the University of California at Davis.

Experimental design

To reduce circulating levels of endogenous estrogens, littermate pairs of boars were treated orally with either a nonsteroidal aromatase inhibitor (0.1 mg letrozole (CGS 20267; 4-4’-(1H-1,2,3-triazol-1-yl-methylene)-bis-benzo-nitrile; Ciba-Geigy, Basel, Switzerland)/kg body weight or canola oil vehicle, weekly starting at 1 week of age. The letrozole dose was shown previously (At-Taras et al. 2006) to effectively reduce circulating estradiol (E2) concentration and to reduce testicular aromatase activity by 90%.
Corpora epididymidis were obtained from boars at 6 and 6.5 weeks of age with animals treated through 5 weeks of age, at 8 weeks of age with treatment continued through 7 weeks of age, at 11 weeks of age with treatment continued through 6 weeks of age and at 16 weeks of age (a peripuberal developmental time point) with animals treated from 11 to 15 weeks of age (Fig. 1). Five littermate pairs were evaluated at each age except four littermate pairs were evaluated at the 8 week timepoint. E2 levels were dramatically reduced as previously reported for these animals but testosterone, follicle-stimulating hormone, luteinizing hormone, inhibin and prolactin were not affected by letrozole treatment (At-Taras et al. 2006, Berger et al. 2012, Berger & Conley 2014a,b).

To block the effects of endogenous estrogen mediated by ESR1 and ESR2, one boar in littermate trios of boars (total of five litters) received at least 125 µg fulvestrant, a nuclear estrogen receptor (ESR1 and ESR2) antagonist and G protein-coupled estrogen receptor (GPER) agonist (Tocris USA, Ellisville, MO, USA)/kg body weight per day via Alzet osmotic pumps (models 2ML4 and 2ML2; Durect Corporation, Cupertino, CA, USA) as described previously (Berger et al. 2013). The remaining two littermates received pumps containing the 50% DMSO:50% PBS vehicle. Letrozole-treated littermates received this aromatase inhibitor in canola oil weekly as previously described and all others received oral treatment with canola oil vehicle weekly with tissue collected at 6.5 weeks of age. The treatment level for fulvestrant was chosen based upon an effective dose in neonatal female pigs and effectively stimulated Sertoli cell proliferation in the testes in these littermates (Tarleton et al. 1999, Berger et al. 2013). E2 was decreased by letrozole treatment but not affected by fulvestrant as previously reported for these animals (Berger et al. 2013). Whole epididymides were weighed upon tissue collection at 6, 6.5, 11 or 16 weeks of age.

To block the androgen receptor, a nonsteroidal anti-androgen, 2-methyl-N-(4-N-3-(trifluoromethyl) phenol)propanamide, (flutamide) was administered orally beginning at 1 week of age at a dose of 10 mg/kg body weight. Effective flutamide dosage was based upon previous evaluations (Neri & Monahan 1972, Juniewicz et al. 1990, Legacki et al. 2011). Daily administration continued through 6 weeks of age and tissues were collected at 6.5 or 11 weeks of age (n=4 for each time point) (Legacki et al. 2015). Littermate controls received the canola oil vehicle orally. Whole epididymides were weighed upon tissue collection.

**Evaluation of tight junctions**

Tissues were processed by immersion fixation as described previously (Cambrosio Mann et al. 2003, Dube et al. 2007). In brief, 1 mm³ sections of corpus epididymides were rinsed with tap water and samples were immersed in 2% glutaraldehyde and 1% lanthanum in 0.1 M cacodylate buffer, pH 7.2 for 15 min. Samples were kept overnight in 0.1 M cacodylate buffer, pH 7.2 for 15 min. Samples were then washed in cacodylate buffer and postfixed with 1% OsO₄ in 0.1 M cacodylate buffer for 1 h followed by dehydration in acetone and resin polymerization. Toulidine blue stained, thick sections were viewed to ensure luminal and epithelial areas. Thin sections were cut with a diamond knife, mounted on copper grids and counterstained with uranyl acetate and lead citrate. Images were taken using a Philips CM120 electron microscope, MegaScan camera and Gatan Digital Micrograph Software and evaluated for lanthanum exclusion.

**Figure 1**

Schematic of experimental treatments. In each experiment, one member from a littermate pair or littermate trio was allocated to each treatment with four or five different litters utilized in each experiment.

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Analysis of cell proliferation

Epididymides (from vehicle and letrozole-treated littersmates, from vehicle, letrozole, and fulvestrant-treated littersmates, or from vehicle and flutamide-treated littersmates) were fixed in 4% paraformaldehyde in PBS, processed for paraffin embedding and subsequently used for immunohistological detection of phosphorylated histone 3 (PH3) as a marker of epithelial cell proliferation (Brenner et al. 2003). Following rehydration, sections were blocked with normal goat serum (Vector Elite ABC Rabbit kit, Vector Laboratories, Burlingame, CA, USA) for 20 min and incubated overnight at 4°C with 1:50 polyclonal rabbit anti-PH3 IgG (Santa Cruz; cat # SC-8656R), then rinsed in Tris-buffered saline. Negative control sections were incubated with normal rabbit IgG. Sections were incubated with biotinylated anti-rabbit secondary antibody (Vector Elite ABC Rabbit kit; cat # PK-6101), followed by 40 min incubation with ABC reagent. Labeling was visualized using AEC Chromagen (Vector Laboratories; cat # SK-4200). Nuclei were lightly stained with hematoxylin and slides were cover slipped prior to examination. Slides were examined with brightfield illumination using a 20× objective. QImaging Micropublisher 3.3 digital camera and QCapture Pro software (QImaging Corporation, Burnaby, BC, Canada) were used to record images of corpus epididymides. Proliferating epithelial cells (PH3 immunolabeled) were counted in 2.43×10^5 μm² for each field of view and nine fields from each tissue section were evaluated. Although PH3 labeled stromal cells (based upon location) were observed, they were not included in the analysis.

Measurement of epithelial height

Sections (5 μm) were stained with hematoxylin and images recorded as previously described. Epithelial height was measured on a single tubule in ten separate fields per animal (792× magnification); when variation in epithelial height within the tubule was present, the visually estimated shortest height was measured to avoid distortion from a nonperpendicular orientation. The estimated coefficient of variation for estimates based on nine fields was 2%.

Detection of GPER

Corpora epididymidis from 6.5-week-old pigs was homogenized in 0.1 M K phosphate buffer, pH 7.4 containing 5 mM mercaptoethanol and 0.5 mM AEBSF and the supernate following centrifugation of the homogenate at 10 000 g for 10 min was stored at −20°C. Ten microgram protein was loaded per well and proteins separated by gel electrophoresis. Following protein transfer to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA) and blocking with 10% normal donkey serum, the membrane was probed with a 0.5 μg/ml of a rabbit anti-GPER antibody (ARP62244, Aviva Systems Biology, San Diego, CA, USA) with a second lane probed with diluted normal rabbit serum containing 1 μg/ml of rabbit IgG. Blots were subsequently incubated with peroxidase-labelled donkey anti-rabbit IgG and visualized with Luminata Forte chemiluminescent reagent (EMD Millipore). Paraffin sections of corpora epididymidis were processed similarly to that previously described for cell proliferation with the following differences. Sections were incubated for 30 min with 0.3% peroxidase in methanol prior to blocking for 20 min with dilute goat serum, incubated overnight with 10 μg/ml of the rabbit anti-GPER antibody (10 μg/ml of normal rabbit IgG for the negative control), rinsed in Tris-buffered saline, incubated for 30 min with an alkaline phosphatase polymer reagent, rinsed, and incubated for 15 min with ImmPACT Vector Red and lightly counterstained with 0.5% methyl green.

Statistical analysis

Three viewers blind to treatment evaluated electron micrographs for presence of blood barrier formation (lanthanum exclusion) and ranked littersmates. These rankings were in agreement with the percentage of intercellular junctions with lanthanum exclusion determined by a fourth viewer. The percentage of barriers formed weighted by number of epithelial cell boundaries examined was subjected to ANOVA using Proc Mixed (SAS Statistical Software, SAS Institute, Inc., Cary, NC, USA). Treatment was considered a fixed factor and litter a random factor. Epithelial height, proliferation data and epididymal weight were similarly subjected to ANOVA with the following differences. Sections were analyzed separately. Values are presented as least squares means ± pooled s.e.m. Differences were considered significant if P ≤ 0.05.

Results

Blood–epididymal barrier formation in the corpus epididymidis

To assess the timeframe for the development of an intact blood–epididymal barrier, epididymal tissue from a 3-month-old boar was evaluated in a preliminary trial. Cells lining the lumen of the corpus epididymidis had
numerous microvilli (Mv) extending into the lumen and a large number of vacuoles were observed in these cells as well (Fig. 2). The intercellular boundaries were black indicative of the presence of the electron dense lanthanum except the lanthanum was not present near the lumen. This absence of lanthanum in the ~1 μm closest to the lumen is generally perceived as indicative of anatomical tight junctions (Martinez-Palomo et al. 1971). In tissues from 6 week and 6.5-week-old boars, the cell surface near the lumen contained a sparse number of quite short Mv and vacuoles were again present (Fig. 3). Intercellular boundaries appeared black from the electron dense lanthanum but only a fraction of these boundaries were not marked by penetration of the lanthanum to the lumen. At 6 weeks of age, approximately one-third of the intercellular junctures blocked lanthanum access to the lumen (32 and 38% in two vehicle-treated boars and their two littermates treated with the aromatase inhibitor to reduce endogenous estrogens, respectively). The 6.5 week age was consequently chosen to determine if endogenous estrogen signaling affected development of the block in these cellular junctures. Sixty-five percent of the epithelial cell–epithelial cell boundaries blocked lanthanum access to the lumen in the animals with reduced endogenous estrogens compared with 32% in their littermates treated with vehicle (s.e.m. = 11, P < 0.05, n = 5 littermate pairs; Fig. 3). In two litters, the third littermate treated with fulvestrant (an antagonist for ESR1 and ESR2 but an agonist for GPER) had apparently reduced barrier formation compared with littermates treated with letrozole or the canola oil vehicle (23% vs 63% or 56%, s.e.m. = 9% or 12%, P = 0.05, P = 0.10, respectively).

Cell proliferation and epithelial height

Increased numbers of proliferating cells were present in the epididymides from letrozole-treated boars compared with vehicle-treated littermates at 6 and at 6.5 weeks of age (P < 0.01; Fig. 4). Treatment with the GPER agonist and ESR1 and ESR2 antagonist fulvestrant reduced the number of proliferating cells compared with littermates treated with letrozole (P < 0.01; Fig. 5). Epithelial height was increased in boars treated with letrozole compared with their vehicle-treated littermates. Similar to the cell proliferation response, littermates treated with fulvestrant had reduced epithelial height compared with their letrozole-treated littermates (Fig. 5). The vehicle-treated boars had a greater proportion of proliferating cells at 8 and 11 weeks of age compared with their letrozole-treated littermates (P < 0.001; Fig. 4). At 16 weeks of age, proliferation in the epididymis did not appear to be responsive to reduced endogenous estrogens during the preceding 5 weeks (P > 0.5).

Proliferation of corpus epididymal cells at 6.5 weeks was not affected by blocking androgen receptors with
flutamide although the number of proliferating cells was numerically higher in littermates treated with flutamide compared with vehicle-treated littermates (P < 0.5; 51 vs 40 proliferating cells/mm², S.E.M. = 17). Similarly, exposure to flutamide did not affect epithelial height either (P > 0.25, data not shown). Treatment with flutamide through 6.5 weeks of age reduced the number of proliferating cells at 11 weeks of age compared with their vehicle-treated littermates (P < 0.05; 28 vs 19 proliferating cells/mm², S.E.M. = 3).

Epididymal weights increased with age but weights were not different between vehicle and letrozole-treated boars at 6, 11, or 16 weeks of age (data not shown). At 6.5 weeks of age, epididymal weights were higher in the letrozole-treated boars compared with their vehicle-treated littermates (4.1 vs 4.7 g, S.E.M. = 0.2; P < 0.05).

Localization of GPER

Immunohistochemical detection of GPER suggested widespread localization in the corpus epididymidis of neonatal boars (Fig. 6). Labelling in the epithelium of the duct appeared stronger than labeling in the stroma with the lightest labeling in the smooth muscle surrounding the duct. Western immunoblot analysis detected a protein of ~41 kDa, the appropriate size for the porcine GPER.

Discussion

The blood–epididymal barrier is becoming an anatomical barrier at 6–6.5 weeks of age in the boar. To our knowledge, this is the first report for timing of barrier development in the pig epididymis. The blood–testis barrier is believed to be functional at 4 months of age in pigs or slightly earlier (Tran et al. 1981). Asynchrony in the formation of the blood–testis and blood–epididymal barriers is not uncommon. In the Sprague–Dawley rat, the epididymal barrier forms later than the testis barrier but is formed earlier than the testis barrier in the mink (Agarwal & Hoffer 1989, Pelletier 1994) as we observed here in the pig. Disparate timing of formation of the physical barrier in the epididymis and the testis has led to the idea that the two are subject to different regulatory mechanisms (Suzuki & Nagano 1978b, Pelletier 1994, Cyr et al. 1999). Differences among species in developmental timing of the blood–epididymal barrier relative to completion of spermatogenesis, including observations of formation by 7 days of age in the Wistar...
Effects of reducing endogenous estrogens on cell proliferation were larger in magnitude than the effect on junctional barrier formation at 6.5 weeks of age with the reduction in estrogens almost doubling the number of proliferating cells. Estrogen levels in the neonatal boar are comparatively high and approximately the same level as in the pubertal boar although an intervening decline to relatively low levels is observed at 6 weeks of age (Ford 1983, Berger et al. 2013). Hence, the letrozole treatment would initially mimic this decrease in E$_2$ but at an earlier age.

Blocking nuclear estrogen receptors with fulvestrant would be expected to have similar effects to reduced estrogens if signaling is mediated by the classic genomic receptors ESR1 and ESR2. However, fulvestrant had the opposite effect, delaying epididymal barrier formation, reducing epithelial cell proliferation, and decreasing epithelial height. In addition to antagonism of ESR1 and ESR2 receptors, fulvestrant acts as an agonist for the

Endogenous estrogens appear to delay formation of the blood–epididymal barrier since reducing endogenous estrogens by inhibiting aromatase slightly accelerated the formation of the epididymal–blood barrier in this study. Although statistically significant, this was not a large effect with the vehicle-treated littersmates exhibiting a wide range of barrier formation between principal cells at 6.5 weeks of age compared with their letrozole-treated littersmates.

Figure 4
Proliferating epithelial cells in the porcine corpus epididymidis. (A) Immunohistochemical labeling with an antibody to phosphorylated histone 3 to detect proliferating cells. Arrows point to labeled cells, most of which are epithelial cells. (B) Open bars represent least squares means and s.e.m. for vehicle-treated boars and black bars represent means and s.e.m. for letrozole-treated boars. Values are derived from five animals at each age except the 8-week values are based upon four animals and ** indicates value for letrozole-treated boars differ from littermates treated with vehicle at $P<0.01$ with *** indicating values differ $P<0.001$.

Figure 5
Epithelial cells in the corpus epididymidis in littermates treated with vehicle (open bars), letrozole (black bars), or fulvestrant (hatched bars). (A) Proliferation of epithelial cells and (B) epithelial cell height were altered by treatment. Value for vehicle-treated boars is less than that for letrozole-treated littersmates at $P<0.05$ (*) and value for fulvestrant-treated boars is less than the value for letrozole-treated littersmates at $P<0.01$ (**). Bars represent means and s.e.m. for animals from five litters. (C) Epithelial cell height was measured as the distance between arrowheads with the shortest distance in the tubule measured to avoid distortion due to orientation. Scale bar represents 10 µm.
membrane-bound estrogen receptor, GPER (Thomas et al. 2005). Therefore, the divergent responses of letrozole and fulvestrant suggest that fulvestrant slows epididymal development through effects on GPER.

Premature reduction of E2 appears to slightly accelerate development (junctional barriers, proliferation, and epididymal weight) but not to have long-term effects. Proliferation rates of the epithelial cells in the letrozole-treated animals were quite high at 6 and 6.5 weeks of age compared with the vehicle-treated littermates followed by an interval of lower proliferation rates than the vehicle-treated littermates. In contrast, the proliferation rate of the vehicle-treated animals appeared fairly consistent through 11 weeks of age. This suggests the effect of these manipulations of estrogen synthesis and signaling may alter timing of epithelial cell proliferation, but may not affect cell numbers in the long term as the epididymal weight difference at 6.5 weeks that presumably reflects the increased proliferation is rapidly eliminated (Pearl et al. 2007). The postpuberal epididymis is functional in treated animals indicating the accelerated development is consistent with normal function (Berger et al. 2008).

We believe that our data support a local response of the corpus epididymidis to testicular E2. Our previous work has indicated the absence of a letrozole effect on circulating gonadotropins, inhibin, prolactin and testosterone in the boar (At-Taras et al. 2006, Berger et al. 2013). Although the corpus epididymidis might be considered to be a local source of E2, and we were able to measure aromatase activity in 80% of the samples evaluated (data not shown), aromatase activity in the corpus epididymidis averaged <2% of the activity in the testis, suggesting the majority of the E2 would originate from the testis. Surprisingly, androgens were not significant regulators of cell proliferation in the early postnatal epididymis, at least in the boar, as blocking androgen receptors with flutamide had no effect. This observation further supports a local response to E2. The presence of GPER in the rat corpus epididymidis (Hess et al. 2011) and in the corpus epididymidis of the young pig is consistent with this hypothesis.

Inhibiting aromatase activity and consequently decreasing estrogens, accelerated timing of blood barrier formation and increased early epithelial cell proliferation in the corpus epididymidis. Blocking the androgen receptor had minimal effect. Since blocking ESR1 and ESR2 exhibited a different response to that observed with reduced endogenous estrogens, estrogens appear to be acting in part via GPER in corpus epithelial cells. The divergent responses of the corpus epididymidis to fulvestrant and reduced endogenous estrogens suggest partial maintenance of the immature state by a GPER mediated mechanism.

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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