Testosterone upregulates anion secretion across porcine vas deferens epithelia in vitro

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Title: Testosterone upregulates anion secretion across porcine vas deferens epithelia in vitro

Short title: Androgen-regulation of vas deferens anion secretion

Summary sentence:
Testosterone modulates the luminal environment to which sperm is exposed in the vas deferens by increasing anion secretion via upregulation of cyclooxygenase expression.

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Abstract

Testosterone induces and maintains prostaglandin-endoperoxide synthase 2 (PTGS2, also known as cyclooxygenase 2) expression in vas deferens epithelial cells, but it remains unknown whether this has a physiological role in the context of male reproductive biology. Prostaglandins (PG) induce concentration-dependent increases in anion secretion in porcine vas deferens epithelial cell monolayers (1ºPVD), where bicarbonate contributes to cAMP-stimulated anion secretion. Moreover, bradykinin (BK) induces anion secretion across 1ºPVD monolayers that is indomethacin-sensitive, and both *PTGS2* and *PTGS1* are expressed in this model system. Therefore, it was hypothesized that testosterone modulates anion secretion across vas deferens epithelia via PTGS-dependent pathways and PG synthesis. Porcine vas deferens epithelial cells were isolated and cultured as monolayers on permeable supports until assayed in modified Ussing chambers. RNA and protein were isolated concurrently for semi-quantitative expression analysis. Testosterone upregulated basal and BK-induced short circuit current across 1ºPVD monolayers, indicative of anion secretion. Testosterone also induced greater transepithelial electrical resistance. Increases in anion secretion were associated with preferential upregulation of PTGS2 at the mRNA and protein levels. In addition, testosterone induced greater basal and BK-induced anion secretion across vas deferens epithelial cells isolated from the distal segment of the duct. Taken together, these results suggest that testosterone upregulates epithelial responsiveness to acute modulations of anion secretion (likely bicarbonate secretion) that ultimately modifies the environment to which sperm are exposed.

Introduction

Early studies reported that testosterone upregulated prostaglandin (PG) synthesis in various segments of the male reproductive duct, and that this occurred most intensively in the vas deferens [1, 2]. PG concentration in the vas deferens was shown to increase with puberty and an increase in PG concentration was induced by testosterone [2-4]. Furthermore, it was shown that vas deferens tissue derived from intact rats converted arachidonic acid into PGs at twice the rate when compared to vas deferens tissues isolated from castrated animals, and that testosterone administration to castrated rats rescued the PG synthesis rate [5]. Subsequently, it was demonstrated that testosterone induced and sustained prostaglandin-endoperoxide synthase 2 (Ptgs2, previously known as cyclooxygenase 2) expression in epithelial cells lining the adult rat vas deferens [6]. Castration eliminated Ptgs2 expression in a time-dependent pattern, and testosterone supplementation rescued that expression in the distal vas deferens of castrated rats [6]. Additionally, testosterone modulates PTGS2 expression in human fetal and adult vas deferens epithelial cells [7].

Vas deferens epithelia exhibit cyclic adenosine monophosphate (cAMP)-stimulated anion secretion that requires bicarbonate in the basolateral medium and depends on sodium-bicarbonate co-transporter activity in the basolateral membrane [8]. Bicarbonate exchangers and the cystic fibrosis transmembrane conductance regulator are also thought to participate in bicarbonate secretion across vas deferens epithelia [9]. Moreover, PGs induce anion secretion across vas deferens epithelial cells via activation of prostaglandin E receptor 4 (PTGER4) and PTGER2, which are known to initiate cAMP generating pathways [10]. In addition, bradykinin (BK)-induced anion secretion is sensitive to the non-selective PTGS blocker indomethacin in vas deferens epithelia, where *PTGS2* and *PTGS1* are highly expressed [10].

Bicarbonate was recently shown to be required for the development of sperm fertilizing capacity and male fertility [11]. In addition, data are available suggesting that in addition to the epididymis, the vas deferens also functions as a sperm storage site [12]. Thus, regulated bicarbonate transport across epididymal and/or vas deferens epithelium likely affects sperm activity and viability.

Experiments were designed to investigate whether testosterone modifies ion transport and/or electrophysiological properties across primary monolayer cultures of porcine vas deferens epithelial cells (1ºPVD). Results reported here support the conclusion that testosterone increases both basal and BK-induced anion secretion across porcine vas deferens epithelia in vitro. Moreover, increases in anion secretion are associated with upregulation of PTGSs at both the mRNA and protein levels. Data are also provided to suggest that testosterone upregulation of anion secretion is of greater magnitude at the distal segment of the boar vas deferens.

Materials and Methods

Vas Deferens Tissue Acquisition and Epithelial Cell Isolation. Porcine vas deferens were surgically excised immediately postmortem from sexually mature boars at a local swine production facility, placed in ice-cold Hank's buffered salt solution (mM composition: 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.6 Na_2HPO_4, 5.5 glucose) and transported to the laboratory where isolation of epithelial cells for primary culture was performed as described previously [13]. This same protocol was employed to isolate epithelial cells from porcine proximal and distal vas deferens. In this case, a single vas deferens was transected in three segments of equal length: proximal, middle and distal. Thereafter, proximal and distal vas deferens segments were subject to the cell isolation procedure while the middle segment was discarded.

Cell Culture. Vas deferens epithelial cells isolated from the entire duct, as well as proximal and distal vas deferens epithelial cells were seeded on 25 cm^2 tissue culture flasks and grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 1% penicillin and streptomycin (Invitrogen), which is here referred to as basal medium. Cell isolates had media changes every other day for 2-5 days. Cells were lifted subsequently with phosphate buffered saline (PBS) containing trypsin and ethylene-diaminetetraacetic acid (Invitrogen), suspended in basal medium, and seeded on 1.13 cm^2 Snapwell permeable supports (Corning-Costar; Cambridge, MA). Monolayers were kept in culture in either the absence or presence of testosterone cypionate (TC; 100 μM; Pharmacia & Upjohn Company, Kalamazoo, MI) with media changes every other day until assay. In a separate subset of experiments, vas deferens epithelial cells were isolated from the entire porcine duct, but the isolation and cell culture procedures were conducted with phenol red-free DMEM (Invitrogen), supplemented with 4,500 mg/l L-Glutamine (Invitrogen) to match this nutrient's concentration to that of basal medium, 10% basal FBS or charcoal-stripped FBS and 1% penicillin and streptomycin. FBS either was used as provided by the supplier or was charcoal-stripped as described previously [14]. Medium containing charcoal-stripped FBS was partitioned and one of the parts was supplemented with TC.

Electrophysiology. Epithelial cell monolayers were mounted in modified Ussing flux chambers (model DCV9, Navicyte; San Diego, CA; model P2300, Physiologic Instruments, San Diego, CA), bathed symmetrically in Ringer solution (composition in mM: 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$), maintained at 39°C and bubbled with 5% CO_2-95% O_2 to provide mixing and pH stability. Monolayers were clamped to 0 mV and short circuit current (I_{SC}) was measured continuously with a voltage-clamp apparatus (model 558C; University of Iowa, Dept. of Bioengineering, Iowa City, IA; VCCMC8, Physiologic Instruments). Data were acquired digitally at 0.1 to 1 Hz with an Intel-based computer using either an MP100A-CE interface and AcqKnowledge software (ver. 3.7.3; BIOPAC Systems, Santa Barbara, CA) or Acquire and Analyze software (version 2.3.159; Physiologic Systems). Once recordings began, this system periodically generated a bipolar voltage pulse. The resulting change in I_{SC} was used to calculate transepithelial electrical resistance (R_{TE}) according to Ohm's law. While in chambers, monolayers were exposed to lysylbradykinin (LBK; Bachem, King of Prussia, PA) and prostaglandin E2 (PGE2; Sigma-Aldrich, Saint Louis, MO).

RNA Isolation and Semi-Quantitative RT-PCR. Total RNA was isolated from epithelial cell monolayers following Ussing chamber protocols, using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. RNA quality was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA concentrations were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and all samples were diluted to 100 ng/µl. One-step RT-PCR was performed using One-step RT-PCR kit (Qiagen) according to manufacturer's recommendations, with 100 ng of RNA per reaction. Single-target reactions were conducted using primers for porcine GAPDH, PTGS1 or PTGS2, as described previously [10]. Briefly, SYBR Green I (Molecular Probes, Eugene, OR) fluorescence signals were detected and quantified by a SmartCycler (Cepheid, Sunnyvale, CA). Transcript abundance measurements in any given RNA sample were performed by reactions carried out in duplicates. Melting analyses were performed and reaction products were subjected to agarose gel electrophoresis. Acquired threshold cycle values for target and reference genes were normalized according to the amplification efficiency method [15]. Means and

standard error of the mean (SEM) were derived from normalized results, within each condition. Results were expressed as the fold-change of *PTGS* expression in cells cultured in TC-supplemented medium, compared to the paired cells cultured in basal medium.

Western Blot Analysis. Epithelial cell monolayers cultured on permeable supports were lysed and solubilized in buffer containing protease inhibitors (Calbiochem, San Diego, CA). After centrifugation at 6000 rpm, 4^{o}C, for 15 min, the supernatant was transferred to a fresh tube and protein quantified with Micro BCA protein assay kit (Pierce, Rockford, IL). Protein sample aliquots were prepared for electrophoresis by addition of loading buffer (Boston Bioproducts, Worcester, MA), β-mercaptoethanol (Sigma-Aldrich) and heated to 95^{o}C for 5 min. Polyacrylamide 4-20% gradient pre-cast gels (Pierce) were loaded with a set of protein samples isolated from 1ºPVD monolayers originating from the same cell isolation and cultured in paired conditions. Gel lanes received either 40 or 20 µg of protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was conducted at 100 V, for approximately 45 minutes or until optimal separation of pre-stained protein standards (Bio-rad Laboratories) was observed. Electro-transfer onto PVDF membranes (Millipore, Billerica, MA) was carried out in methanol-containing transfer buffer (20% v/v), at 30 V for 3 h. Gels were stained by GelCode Blue Stain (Pierce) for assessment of protein transfer efficiency. Two blots were obtained from each 1ºPVD isolation, in a paired manner. Blocking was conducted with blotting grade non-fat dry milk (Bio-rad Laboratories, 5% w/v). Antibody treatment of each blot was conducted so that, within each blot pair, one was exposed to 0.5 µg/ml monoclonal anti-human PTGS2 (Cayman, Ann Arbor, MI), while the other was exposed to 0.5 µg/ml monoclonal anti-mouse Ptgs1 (Abcam, Cambridge, MA). None of these PTGS antibodies exhibits PTGS isoform cross-reactivity, as tested by their respective manufacturers. Specificity of the anti-PTGS2 sera was demonstrated previously [16]. Secondary detection of both PTGS1 and PTGS2 was conducted with 0.14 µg/ml anti-mouse Enhanced Chemiluminesence antibody (GE Healthcare, Piscataway, NJ), the substrate SuperSignal West Femto (Pierce) and a FluorChem HD2 imager (Alpha Innotech, San Leandro, CA). After PTGS detection, each blot was stripped with Restore Plus western blot stripping buffer (Pierce), washed and incubated with 0.16 µg/ml anti-ACTIN

(Sigma-Aldrich) for 1 hour. Secondary detection of ACTIN was conducted with the same reagents and equipment as for PTGSs, with the exception that 0.05 µg/ml anti-rabbit Enhanced Chemiluminesence antibody (GE Healthcare) was employed. Densitometry was carried out on the band of expected mobility for each of these targets, resolved on lanes where 40 µg of protein had been loaded, using the AlphaEase FC software (version 6.0.0; Alpha Innotech). Each PTGS band density value was divided by the density of the respective ACTIN band, within each condition and cell isolation, so that an ACTIN-normalized PTGS density was determined. Means and SEM were derived from ACTIN-normalized PTGS densities, within each condition. Results are presented as the fold-change of PTGS expression in TC-supplemented cells compared to the paired untreated cells.

Statistical Analysis. Paired and unpaired *t*-tests and analysis of variance (ANOVA) were performed as appropriate. These tests and the calculation of means and SEM were performed with Excel Microsoft Office Suite 2003 (Microsoft Corporation, Redmond, WA). All graphs were made with SigmaPlot (version 6.0, Systat Software Inc., Point Richmond, CA).

Results

Testosterone modulates anion secretion across porcine vas deferens epithelia in vitro

Initial experiments to determine whether testosterone modulates ion transport across 1ºPVD were performed using monolayers derived as reported previously [13]. Paired 1ºPVD monolayers were cultured in either the absence (basal media) or presence of TC (TC-supplemented). Monolayers were maintained in these conditions for 11-19 days and then assayed in modified Ussing flux chambers. Monolayers cultured in TC-supplemented medium exhibited greater basal I_{SC} and R_{TE} than monolayers maintained in basal medium (Figs. 1A and 1B). Basal I_{SC} was nearly doubled in TC-supplemented cells, while R_{TE} was approximately 20% greater. These results indicate that testosterone exposure modifies the composition and/or volume of secretion and that the ability of these cells to separate fluid compartments of different compositions is enhanced.

Once exposed to LBK (1 nM) in both the apical and basolateral compartments, all monolayers responded with rapid increases in I_{SC} and decreases in R_{TE} that were suggestive of anion secretion (Fig. 2A). TC-supplemented monolayers exhibited greater maximal changes in I_{SC} ($\Delta I_{SC\text{-}MAX}$) than those of monolayers cultured in basal medium (Fig. 2B). Changes in I_{SC} were also assessed over 900 sec to test independently for differences in net ion flux, in addition to a difference in the maximal rate of flux. Like in $\Delta I_{SC\text{-}MAX}$, net ion flux across TC-supplemented monolayers over 900 sec was approximately 65% greater, when compared to that of monolayers cultured in basal medium (Fig. 2C). Monolayers responded to LBK stimulation in a pattern where large decreases in R_{TE} occurred rapidly in both conditions. Maximal changes in R_{TE} ($\Delta R_{TE\text{-}MAX}$) were also greater in TC-supplemented monolayers (Fig. 2D).

To test if the lack of testosterone impairs epithelial cell monolayers capacity to respond to PGs, monolayer pairs (n = 4) were exposed symmetrically to PGE2 (0.5 or 1 μM). PGE2 induced $\Delta I_{SC\text{-}MAX}$ of 4.6 ± 0.9 and 4.7 ± 0.6 $\mu A*cm^{-2}$ in basal media and TC-supplemented, respectively, indicating that the underlying mechanisms accounting for the ion transport were not changed by TC exposure (data not shown).

Additional experiments were conducted in conditions optimized to minimize steroid effects in basal medium. Phenol red, a pH indicator present in most culture media

preparations, reportedly has estrogenic effects [17] and steroid hormones can be present in FBS. Thus, experiments were conducted where phenol red-free medium (PR-free) and charcoal-stripped fetal bovine serum (CS-FBS) or basal FBS were used to formulate 3 experimental conditions: PR-free plus CS-FBS, PR-free plus CS-FBS plus TC (100 μM), and PR-free plus FBS. Paired 1ºPVDs were cultured in these conditions for 19-21 days and then assayed in modified Ussing flux chambers. Consistent with the initial observations, testosterone upregulated basal I_{SC} (Fig. 3A). However, no statistically significant differences in R_{TE} were observed (Fig. 3B). PR-free plus CS-FBS plus TC exhibited statistically greatest LBK-induced net ion flux (Fig. 4B). $\Delta I_{SC\text{-}MAX}$ and $\Delta R_{TE\text{-}MAX}$ were also greater in PR-free plus CS-FBS plus TC, when compared with PR-free plus CS-FBS, but no statistically significant differences were noted between these experimental groups (Figs. 4A and 4C). All monolayers were exposed subsequently to symmetrical PGE2 (1 μM). PGE2 induced $\Delta I_{SC\text{-}MAX}$ of 3.9 ± 0.9, 3.5 ± 0.7 and 5.2 ± 1.8 $\mu A*cm^{-2}$ across PR-free plus CS-FBS, PR-free plus CS-FBS plus TC and PR-free plus FBS, respectively, with no statistically significant differences among these responses. This outcome suggests that in a condition where steroid presence is minimal, such as in PR-free plus CS-FBS, 1ºPVD monolayers still preserve their capacity to respond to PGE2 at a magnitude comparable to those where slightly greater levels of steroids are present.

Taken together, these results support the notion that testosterone increases basal I_{SC} and R_{TE} of porcine vas deferens epithelial cells in vitro, and upregulates bradykinin-induced ion transport.

Testosterone induces preferential PTGS2 upregulation when compared to PTGS1 at the mRNA and protein levels in vas deferens epithelial cells in vitro

Testosterone was shown to induce and maintain PTGS2 expression in vas deferens epithelial cells of rats and men [6, 7]. In addition, *PTGS2* and *PTGS1* mRNAs are expressed in 1ºPVD, where they are thought to increase anion secretion by synthesizing PGs upon BDKRB2 activation [10]. To determine if the 1ºPVD testosterone-induced upregulation of anion secretion reported here is caused by a transcriptional testosterone effect that upregulates *PTGS2* and/or *PTGS1* mRNA copy numbers and, subsequently, the amount of the respective proteins, total RNA and protein samples were isolated from the monolayers employed in the functional assays.

RNA isolates derived from paired epithelial cell monolayers cultured in the absence or presence of TC were employed in SYBR-green-based qRT-PCR targeting the *PTGS2*, *PTGS1* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) transcripts. *GAPDH*-normalized results revealed a certain degree of *PTGS2* upregulation in the TC-supplemented group (Fig. 5A). Further quantitative analysis of these transcripts was sought in RNA isolates derived from monolayers cultured in steroid-free conditions as described above. Outcomes from these experiments were similar to those previously acquired as *PTGS2* mRNA was approximately one and a half times more abundant in PR-free plus CS-FBS plus TC than in PR-free plus CS-FBS. Alternatively, *PTGS1* mRNA abundance did not change or was slightly decreased in PR-free plus CS-FBS plus TC (Fig. 5B).

PTGS2 and PTGS1 protein expression levels were measured in 1ºPVD cultured in the presence and absence of TC supplementation by western blot analysis. The anti-PTGS2 sera employed in these experiments was previously shown to selectively detect human PTGS2 [16], and has no cross-reactivity with PTGS1 as reported by the manufacturer. Results from immunoblots generated with 1ºPVD protein and probed with anti-PTGS2 revealed either a single band of mobility at approximately 75 kDa or two prominent bands, that included this band at 75 kDa and a second band with a mobility at approximately 210 kDa (Fig. 6A). The 75 kDa band is consistent with published data for porcine PTGS2 [18]. This band was subjected to densitometric analysis that included ACTIN immunoreactivity as a reference for normalization (Fig. 6A). PTGS2 was upregulated by 2-fold in monolayers cultured in the presence of TC-supplementation, both in regular media conditions and in the optimized cell culture conditions that included phenol-red free medium and charcoal stripped FBS (Fig. 6A). Although the mobility of porcine PTGS1 was previously demonstrated at 69 kDa [18], immunoblots generated with 1ºPVD protein exhibited a band at approximately 60 kDa, as the signal of greatest molecular weight. One or two bands at the range of 35 to 55 kDa were also present in lanes loaded with 1ºPVD protein, both in TC-supplemented or not. The 60 kDa band was detected in 6 out of 8 western blots conducted for PTGS1. The ~ 60 kDa band was also the form of greatest apparent molecular weight detected when 1ºPVD protein was subjected to electrophoresis side by side with protein isolated from porcine kidney

and a platelet-enriched porcine blood cell preparation (Fig. 6B). Densitometry conducted with the signals derived from the ~ 60 kDa band and normalized to ACTIN revealed that PTGS1 expression was unchanged in TC-supplemented monolayers, when compared to basal media and/or CS-FBS.

These results suggest that vas deferens epithelial cells cultured in the presence of testosterone supplementation exhibited a greater number of *PTGS2* transcripts and increased levels of PTGS2.

Testosterone-regulated anion secretion is more prominent in distal porcine vas deferens epithelial cells

Testosterone was shown to induce and support PTGS2 expression in the rat distal vas deferens [6], although the physiological implications of this site-specific expression are unknown. To test whether TC-supplementation has differential effects in proximal versus distal vas deferens, epithelial cells were isolated from the proximal and distal thirds of the porcine vas deferens. Paired proximal and distal cell isolates derived from the same duct were cultured as epithelial monolayers in basal or TC-supplemented media for 18-19 days and assayed in modified Ussing flux chambers. Basal I_{SC} was greatest in TC-supplemented distal epithelial cell monolayers and distal monolayers maintained in basal medium had the lowest basal I_{SC} (Fig. 7A). R_{TE} was high and similar across the different experimental groups (Fig. 7B).

Exposure to LBK (1 nM) induced greatest I_{SC}-changes in distal epithelial cell monolayers that had been TC-supplemented. Net ion flux over 900 sec was significantly greater in this group, suggesting a response to LBK that was more sustained (Figs. 8A and 8B). Maximal changes in R_{TE} ($\Delta R_{TE\text{-}MAX}$) were 3800 ± 270, 4400 ± 700, 3800 ± 900 and 4600 ± 600 Ω*cm^2 in proximal-basal media, proximal-TC supplemented, distal-basal media and distal-TC supplemented, respectively, with no significant differences being observed.

Outcomes from these assays suggest that epithelial cells lining the distal vas deferens present greater basal net anion secretion that is testosterone-dependent. In addition, these results suggest that anion secretion elicited by BK is upregulated to greater levels by testosterone acting in distal vas deferens epithelia.

Discussion

Results presented here demonstrate that testosterone modulates ion transport across vas deferens epithelial cells. Functional data derived from porcine vas deferens epithelial cell monolayers reveal that testosterone upregulates the basal levels of net anion secretion across vas deferens epithelia. Moreover, these data suggest that rapid increases in anion secretion induced by BK are of greater magnitude if vas deferens epithelial cells are maintained in the presence of testosterone supplementation. Results from molecular assays that quantified PTGSs at both the mRNA and protein levels, indicate that modulation of ion transport by testosterone is exerted through upregulation of PTGS2. These data support that, in addition to its widely known effects on the male reproductive tract and other tissues, testosterone has both direct and indirect roles in modifying the luminal environment to which sperm cells are exposed. It was recently shown that bicarbonate is required for the development of sperm cell fertilizing capacity [11]. In addition, vas deferens epithelia anion secretion, shown here to be upregulated by testosterone, was shown previously to be bicarbonate-dependent [8]. Thus, these data provide evidence for an additional pathway by which testosterone can modify male fertility.

Monolayers of 1ºPVD, whether maintained in basal medium or phenol red-free medium, exhibited a consistently greater level of basal I_{SC} when cultured in the presence of testosterone supplementation. This is expected to generate greater amounts of solute and water transported onto the lumen of the post-pubertal vas deferens. Assuming the porcine vas deferens luminal diameter is ~ 1 mm and that epithelial secretion is approximately 150 mM in monovalent salts, then the basal I_{SC} level (0.8 μA) carried out by epithelia maintained without testosterone supplementation would add approximately 0.2% per day to luminal contents. Following testosterone exposure, the contribution of vas deferens epithelial cells to luminal content would double. Testosterone supplementation was also associated with an increase in basal R_{TE}. The elevated R_{TE} likely results in a decrease in passive ion and water fluxes driven by concentration or electrical gradients. Thus, the combination of increased basal I_{SC} and R_{TE} likely empowers this epithelial system to more efficiently separate and modify the luminal compartment. Such observation of concomitantly increased I_{SC} and R_{TE} stands out of the

classical scenario where increased I_{SC} is usually detected along with lower levels of R_{TE} that reflects a greater number of open ion channels in one or both cell membranes. No data are yet available to offer an explanation as to how testosterone is upregulating both I_{SC} and R_{TE} in 1ºPVD, but it has been demonstrated that testosterone has an androgen receptor (AR)-independent antagonistic effect on voltage-gated calcium channels expressed in vascular smooth muscle [19]. Thus, testosterone could be inducing increases in R_{TE} through either direct action on specific ion transporting proteins or through signaling pathways that are not investigated here. In a previous study, where 1ºPVD cells were cultured in the presence or absence of 100 nM testosterone, no upregulation of basal I_{SC} or R_{TE} was detected [20], which suggests the possibility of a concentration-dependent factor in these observations.

BK induces rapid and profound changes in I_{SC} and R_{TE} that are largely inhibited by indomethacin in 1ºPVD, where *PTGS2* and *PTGS1* are expressed and proposed to be activated upon binding of the BDKRB2 [10]. In addition, it was reported that testosterone induces expression of PTGS2 in the rat vas deferens [6]. Data reported here demonstrate that testosterone upregulates BK-induced anion secretion across 1ºPVD monolayers. Upregulation of responses to LBK by testosterone took place not only in terms of the magnitude of rapid and maximal I_{SC} changes ($\Delta I_{SC\text{-}MAX}$), but also over the course of 900 sec. Thus it is proposed that greater levels of BK-induced anion secretion, including that occurring over an extended period of time, is brought about in 1ºPVD by greater expression of PTGSs. Greater abundance of PTGSs would take place through a genomic effect and could account for greater levels of PG synthesis. Increased concentration of PGs in this system ultimately would be responsible for greater or longer lasting anion secretion. Corroborating this, at least to some extent, are data revealing that PGs induce changes in I_{SC} in a concentration-dependent fashion in 1ºPVD [10]. Consistent with greater I_{SC} changes, testosterone also induced greater reductions in R_{TE} elicited by LBK. As previously reported, BK-induced changes in R_{TE} in this system tend to be maximal at the onset of I_{SC} responses and return, over the course of approximately 15 minutes, to levels similar to those of baseline [10]. Such a response profile is consistent with a rapid and robust channel-opening process that might be triggered by increased cAMP levels once PGs increase in abundance and bind to PTGER4 and/or PTGER2, which are

expressed in 1ºPVD [10]. Similarly, it is proposed that greater levels of PG synthesis elicited by LBK and brought about by greater PTGSs abundance in testosterone-treated monolayers are factors contributing to greater reductions in R_{TE}.

It was proposed initially that BK, acting on the basolateral membrane of epididymal epithelia induces or stimulates anion secretion through a prostanoid-producing pathway, as opposed to a prostanoid-independent pathway when BK is applied to the apical membrane [21-23]. It was reported subsequently that the prostanoid-producing pathway resides in epididymal basal cells, where LBK induces activity of Ptgs1 only [24]. However, a recent report from another laboratory indicates that BDKRB2 is localized at the apical membrane of principal cells of the cauda epididymis [25]. These aspects regarding bradykinin receptor localization, as well as the involvement of specific PTGSs and their localization have particular importance here, where an attempt to correlate testosterone modulation of anion secretion in epididymal epithelia [22] versus that in vas deferens epithelia is made. In epididymal epithelia, testosterone supplementation failed to alter responses to apical LBK but induced substantial LBK-responsiveness when LBK was applied basolaterally [22]. Confocal microscopy with immunodetection showed that BDKRB2 is localized at the apical membrane of principal cells in porcine vas deferens epithelium [10]. Moreover, immunocytochemistry derived from the intact rat and human vas deferens epithelium reveals that PTGS2 is expressed widely throughout principal cells of the distal vas deferens [6, 7]. PTGS2 is more abundant in 1ºPVD at the mRNA level than PTGS1 [10]. Taken together, these data suggest that the testosterone modulation of anion secretion in vas deferens epithelia reported here might occur via an apically expressed BDKRB2 that activates PTGS2 and PTGS1.

PTGS2 expression in human and rat vas deferens epithelial cells was shown to be testosterone-dependent [6, 7]. Epithelial cell monolayers that were derived from the entire extent of the porcine vas deferens and employed in the functional assays reported here, were also subject to gene and protein expression analysis that targeted PTGSs. The outcomes reveal that testosterone upregulated expression of PTGS2 at the mRNA and protein levels. Thus these data suggest that the functionally detected testosterone effect on 1ºPVD was carried out, at least in part, through transcriptional and translational

upregulation of PTGS2. Greater PTGS2 abundance would most likely contribute to greater PG synthesis and, ultimately, greater anion secretion. Testosterone was reported to upregulate both *Ptgs1* and *Ptgs2* expression in rat epididymal epithelia [22]. Those data, combined with the reported here, are apparently a first indication that PTGS expressions are differentially regulated by testosterone in the epididymis and vas deferens.

Results reported here also show that basal anion secretion is enhanced to a greater extent by testosterone in the distal porcine vas deferens when compared to the proximal segment. Testosterone supplementation upregulated basal anion secretion in distal epithelia and absence of testosterone supplementation brought basal anion secretion to a significantly lower level than that of proximal vas deferens epithelia. This suggests that net anion secretion is quantitatively increased in more distal portions of the duct. In addition, responses to LBK were greater in testosterone-supplemented distal porcine epithelia. From a fundamental perspective, these outcomes align with expectations, as PTGS2 is a major component of the BK-stimulated pathway that culminates in anion secretion by 1ºPVD [10], considering that PTGS2 expression is induced and supported by testosterone at the distal rat vas deferens [6], and that PGs induce anion secretion in 1ºPVD [10]. These outcomes from segmental 1ºPVD isolates might also serve as further evidence that increases in anion secretion observed in the experiments employing whole vas deferens cell isolates are due, in large part, to testosterone-induced PTGS2 upregulation. Early investigation on the ultra-structure of epithelial cells lining the rat vas deferens revealed marked differences between the proximal and distal segments and suggested that steroidogenic capacity would be present at the distal segment [26]. It was shown subsequently that the vas deferens of rats, dogs and men are capable of generating several testosterone metabolites, including 5α-dihydrotestosterone [27, 28]. In addition, the proximal and distal segments of the rat vas deferens were shown to present equivalent rates of testosterone metabolism [28]. Thus, it seems unlikely that the effect of testosterone supplementation, which induced greater anion secretion in distal 1ºPVD, is due to different and intrinsic capacities in proximal and distal cells to process TC into metabolites of greater affinity for the androgen receptor.

Data reported here are from an in vitro system and largely composed by electrophysiological measurements. However, these data might well be of relevance for further understanding of male fertility regulation. For instance, the vas deferens lumen has been shown to function as a sperm storage site [12]. More than 20% of sperm cells are found in the vas deferens and close to half of these are found at the middle and distal thirds of its length [12]. Sperm cells stored in the vas deferens lumen, located distally from a vasectomy site, and/or seminal vesicles constitute a contraceptive concern that require patients to observe a period of several weeks until sperm clearance is complete [29, 30]. These observations demonstrate that sperm cells remain in the vas deferens lumen, exposed to its luminal composition, for much longer than generally thought. Obviously, the vas deferens luminal environment reflects activity of the epithelia lining this compartment, as well as contents arriving from more proximal portions of the duct. As indicated previously, vas deferens epithelia carry out anion secretion that is largely bicarbonate-dependent [8] and expression of a subset of bicarbonate transporters has been demonstrated in vas deferens epithelial cells [8, 9]. Moreover, sperm cells take up bicarbonate from the extracellular environment, in a process that is required for the development of fertilizing capacity [11]. Hence, there is great likelihood that testosterone participates in sperm maturation by modifying anion secretion across vas deferens epithelia.

In conclusion, the results show that testosterone upregulates both basal and BK-induced anion secretion across porcine vas deferens epithelial cells in vitro. Moreover, distal vas deferens epithelial cells are more responsive to testosterone modulation and exhibit greater anion secretion in the presence of testosterone supplementation. It is also reported that upregulation of PTGSs, markedly PTGS2, is associated with testosterone supplementation, which suggests that testosterone induces the effects described here via increased PG synthesis. Increased anion secretion is expected to modify the luminal content to which sperm is exposed and modulate male fertility.

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Figure Legends

Figure 1. Testosterone (testosterone cypionate, TC) upregulates basal short circuit current (I_{SC}) and basal transepithelial resistance (R_{TE}) in primary cultures of vas deferens epithelial cells (1ºPVD). Basal I_{SC} (**A**) and basal R_{TE} (**B**) are greater in monolayers exposed to testosterone (100 μM). n = 17 in each condition. * indicates significant difference from basal medium ($P < 0.05$).

Figure 2. Testosterone upregulates bradykinin (BK)-induced anion secretion across 1ºPVD. **A**. Typical 1ºPVD responses to LBK (1 nM) derived from paired monolayers cultured in the absence or presence of TC-supplementation (100 μM). Summarized observations (n = 16-17) reveal that TC-supplementation upregulates LBK responses, as measured by maximal changes in I_{SC} ($\Delta I_{SC\text{-}MAX}$; **B**), net ion flux over 900 sec (**C**) and maximal changes in R_{TE} ($\Delta R_{TE\text{-}MAX}$; **D**). * indicates significant difference from basal medium ($P < 0.05$).

Figure 3. Testosterone upregulates basal I_{SC} in 1ºPVD cultured in conditions that included phenol red-free (PR-free) media, charcoal-stripped fetal bovine serum (CS-FBS) or typical fetal bovine serum (FBS). Basal I_{SC} (**A**) is greater in the PR-free + CS-FBS + TC -group, when compared to PR-free + CS-FBS or PR-free + FBS. Basal R_{TE} (**B**), although slightly greater in PR-free + CS-FBS + TC, was not significantly different from those of the two other conditions. * indicates significant difference ($P < 0.05$, n = 7).

Figure 4. Testosterone upregulates BK-induced anion secretion across 1ºPVD cultured in conditions optimized to reduce the effect of other steroids. Summarized observations (n =

565 7) reveal that TC upregulates LBK responses, as measured by maximal changes in I_{SC} ($\Delta I_{SC\text{-}MAX}$) (**A**), net ion flux over 900 sec (**B**) and maximal changes in R_{TE} ($\Delta R_{TE\text{-}MAX}$) (**C**). * indicates significant difference from PR-free + CS-FBS ($P < 0.05$).

Figure 5. Testosterone upregulates *PTGS2* mRNA abundance in 1ºPVD. **A**. Cells
570 cultured in TC-supplemented media exhibit *PTGS2* upregulation, compared to paired cells cultured in basal medium (n = 10 and 12 reactions for PTGS1 and PTGS2, respectively. RNA derived from 5 and 6 animals, respectively). **B**. A more prominent *PTGS2* upregulation was detected in 1ºPVD cultured in PR-free + CS-FBS + TC, when compared to 1ºPVD cultured in PR-free + CS-FBS (n = 10 and 12 reactions for PTGS1
575 and PTGS2, respectively. RNA derived from 5 and 6 animals, respectively). * indicates significant difference from PR-free + CS-FBS ($P < 0.05$).

Figure 6. Testosterone upregulates apparent PTGS2 protein abundance in 1ºPVD. **A**. Immunoblots exhibited a band of approximately 75 kDa that is consistent with porcine
580 PTGS2, and of greater density in protein samples derived from TC-supplemented 1ºPVD. After normalization to ACTIN, PTGS2 expression was 2-fold greater in TC-supplemented monolayers assayed in two cell culture systems: regular medium (n = 5) and optimized medium (n = 6). **B**. PTGS1 immunoreactivity, which in porcine kidney and white blood cells exhibits the same mobility as in 1ºPVD, is unchanged by
585 testosterone (n = 6).

Figure 7. Testosterone induces the greatest basal I_{SC} in the distal segment of porcine vas deferens. **A**. Basal I_{SC} reaches the greatest levels in TC-supplemented distal 1ºPVD monolayers. Additionally, distal 1ºPVD cultured in basal medium exhibit lower basal I_{SC} than that of proximal 1ºPVD cultured in basal medium. **B**. Monolayers presented similar levels of basal R_{TE} across experimental groups and treatments. Summarized results (n = 6) of paired cell isolates derived from the same vas deferens for each animal. ***** indicates significant difference compared to proximal, basal medium ($P < 0.05$); # indicates significant difference compared to proximal, TC-supplemented ($P < 0.05$) or compared to all other groups ($P < 0.05$).

Figure 8. Testosterone induces greater upregulation of BK-induced anion secretion across distal 1ºPVD. Summarized results (n = 6) reveal that BK-induced $\Delta I_{SC\text{-}MAX}$ is greatest in distal TC-supplemented 1ºPVD (**A**). Net ion flux over 900 sec is greater in distal TC-supplemented (**B**). ***** indicates significant difference ($P < 0.05$) from proximal TC-supplemented (t-test) and/or from the three other experimental groups (ANOVA).