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Testosterone up-regulates vacuolar ATPase expression and functional activities in vas deferens of orchidectomized rats

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ABSTRACT

Precise regulation of vas deferens fluid pH is essential for sperm. However, the mechanisms underlying effect of testosterone on vas deferens fluid pH have never been identified, which could involve changes in expression and functional activity of vacoular (V)-ATPase.

Methods: Orchidectomized, adult male Sprague-Dawley rats were treated subcutaneously with 125 μg/kg/day and 250 μg/kg/day testosterone with or without flutamide (androgen receptor blocker) and finasteride (5α-reductase inhibitor) for seven (7) days. Following treatment completion, *in vivo* perfusion of vas deferens lumen was performed and changes in fluid secretion rate, pH and HCO_3^- content were measured with and without bafilomycin, a V-ATPase inhibitor. Rats were then sacrificed and vas deferens were harvested and subjected for V-ATPase A1 and B1/2 protein expression and distribution analysis by western blotting and immunohistochemistry, respectively.

Results: In sham-operated and testosterone-treated orchidectomized rats, higher fluid secretion rate, which was not antagonized by bafilomycin but lower HCO_3^- content and pH which were antagonized by bafilomycin were observed when compared to orchidectomized-only and orchidectomized, testosterone-treated rats receiving flutamide or finasteride, respectively. Bafilomycin had no effect on fluid secretion rate, HCO_3^- content and pH in orchidectomized and testosterone-treated orchidectomized rats receiving flutamide and finasteride. V-ATPase A1 and B1/2 proteins were expressed at high levels in vas deferens and were highly distributed at the apical membrane of luminal epithelium and in muscle layer of this organ, mainly in sham and testosterone-treated orchidectomized rats.

Conclusions: V-ATPase is involved in acidification of vas deferens fluid under testosterone influence.

1. Introduction

Testosterone plays important role in the development and function of the male reproductive tract. However, its role in the regulation of vas deferens fluid pH remains unknown. Vas deferens plays important role in transporting the sperm from the epididymis to the ejaculatory ducts with pH and electrolytes content of its fluid were precisely regulated. These regulation is crucial for sperm survival [1]. The pH of epididymal and vas deferens fluid is reported to be acidic [2]. Acidification of the luminal fluid pH in general is reported to involve the membrane transporter proteins including vacuolar (V)-ATPase [2,3]. The acidic pH of vas deferens fluid is necessary to maintain the sperm quiescence and to prevent premature activation of sperm' acrosomal enzymes during their storage in cauda epididymis and vas deferens [4].

V-ATPase is a complex enzyme that is composed of several subunits, which are assembled into two domains: *trans*-membrane (V0) and cytosolic (V1) domains [3,5]. Epididymal epithelial cells of rats has been reported to contain V-ATPase which is distributed at the apical pole of the narrow and clear cells [2]. These cells are known to be involved in generating low epididymal fluid pH [6]. Some of V-ATPase subunits have more than one isoform [7]. As an example, four "a" isoform and two "b" isoform of V-ATPase subunit are present in the human genome [8,9]. Subunit a1 was expressed ubiquitously while subunit B1 was found in the epididymis and other tissues, while B2 was found to be ubiquitously expressed in the intracellular organelles [9]. The importance of V-ATPase to male reproduction has been demonstrated in mice in which lack of transcription factor, Foxil, a master regulator of V-ATPase in clear cells of epididymis affects male fertility due to inability of the

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sperm to move up the female genital tract to fertilize the oocyte [10–12].

In epididymal clear cells, V-ATPase function is under hormonal controls with hormone known to influence V-ATPase activity include angiotensin II (AngII). Ang II stimulates V-ATPase function via binding to Ang II type II receptors (AGTR2), which are found in the adjacent basal cells [13]. Once binding to AGTR2, nitric oxide (NO) is released from the basal cells which then diffuses out to stimulate proton secretion in clear cells via activation of cGMP pathway [14]. In vas deferens, the role of hormones in controlling V-ATPase expression and functional activity is largely unknown. Therefore, in this study, we hypothesized that testosterone affects V-ATPase expression and functional activity in vas deferens through which this hormone control fluid pH of this organ. Thus the objectives of this study were to identify the functional activity, expression level and distribution of V-ATPases in vas deferens under testosterone influence. The finding of this study could help to explain the mechanisms underlying testosterone-mediated effect on vas deferens fluid pH where dysregulation could adversely affect male fertility.

2. Materials and methods

2.1. Animal preparation and hormonal treatment

All experimental protocols were approved by the Institutional Animal Ethics Committee, University of Malaya with ethics number: 201405-07/PHYSIO/R/NS (2014/85). Adult male Sprague–Dawley rats weighing 200–225 g were obtained from Animal House, Faculty of Medicine, University of Malaya and were housed under standardized housing condition (temperature: 23 ± 2 °C, 12/12-h light–dark cycle, 30%–70% humidity). The rats had free access to rodent diet (Harlan, Rossdoff, Germany) and tap water *ad libitum*. Orchidectomy was performed following the previously described method [15,16]. Following orchidectomy, rats were given intramuscular injection of 0.1 ml Kombitrim antibiotic to avoid post-surgical wound infection.

Testosterone propionate (Sigma–Aldrich, MO, USA) was dissolved in ethanol prior to mixing with peanut oil. Three weeks after orchidectomy, drugs were subcutaneously administered for seven (7) days at the neck scruff. The doses of testosterone were selected on the basis of the previously reported doses [17–19]. Animals were then divided into eight groups with n = 6 per group:

Group 1: sham operated (S)
Group 2: orchidectomized, receiving peanut oil (control) (O)
Group 3: orchidectomized, receiving testosterone at 125 μg/kg/day (T125)
Group 4: orchidectomized, receiving testosterone at 250 μg/kg/day (T250)
Group 5: orchidectomized, receiving 125 μg/kg/day of testosterone and flutamide at 1 mg/kg/day (T125 + FU)
Group 6: orchidectomized, receiving 250 μg/kg/day of testosterone and flutamide at 1 mg/kg/day (T250 + FU)
Group 7: orchidectomized, receiving 125 μg/kg/day of testosterone and finasteride at 5 mg/kg/day (T125 + FN)
Group 8: orchidectomized, receiving 250 μg/kg/day of testosterone and finasteride at 5 mg/kg/day (T250 + FN).

24 h after the last drug injection, *in-vivo* perfusion of vas deferens lumen was performed with the rats under anesthesia. Following completion of the perfusion experiment, rats were sacrificed via cervical dislocation and vas deferens were immediately removed and then stored in an appropriate medium prior to analyses of protein expression and distribution. Following perfusion experiment, histology was performed to determine the integrity of vas deferens epithelium, where the epithelial lining was found to be intact (data not shown). In the meantime, blood was collected via direct heart puncture in order to determine the plasma testosterone levels using ELISA kit (ALPCO Diagnostic, Salem, NH, USA).

2.2. In vivo perfusion of vas deferens lumen

In order to investigate the changes in secretion rate, pH and HCO_3^- content of vas deferens fluid, *in vivo* perfusion of vas deferens lumen was performed according to the methods as previously described [20,21], but with a slight modification. In brief, anesthetized rats were placed on a heat pad to maintain their constant body temperature. An incision was made in the genital area in order to expose the cauda epididymis and vas deferens. Then, a blunt-end 27G needle, connected to a 3 cc syringe attached to a perfusion pump (Harvard apparatus, MS, USA) was inserted into the proximal end of vas deferens. The distal end of vas deferens (at the junction with epididymis) was cut open and directly placed above the opening of an eppendorf tube. Perfusion was conducted over the period of 3 h, at a rate of 0.25 ml/h.

The perfusate contains the following compositions: 50mosmol/l NaCl, 50mosol/l K gluconate, 1.2 mosmol/l $MgSO_4$, 0.6 mosmol/l $CaCl_2$, 4 mosmol/l Na acetate, 1 mosmol/l trinatrium citrate, 6.4 mosmol/l NaH_2 PO_4 and 3.6 mosmol/l Na_2HPO_4. The pH of the perfusate was adjusted to 6.8, using 350–360 mosmol (kg $H_20)^{-1}$ raffinose In order to detect the functional activity of V-ATPase, bafilomycin A1 (Santa Cruz, CA, USA), a V-ATPase inhibitor was dissolved in the perfusate at 2 μM, and then perfused. To determine the changes in fluid secretion rate, net weight of the collected fluids was divided by the total perfusion time (180 min). All tubes were weighed by using an electronic balance prior to and after perfusate collection.

The difference between tube weight before and after perfusate collection was considered as the rate of fluid secretion. pH of the perfusate was directly measured by using aquatwin pH meter (Horiba Scientific, Japan). HCO_3^- concentration was determined by using enzymatic assay of phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH) where the end products were measured by using a spectrophotometer at wavelength 405 or 415 nm. Changes in the colour were directly proportional to the HCO_3^- concentration in the samples.

2.3. Detection of V-ATPases protein distribution by immunohistochemistry

Immunohistochemistry was performed following the methods as previously described [22]. Firstly, immediately after harvesting, vas deferens was fixed in 10% formalin overnight prior to processing, following then, tissues were dehydrated through increasing concentrations of ethanol, cleared in chloroform and blocked in paraffin wax. Tissues were then sectioned into 5-μm thickness, deparaffinized in xylene followed with rehydration in reducing concentrations of ethanol. Antigen retrieval was performed by using Tri-EDTA buffer (10-mM Tris base, 1-mM EDTA, 0.05% Tween 20, pH 9.0). Endogenous peroxidase was neutralize by using 1% H_2O_2 in methanol. The sections were then blocked with a blocking serum to prevent the non-specific binding. This was performed prior to incubation with VATPase A1 (sc-28801, Santa Cruz, CA, USA) and VATPase B1/2 (sc-20943, Santa Cruz, CA, USA) antibodies, at 1:100 dilution. Sections were incubated with these antibodies at 4 °C overnight. 24 hrs later, sections were rinsed with PBS, three times, 5 min each, prior to incubation with biotinylated secondary antibody for 1 h at room temperature. Proteins were