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EFFECTS OF PROTEIN SYNTHESIS INHIBITOR AND ANTIMICROTUBULAR AGENT ON TRANSEPITHELIAL MOVEMENT OF ^{3}H-ANDROGENS IN THE RAT CAPUT EPIDIDYMIS

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ABSTRACT

The effects of protein synthesis inhibition and disassembly of microtubules in the epididymal epithelia on proluminal movement of ^{3}H-androgens were investigated by using in vivo microperifusion of ^{3}H-testosterone and subsequent micropuncture to obtain peritubular and intraluminal fluids of caput epididymal tubules. Cycloheximide (100 μg/ml) was used as protein synthesis inhibitor. Nocodazole (3 μg/ml) was used to depolymerize microtubules in the cell. The perifusion fluid was Minimum Essential Medium containing 26.7 μCi/ml ^{3}H-testosterone and 1.3 μCi/ml ^{14}C-polyethyleneglycol (^{14}C-PEG), or the same fluid supplemented with cycloheximide or nocodazole. Radioactivity of ^{3}H-androgen and ^{14}C-PEG in perifusion and intraluminal fluids was determined at one hour after initiation of the sustaining perifusion, and the percentage of radioactivity of ^{3}H-androgen and ^{14}C-PEG appearing in the intraluminal fluid to that in the peritubular fluid was determined. Proluminal movement of ^{3}H-androgens into the caput epididymal tubules in the control rats was 323.4±73.2%. This value was significantly reduced to 121.8±13% by addition of cycloheximide to the perifusion fluid ($p<0.01$). Transepithelial movement of ^{3}H-androgen in the caput epididymis was significantly decreased to 86.6±5.3% by exposure of the epididymal tubules to nocodazole ($p<0.01$). Inhibition of protein synthesis and disassembly of microtubules in the epididymal epithelial cells completely eliminated antigrade proluminal movement of ^{3}H-androgen into the tubules. Study of the incorporation of ^{35}S-Methionine into epididymal tissue protein revealed significant reduction of the quantity of radiolabeled proteins in the perifused tissue with fluid containing cycloheximide ($p<0.01$). These results suggest that antigrade proluminal androgen movement may be related to the synthesis of intracellular cytoplasmic proteins.

Key Words: Androgen, Epididymis, Cytoplasmic receptor, Methionine, Cycloheximide, Nocodazole.

INTRODUCTION

The epididymal luminal fluid milieu or microenvironment appears to play an important role in the maturation and survival of spermatozoa.[1,2] This microenvironment is a product of a complex series of processes which include testicular secretion, epididymal secretion and absorption and sperm metabolism.[1,2] Androgen plays an important role in the maintenance of the epididymal microenvironment.[1,2] Androgen concentrations in the caput epididymal lumen are high (approximately 225 nM) relative to androgen concentrations in the seminiferous tubules (approximately 150 nM) and higher still than androgen concentrations in the surrounding caput epididymal vascular supply (approximately 5 nM).[3] Intraluminal androgen-binding protein is concentrated in the caput epididymal lumen, and the intraluminal ratio of androgen-binding protein to androgen is approximately 1:1.[3] This result is consistent with the speculation that androgen-binding protein sets the intraluminal androgen concentration.

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^{3}H-androgens appear in caput epididymal fluid in concentrations that are approximately 250% to 300% of the concentrations present in interstitial fluid.[4,5] This antigrade, proluminal ^{3}H-androgen movement is subject to inhibition with unlabeled testosterone.[6] These results and others led us to hypothesize that ^{3}H-androgens diffuse across the epididymal epithelia, but bind to intraluminal androgen-binding protein in the epididymis, thus accounting for the antigrade movement of ^{3}H-androgens and the maintenance of high androgen concentrations in the caput lumen.

We have recently obtained two differing and confusing results. One result is that androgen-binding protein is required to maintain antigrade proluminal movement of ^{3}H-androgens in the caput epididymis.[7] The other result is that metabolic inhibitors (dinitrophenol or potassium cyanide) reduce antigrade proluminal androgen movement.[8] The latter finding is an enigma, but is subject to a testable hypothesis. The data might be explained by the inhibition by a metabolic inhibitor of a high affinity androgen transport receptor (protein) in the epididymal epithelial cells. This receptor can be transported along the intracellular microtubules from basal to apical cell surface. Therefore, the present study was undertaken to determine whether or not a protein synthesis inhibitor or antimicrotubular agent decreases antigrade proluminal androgen movement in the caput epididymis.

MATERIALS AND METHODS

In Vivo Microperifusion and Subsequent Micropuncture

Mature male Sprague-Dawley rats (420 to 650 gm) were housed in the vivarium in the Nagoya University School of Medicine under a 12-h light/12-h dark cycle and had free access to food and water. They were allowed to acclimate after shipment for at least one week before entering the experiment. The following isotopes were purchased from New England Nuclear (Boston, Massachusetts): ^{3}H-testosterone (specific activity: 55.2 Ci/mmol, M.W. 288.4); and ^{14}C-polyethyleneglycol (^{14}C-PEG; specific activity: 15.0 mCi/g, M.W. 4000). Micropipettes for perifusion and puncture were drawn on a vertical pipette puller (David Kopf Instruments, La Grange, IL) from constant-bore flint glass tubing with an inside diameter of 0.6 mm and an outside diameter of 0.9 mm. To facilitate penetration of the tubule wall, the pipette tips were sharpened on a rotating wet stone grinder (Bausch & Lomb Incorporated, Rochester, NY) to a diameter of 75 μm to 100 μm. Perifusion micropipettes were approximately 100 μm, while micropuncture tip sizes were 75 μm. After pipettes had been ground, they were cleaned with acetone and distilled water, and rendered hydrophobic by rinsing in a 1% aqueous solution of Siliclad (Clay Adams, Parsippany, NJ).

The animals were anesthetized with intraperitoneal injections of inactin (sodium 5-ethyl-5-(1-methylpropyl)-2-thiobarbiturate, Byk Guilden Konstanz, Hamburg, Germany; 100 mg/kg body weight) and subjected to in vivo microperifusion and micropuncture, as previously described.[9] Briefly, the epididymis was externalized through a scrotal incision, placed in a 35°C testicle warmer, and immobilized in 2% agar. A small area of the immobilized caput epididymis was exposed through the agar and was covered with water-equilibrated mineral oil. A 100-μm tip micropipette was placed in a micromanipulator (Leitz, Hamburg, Germany), connected to a 3-ml glass syringe with PE-60 tubing, filled with perifusion fluid, and attached to a perifusion pump (model 341B, Sage Instruments, Cambridge, MA).

The perifusion fluid was 0.3% lissamine-green-dyed Minimum Essential Medium (MEM) (pH 7.0–7.4; Gibco Laboratories, Grand Island, NY) containing 26.7 μCi ^{3}H-testosterone/ml

and 1.3 μCi ^{14}C-PEG/ml, or the same fluids supplemented with cycloheximide (100 μg/ml), 0.1% (v/v) dimethyl sulfoxide (DMSO), or 0.1% (v/v) DMSO and nocodazole (3 μg/ml). These four different fluids were used in different experiments on the caput epididymis. The perifusion micropipette was used to puncture directly through the transparent epididymal tunica, and the pipette tip was left in the interstitial space. The perifusion pump was set for a priming infusion at a rate of 6 μl/min for 15 min. Subsequently, a sustaining perifusion rate was set at 2 μl/mín for the remainder of the experiment. The perifusions continued for one hour, and samples of fluid from the epididymal interstitial space and intraluminal fluids of the epididymal tubule were collected by in vivo micropuncture methods. After the samples were collected they were sandwiched between columns of mineral oil in the collection pipette. One end of the pipette was sealed with Critoseal (Monoject Scientific, St. Louis, MO) and the pipette was inserted into a standard capillary hematocrit tube. This tube was inserted into a special plexiglass adapter which was inserted into a rotor head on an IEC model B-20 refrigerator centrifuge. The sample was centrifuged at 10,000 x g for 20 min at 0°C. The centrifuged sample in the original micropipette was placed into a vertical transfer apparatus (Bunton Instruments Co., Rockville, MD) and aliquots of cell-free fluid were transfered into scintillation vials, and each fluid sample was analyzed in triplicate for ^{3}H and ^{14}C radioactivity by scintillation spectrophotometry (LS 7000, Beckman Instruments, Inc. Fullerton, CA).

Since ^{14}C-PEG is excluded by the blood epididymal barriers[4] (little or no entry into the intraluminal space), the appearance of ^{14}C-PEG in the intraluminal fluid was taken as an indicator of inadvertent contamination from the extratubular compartment. The proportion of intratubular ^{14}C-PEG appearing in the intraluminal fluid was used to correct the ^{3}H-androgen data. ^{14}C-PEG was also used as a dilution marker for dilution of perifusion fluid by native interstitial fluid. The concentration of radioactive substances in the tubule lumina was expressed as a percentage of the radioactivity in the tubule lumina to that in the same volume of interstitial fluid surrounding the same tubule at one hour after perifusion.

Estimation of the Incorporation of ^{35}S-Methionine into Epididymal Tissue Proteins

To confirm the effect of cycloheximide in the perifusion fluid on epididymal protein synthesis, the proteins labeled following exposure of ^{35}S-Methionine and cycloheximide to the tubules were examined. For estimation of the amount of ^{35}S-Methionine incorporated into the proteins, MEM perifusion fluid containing 0.2 mCi ^{35}S-Methionine/ml with or without cycloheximide (100 μg/ml) was used. This perifusion fluid was perifused around the caput epididymal tubules for one hour. After completion of perifusion, the perifused region of the epididymis was dissected, rinsed briefly in normal saline to remove blood and excess perifusion fluid, blotted once to remove excess normal saline, and weighed. The tissue was homogenized and centrifuged at 1500 rpm for 15 min. Small aliquots (5 to 20 μl) of the homogenates were added to 0.5 ml distilled water containing 50 μg bovine serum albumin and 10 mM methionine. Proteins were precipitated with trichloroacetic acid (TCA) at a final concentration of 10% (w/v). The precipitate was collected on Millipore filters (Millipore Corp., Bedford, MA) and washed twice with 20 ml 5% TCA containing 10 mM methionine. After drying, radioactivity of the filter was determined.

Data Analysis

Chauvenet's criterion was applied to all the data.[10] The results are presented as mean and SEM. All multiple comparisons were made by the Kruscal Wallis test[11] for nonparametric data followed by the Wilcoxon rank sum test ($p<0.05$).

RESULTS

Proluminal Movement of ^{3}H-androgen into the Caput Epididymal Tubules

^{14}C-PEG concentrations remaining in peritubular fluids were generally stable in all groups. These values were approximately 85% of those in the original perifusion fluid (Fig. 1). ^{3}H-androgen concentrations remaining in the peritubular fluids in the control group and the 0.1% DMSO-alone group were 22.5±3.7% and 21.9±3.3%, respectively, of those in the original perifusion fluid (Fig. 2). These values were significantly increased in the cycloheximide (43.8±3.7%) and nocodazole (47.5±2.2%) experiment $p<0.05$ (Fig. 2).

Intraluminal ^{3}H-androgen concentrations in the epididymal tubule fluid in the control group and in the 0.1% DMSO-alone group were 323.4±73.2% and 304±39.8%, respectively, of those in peritubular fluid (Fig. 3). These values were significantly decreased in cycloheximide (121.8±13%; $p<0.05$) and nocodazole (86.6±5.3%; $p<0.01$) experiments (Fig. 3). Cycloheximide or nocodazole incorporated in the perifusion fluid completely eliminated antigrade proluminal movement of ^{3}H-androgen in the caput epididymis.

Incorporation of ^{35}S-Methionine into Epididymal Tissue Protein

Total tissue radioactivity of ^{35}S-Methionine after perifusion with MEM alone was 604.8±85 (cpm/mg wet weight) (Table 1). This value significantly declined to 175.6±21.2 after perifusion with MEM containing cycloheximide ($p<0.05$; Table 1). Cycloheximide significantly reduced protein synthesis in the epididymal tissue.

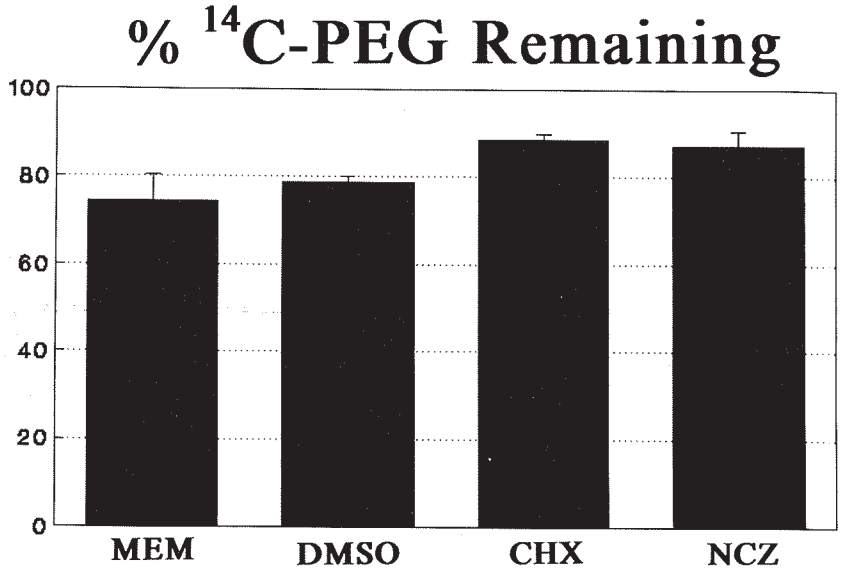

Fig. 1. ^{14}C-polyethyleneglycol remaining in the interstitial space after perifusion. Percentages of the retained ^{14}C-polyethyleneglycols to those in original perifusion fluids were measured using 1 μl of the fluids from the interstitial space obtained at one hour after the sustaining perifusion with Minimum Essential Medium (MEM; n=12)#, MEM containing 0.1% dimethyl sulfoxide (DMSO; n=8), 100 μg/ml cycloheximide (CHX; n=9) and 0.1% DMSO plus 3 μg/ml nocodazole (NCZ; n=10), as described in Materials and Methods. #Numbers in parentheses indicate the number of animals.

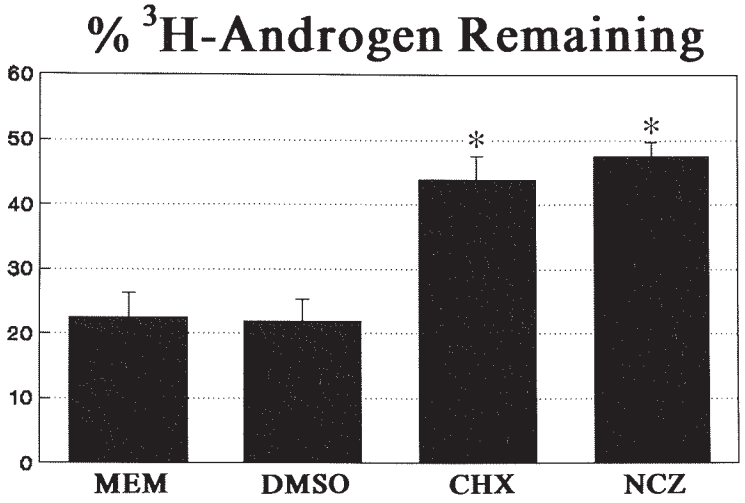

Fig. 2. ^{3}H-androgen remaining in the interstitial space after perifusion in the same materials used for Fig.1. Percentages of the retained ^{3}H-androgens to those in the original perifusion fluids were measured using 1 μl of the fluids from the interstitial space obtained at one hour after the sustaining perifusion with Minimum Essential Medium (MEM; n=12)#, MEM containing 0.1% dimethyl sulfoxide (DMSO; n=8), 100 μg/ml cycloheximide (CHX; n=9) and 0.1% DMSO plus 3 μg/ml nocodazole (NCZ; n=10), as described in Materials and Methods. #Numbers in parentheses indicate the number of animals. *Denotes significant change ($p<0.01$).

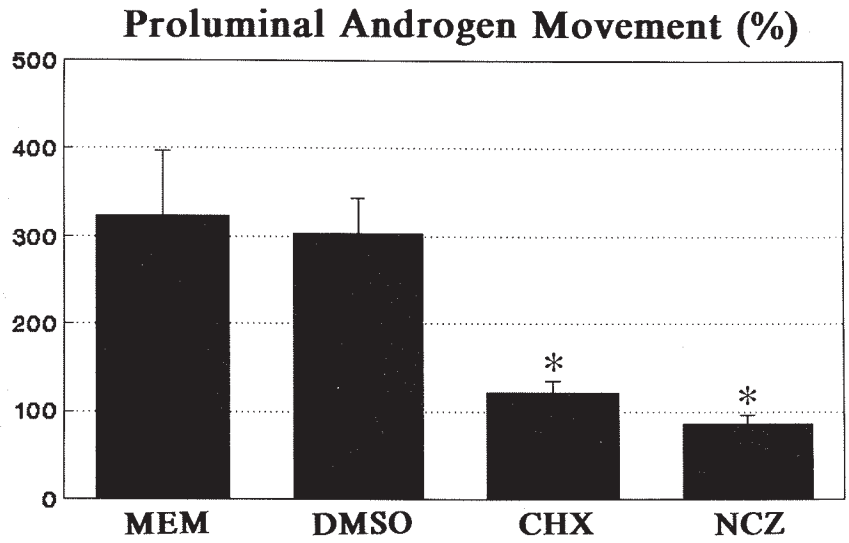

Fig. 3. Proluminal movement of ^{3}H-androgens perifused around the caput epididymal tubules after perifusion in the same materials used for Fig. 1. Percentages of the intraluminal ^{3}H-androgens to those in the original perifusion fluids were measured using 1 μl of the fluids from the epididymal tubules obtained at one hour after the sustaining perifusion with Minimum Essential Medium (MEM; n=12)#, MEM containing 0.1% dimethyl sulfoxide (DMSO; n=8), 100 μg/ml cycloheximide (CHX; n=9) and 0.1% DMSO plus 3 μg/ml nocodazole (NCZ; n=10), as described in Materials and Methods. #Numbers in parentheses indicate the number of animals. *Denotes significant change ($p<0.01$).

Table 1. The Quantity of Newly Synthesized Proteins Labeled with ^{35}S-Methionine in the Perifused Epididymal Regions at One Hour after Perifusion with MEM in the Presence or Absence of Cycloheximide.

Perifusion Fluid	Total Tissue (cpm/mg wet weight)
MEM fluid	604.8±85
MEM fluid + cycloheximide	175.6±21.2

Values are the mean ± standard error for five animals.

DISCUSSION

It has been accepted for years that steroids diffuse across the cell membranes. We have hypothesized that ^{3}H-androgens diffuse across the epididymal epithelia but bind to intraluminal androgen-binding protein in the epididymis, thus accounting for the antigrade movement of ^{3}H-androgens and the maintenance of high androgen concentrations in the caput lumen.[3,4,5] Nevertheless, we have recently reported that metabolic inhibitors (dinitrophenol or potassium cyanide) completely eliminated the uphill aspect of proluminal ^{3}H-androgen movement in the epididymis and suggested that an energy-dependent mechanism may be involved in the epididymal androgen uptake.[8] More recently, we have reported that antigrade proluminal movement of ^{3}H-androgen in the caput epididymis does not occur in the absence of androgen-binding protein in the epididymal lumen.[7]

The concept that antigrade proluminal androgen movement into the epididymal lumen is maintained by the presence of intraluminal androgen-binding protein and an energy-dependent transport process has never been proposed. If cytoplasmic receptors move androgens from the basal aspect of the epithelium to the luminal aspect and then androgens are released into the lumen, suppression of proluminal androgen movement in the epididymis by a metabolic inhibitor could be explained by its inhibition of synthesis of cytoplasmic receptors in the epididymal epithelial cells. Therefore, we examined the effect of a protein synthesis inhibitor on proluminal androgen movement into the epididymal lumen. The present study has shown that antigrade proluminal movement of ^{3}H-androgen in the caput epididymis is inhibited by addition of a protein synthesis inhibitor to the perifusion fluid. Inhibition of protein synthesis by cycloheximide[12] was confirmed by a ^{35}S-Methionine incorporation study. Of course, plenty of intracellular protein remains in the cell after one-hour perifusion with fluid containing cycloheximide. Any intracellular protein important to proluminal androgen movement, and responsive to the cycloheximide, would have to be one with a very short half life, because it would already have to be missing by one hour after beginning sustaining perifusion. When testosterone is taken up by epithelial cells of the epididymis, it is rapidly metabolized to dihydrotestosterone and other androgens. Dihydrotestosterone is bound to cytoplasmic receptors, translocated into nuclei as dihydrotestosterone-receptor complexes, and bound to nuclear chromatin.[13] Maybe in some cells cytoplasmic receptors move androgens around the basal cell surface to other places in the cell.

We have reported that as the concentration of the transported ^{3}H-androgen in the epididymal peritubular space is increased, the rate of transport at first increases, but eventually a concentration is reached after which the transport rate increases no further.[14] This finding supports receptor-mediated active transport. It is conceivable that proluminal movement of ^{3}H-androgen in the

epididymis is not accurate transport or a membrane transport system. Perhaps there is some other metabolic command or some other process in the cell that demands energy or depends upon energy that is important to the access of the androgens from one side of the epithelium to the other.

Another hypothesis which is related to antigrade proluminal movement of ^{3}H-androgen into the epididymal lumen is that ^{3}H-androgens around the epididymal tubules diffuse across the cell membrane but bind to intracellular receptors, and are transported along the intracellular microtubules to the apical side of the cell and finally released into the lumen. During this movement, microtubules provide the tracks for transport of androgen-receptor complexes. These cytoplasmic receptors may be high affinity androgen transport receptors in the epididymal epithelial cell which will not release androgen in the absence of a higher affinity intraluminal molecule like androgen-binding protein. Nocodazole, which is a potent microtubule-depolymerizing agent,[15] significantly reduced proluminal movement of ^{3}H-androgen in the epididymis. The microtubules can be made to disappear, or to depolymerize into constituent subunits, by exposure to low temperatures or to a depolymerizing agent. On removal of such an agent by washing, or on raising the temperature, the microtubules reappear. However, we could not confirm this reversibility of the assembly-disassembly process for microtubules by our in vivo perifusion system. Although another proof of effect of nocodazole on proluminal androgen movement is needed (for example, immunohistochemical study of epididymal tissues by using antimicrotubule antibody before and after perifusion with fluid containing antimicrotubular agent), it is suggested that disassembly of epididymal intracellular microtubules causes significant reduction of proluminal movement of ^{3}H-androgens.

In conclusion, cycloheximide, which blocks protein synthesis, significantly reduced antigrade proluminal movement of ^{3}H-androgen in the epididymis. ^{35}S-Methionine incorporation study demonstrated a significant reduction of the quantity of radiolabeled proteins after one-hour perifusion with cycloheximide. Nocodazole, which depolymerizes microtubules, also completely eliminated this uphill proluminal movement of ^{3}H-androgens. The energy-requiring events for compartmentalization of ^{3}H-androgens in the rat caput epididymis are unknown. They may be related to the synthesis of intracellular proteins and transportation of these proteins along microtubules. Furthermore, intraluminal androgen-binding protein also plays an important role in the maintenance of antigrade proluminal movement of ^{3}H-androgens in the caput epididymis.[6,7,16] Further investigation will be needed to understand these complex mechanisms by which the intraluminal microenvironment of concentrated androgens is maintained in the epididymis and to understand why such high intraluminal androgen concentrations are necessary for normal epithelial functions.

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