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Reduction of Testosterone Availability to 5α-Reductase by Human Sex Hormone-Binding Globulin in the Rat Ventral Prostate Gland In Vivo

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The present studies assess the effects of human sex hormone-binding globulin (SHBG) on the conversion of [^{3}H]testosterone (T) into dihydrotestosterone (DHT) in rat ventral prostate gland in vivo using a constant aortic infusion technique. The DHT/T ratio was determined using two-dimensional thin-layer chromatography (TLC), and these results were confirmed with reverse-phase high-performance liquid chromatography. The prostatic gland DHT/T ratio was 2.1 ± 0.4, 1.3 ± 0.2, 0.24 ± 0.02, or 1.1 ± 0.2, following a 60 sec aortic perfusion of [^{3}H]testosterone dissolved in either Krebs-Henselite buffer (KHB), 5 g/dl human serum albumin (HSA), human pregnancy serum (HPS), or heat inactivated HPS, respectively. Heat inactivation (60°C, 60 min) selectively denatured SHBG in HPS. The distribution of [^{3}H]testosterone in rat ventral prostate was examined with thaw-mount light in microscopic autoradiography. Following an aortic perfusion of [^{3}H]testosterone in buffer alone, the radiolabeled steroid was uniformly distributed among the epithelial and stromal compartments. However, the [^{3}H]steroid hormone was selectively sequestered in the stromal compartment following aortic perfusion of HPS. In conclusion, these studies demonstrate that human SHBG markedly restricts the availability of circulating testosterone to 5α-reductase in the prostate gland in vivo and that the presence of SHBG in serum causes the selective sequestration of the steroid hormone within the stromal compartment of the gland in vivo.

Key words: dihydrotestosterone, microcirculation, DHT

INTRODUCTION

Prostate gland 5α-reductase activity has been implicated in the etiology of both benign prostatic hypertrophy (BPH) and prostatic cancer [1–5], which are the two principal disorders of the human prostate gland [6]. Prostatic dihydrotestosterone (DHT) is concentrated primarily in the stromal tissue as opposed to the glandular epithelium [3]. The preferential accumulation of DHT in stromal tissue correlates with other observations showing that 5α-reductase is primarily localized in stromal cells as opposed to epithelium [7] and that BPH arises largely from overgrowth of stromal tissue [8]. The role of sex hormone-binding globulin (SHBG), the major

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androgen binding protein in blood, in the regulation of androgen metabolism in the prostate gland is not clear. However, several observations suggest that circulating SHBG is extensively taken up by prostate gland. First, SHBG is primarily localized in the stromal compartment [9]. Second, rabbit testosterone-binding globulin (TeBG), which has a 70% amino acid homology with human SHBG or rat androgen binding protein (ABP) [10–14], is rapidly transported across rat prostatic gland microvascular barriers in vivo [15]. Moreover, human SHBG-bound testosterone (T) or estradiol is also rapidly available for transport through rat prostatic gland microvascular barrier, possibly, as a complex with the binding globulin [15,16].

The observation that SHBG is rapidly transported across prostate gland capillaries in vivo [15] suggests that the mechanism by which SHBG regulates testosterone availability to 5α-reductase occurs distal to the microvascular barrier in prostate gland. Under these conditions, SHBG may serve to alter the distribution of circulating testosterone to cellular compartments in prostate gland. If SHBG delivers testosterone to stromal compartments containing 5α-reductase, then the tissue DHT/T ratio might increase in parallel with high serum SHBG. Conversely, if SHBG sequesters blood-borne testosterone within compartments lacking 5α-reductase, then the tissue DHT/T ratio might decrease when serum SHBG is elevated. The role of SHBG in prostate gland androgen metabolism gains added significance with the observation that serum SHBG concentrations in males normally increase with aging [17]. Therefore, the purpose of the present studies was to measure DHT/T ratios in vivo in rat ventral prostate gland following the aortic perfusion of human pregnancy serum containing high concentrations of SHBG and [^{3}H]testosterone. Reverse-phase high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) methodologies were used to measure the conversion of [^{3}H]testosterone to [^{3}H]DHT [18]. The present studies also employed thaw-mount autoradiography of rat prostate gland following perfusion with human pregnancy serum and [^{3}H]testosterone to determine the relative distribution of androgen between stromal and epithelial tissues following perfusion with SHBG.

MATERIALS AND METHODS

Materials

[1,2,6,7,17,17-^{3}H(N)]testosterone, 168 μCi/nmol, and [4-^{14}C]dihydrotestosterone, 55 μCi/μmol, were purchased from DuPont-NEN Corporation (Boston, MA). The radiochemical purity of the isotopes was assessed by TLC and radioscanning and was >98%. Human pregnancy serum was obtained from third trimester pregnant women. The steroid binding protein concentrations in human pregnancy serum have been reported previously, [SBHG] = 323 ± 28 nM and [albumin] = 4.0 ± 0.1 g/100ml (mean ± S.E.), respectively [19,20]. Male, Sprague-Dawley rats weighing 300–400 g were purchased from Harlan Sprague-Dawley (Indianapolis, IN), and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Single Aortic Injection Technique

Following anesthesia with ketamine (135 mg/kg) and xylazine (1.3 mg/kg), injected intraperitoneally, the descending aorta was exposed and an approximately 200 μl volume of Hepes-buffered Ringer's solution (pH 7.4; RHB) was rapidly injected through a 27-gauge needle. The injection solution contained 25 μCi/ml of

[^{3}H]testosterone and, at 2, 10, 30, and 60 sec after injection, the aorta was transsected and the rat ventral prostate gland was quickly removed. After homogenization of the tissue in cold acetone, the steroid hormones were extracted in 90% methanol and evaporated to dryness as described previously [21].

Chromatography

The extracts were redissolved in benzene/ethylacetate (1:1) containing 30 μg of unlabeled testosterone or DHT, and 50 μl aliquots of the sample were applied to 20 × 20 cm, 500 μm alumina plates (Analtech, Inc., Newark, DE) and separated by two-dimensional TLC. TLC plates were pretreated in both solvents and activated at 60°C for 60 min before application of sample. As described previously [22], this system gives complete separation of testosterone, DHT, androstanedione, androstenedione, epiandrosterone, estradiol, and 5α-androstan-3α,17β-diol. Approximately 85–90% of the tissue radioactivity was recovered with the testosterone plus dihydrotestosterone standards, with about 7% of tissue radioactivity recovered with the 5α-androstan-3α-17β-diol standard and less than 5% of tissue radioactivity was recovered with the remaining standards. The DHT/testosterone ratio was determined from the ratio of [^{3}H]DPMs comigrating with the DHT and testosterone standards. The [^{3}H]radioactivity was determined by liquid scintillation spectrometry with quench corrections. The identity of the [^{3}H]radioactive material comigrating with the DHT standard was confirmed using reverse-phase HPLC. In these experiments, the radioactivity migrating with the DHT standard was placed in 5 ml of methanol for extraction of radioactive steroids from the alumina. Following centrifugation, the supernatant was evaporated to dryness and redissolved in 20% acetonitrile in water containing 2,000 DPMs of [^{14}C]DHT. This material was injected onto a C18 reverse-phase HPLC column (Vydac, Hisperia, CA), followed by elution in a stepwise acetonitrile gradient running from 20% to 40% over 10 min with an isocratic elution at 40% for 10 min, followed by a gradient from 40% to 100% acetonitrile over 5 min, followed by a descending gradient from 100% to 20% acetonitrile over 6 min at 1 ml/min. Fractions (0.5 ml) were collected and [^{3}H] and [^{14}C] radioactivities were determined by double isotope liquid scintillation spectrometry. Migration of the unlabeled testosterone was detected by measurements of A_{240}, and the migration of DHT was detected with [^{14}C]DHT.

Constant Aortic Infusion Technique

Following cannulation of the descending aorta in anesthetized rats with PE-50 tubing, 1.5 μCi/ml of [^{3}H]testosterone was perfused at a rate of 2 ml/min for 30, 60, or 120 sec. The testosterone was dissolved in one of the following buffers: 1) Kreb's-Henselite buffer (KHB) [23] containing 0.1 g/dl human serum albumin (HSA), 2) KHB containing 5 g/dl HSA, 3) rat serum, 4) human pregnancy serum (HPS), or 5) HPS heated to 60°C for 30 min to selectively denature SHBG [24]. Each of the perfusion solutions was warmed to 37°C and was bubbled with 95% O_2/5% CO_2. The perfusate was driven with a peristaltic pump (Sage Instruments, Cambridge, MA) immediately following proximal ligation of the descending aorta. At the end of the perfusion, the rat ventral prostate was quickly removed and processed as described above. Control experiments determined that 23%, 5%, and 0% [^{3}H]testosterone radioactivity were lost during the perfusion owing to absorption to the PE-50 tubing following the perfusion of KHB (0.1 g/dl HSA), KHB (5 g/dl HSA), and HPS,

respectively. The loss of [^{3}H]radioactivity in the infusions with KHB (0.1 g/dl HSA) did not affect interpretation of the data since DHT/T ratios were measured.

Thaw-Mount Autoradiography

The rat ventral prostate was labeled using the constant infusion technique described above, except the concentration of [^{3}H]testosterone was increased to 5 μCi/ml. Two types of perfusions were used: 1) KHB (0.1 g/dl HSA) and 2) HPS. At the end of the perfusion, the rat ventral prostate was rapidly removed and snap-frozen in liquid nitrogen-cooled isopentane and embedded in Tissue-Tek OCT compound [21]. Four micrometer sections were cut with a Bright cryostat at −20°C and were thaw-mounted onto emulsion-coated slides and stored at −70°C for 2–4 months. The slides were developed by thawing to 4°C and fixing in 2.5% paraformaldehyde in 0.5 M Na_2HPO_4 (pH 7.4) for 30 sec at 4°C. Following two 30 sec washes in the same buffer at 4°C, the slides were developed for 60 sec at room temperature in the darkroom with Kodak D19 developer, washed for 1 min, and fixed for 5 min in dilute Kodak fixer followed by a final water wash. The slides were air dried for approximately 1 hr and stained with a drop of methylgreen-pyronin for 1–2 min, washed, air dried, and coverslipped. Both dark-field and bright-field photomicrographs were obtained from these slides.

RESULTS

Using the single injection technique, it was found that the [^{3}H]testosterone was instantly converted to DHT; the DHT/T ratio was 1.5 ± 0.3 by 2 sec after a single aortic injection (Fig. 1). However, the ratio did not rise with time after the single aortic injection, and this ratio actually decreased nonsignificantly during the period up to 60 sec after the single aortic injection (Fig. 1). The DHT/T ratio decreased from 1.5 ± 0.3 to 0.20 ± 0.05 when the [^{3}H]testosterone was mixed in HPS prior to injection (Fig. 2). The DHT/T ratio following a single injection of HPS increased in the time periods subsequent to 2 sec using the single injection technique protocol (data not shown) due to wash-out of the SHBG from the prostate gland following a single injection. Therefore, a constant infusion protocol was developed to quantitate more accurately the effects of serum binding proteins on conversion of T to DHT in rat prostate gland in vivo. As is shown in Figure 3, the DHT/T ratio was 2.1 ± 0.4 following a 60 sec infusion of KHB (0.1% HSA). This ratio was 1.1 ± 0.2 and 1.9 ± 0.5 following 30 and 120 sec of perfusion (mean ± S.E., n = 3), respectively, indicating equilibrium was reached by 60 sec of perfusion. The DHT/T ratio decreased to 1.3 ± 0.2, 0.24 ± 0.02, and 1.1 ± 0.2 following the 60 sec perfusion of KHB (5 g/dl HSA), HPS, and heat-treated HPS, respectively (Fig. 3). The DHT/T ratio was decreased to 1.2 ± 0.3 following a 60 sec infusion of rat serum (Fig. 3).

The identity of the ^{3}H radioactivity comigrating with the DHT standard in the two-dimensional TLC system (used to compute the DHT/T ratios in Figs. 1–3) was confirmed with reverse-phase HPLC as described Materials and Methods. As is shown in Figure 4, the ^{3}H radioactivity comigrating with the DHT standard in the TLC system comigrated exactly in the HPLC system with the [^{14}C]DHT internal standard.

The thaw-mount autoradiography studies are shown in Figure 5. Both the dark-field (Fig. 5A) and the bright-field (Fig. 5B) micrographs demonstrate a preferential

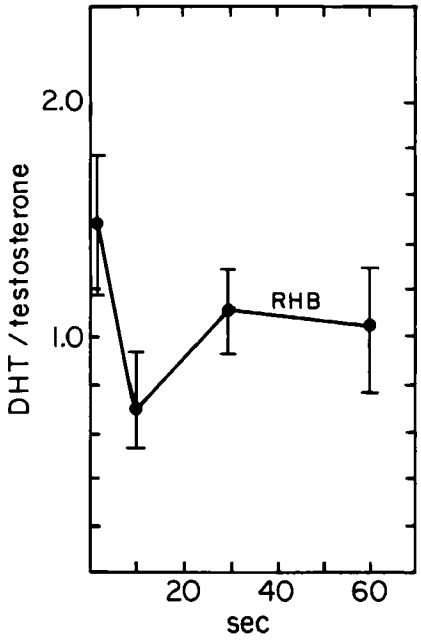

Fig. 1. Ratio of dihydrotestosterone (DHT) to testosterone (T) in rat ventral prostate at 2, 10, 30, and 60 sec following a single aortic injection of [^{3}H]testosterone dissolved in Ringer-Hepes buffer (RHB). Data are mean ± standard error (n = 5–7 rats per point).

sequestration of the [^{3}H]testosterone and its principal metabolite, [^{3}H]dihydrotestosterone, in the stroma of rat ventral prostate following 60 sec aortic perfusion of HPS. Conversely, the ^{3}H radioactivity distributed uniformly in both the stromal and epithelial compartments following 60 sec aortic infusion of the radiolabeled steroid in KHB (0.1 g/dl HSA) (Fig. 5C).

DISCUSSION

The present studies are consistent with the following conclusions. First, the conversion of testosterone into DHT in the prostate gland in vivo is instantaneous and is limited by the transport of testosterone into the tissue (Fig. 1). Second, there is tissue compartmentalization of the metabolism of testosterone in rat ventral prostate, since the DHT/T ratio is constant following a single aortic injection (Fig. 1). Third, SHBG restricts the availability of circulating testosterone to 5α-reductase in the rat ventral prostate gland in vivo and this protein causes 1) a reduction in the DHT/T ratio and 2) the sequestration of testosterone and dihydrotestosterone within the stromal compartment of the gland in vivo (Fig. 5).

The present finding that the DHT/T ratio is 1.5–2.1 following single injection or constant infusion of [^{3}H]testosterone in vivo in the rat ventral prostate parallels observations of Mercier-Bodard et al. [25], who showed that the DHT/T ratio was approximately 3.1 in the human prostate gland following 4 hr of superfusion. Therefore, the present studies regarding the activity of human SHBG in rat ventral prostate may be extrapolated with caution to the human prostate gland after considering

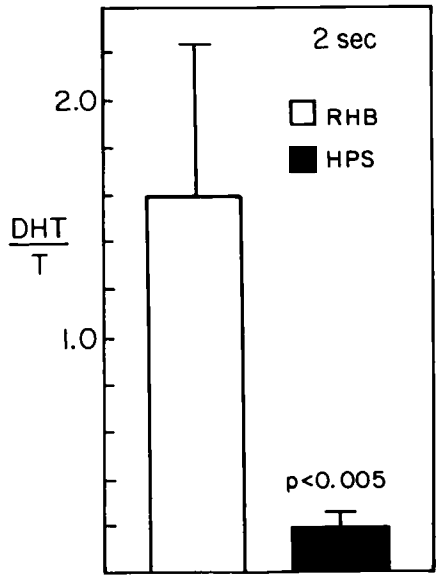

Fig. 2. Ratio of dihydrotestosterone (DHT) to testosterone (T) in rat ventral prostate 2 sec following a single aortic injection of [^{3}H]testosterone dissolved in either Ringer-Hepes buffer (RHB) or human pregnancy serum (HPS). Data are mean ± standard error (n = 5–7 rats per point).

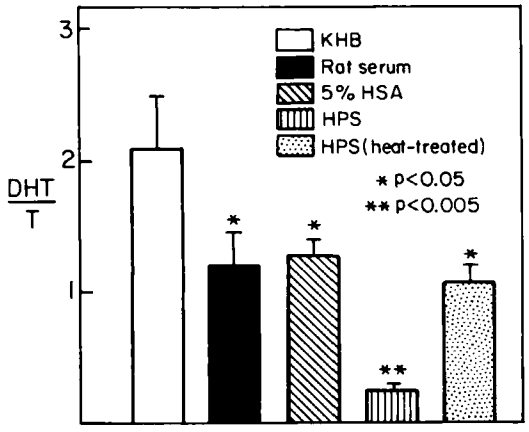

Fig. 3. Ratio of dihydrotestosterone (DHT) to testosterone (T) in rat ventral prostate following 60 sec constant aortic infusion of [^{3}H]testosterone dissolved in Krebs-Henselite buffer (KHB), rat serum, 5% HSA in KHB, human pregnancy serum (HPS), or heat-treated HPS [24]. The heat treatment (60°C, 30 min) selectively denatures SHBG [21]. Data are mean ± standard error (n = 3–5 animals per point).

important species differences. First, the rat ventral prostate is composed primarily of epithelium, with the stromal tissue occupying approximately 20% [26], whereas human prostate gland has a stroma:epithelium ratio of approximately 1:1 [27]. Second, the rat homologue of SHBG is ABP, which is approximately 70% homologous to human SHBG [11]. Therefore, if ABP receptors are present in rat prostate gland, it is possible that these receptors recognize human SHBG [15].

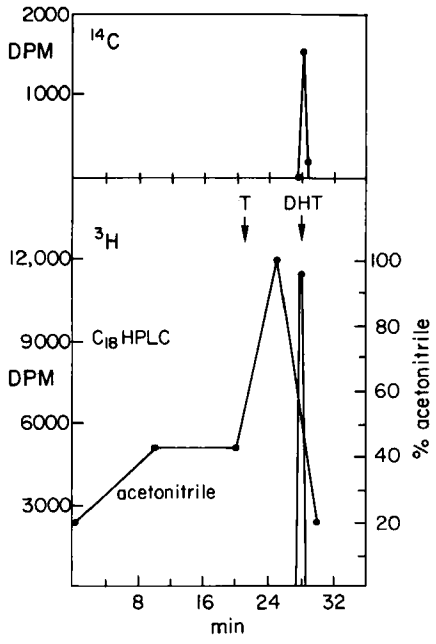

Fig. 4. C18 reverse-phase high performance liquid chromatography (HPLC) of [^{3}H]radioactivity isolated from rat ventral prostate following a 60 sec infusion of [^{3}H]testosterone. Tissue [^{3}H]radioactivity was separated by two-dimensional thin-layer chromatography (TLC) and the radioactivity comigrating with the dihydrotestosterone (DHT) standard was eluted, extracted, enriched with [^{14}C]DHT standard, and injected onto the reverse-phase column. Following elution with an acetonitrile gradient, the fractions were counted for ^{14}C and ^{3}H radioactivity. The experiments show that the tissue ^{3}H radioactivity presumed to be DHT comigrated exactly with the [^{14}C]DHT standard.

The kinetic analysis in Figure 1 indicates that testosterone transport into the rat ventral prostate is rate limiting for conversion of T into DHT by tissue 5α-reductase [18]. This conclusion is drawn from the observation that the DHT/T ratio reaches the maximal value within 2 sec after a single aortic injection (Fig. 1). Moreover, the failure to observe an increased DHT/T ratio in the 60 sec interval following a single aortic injection (Fig. 1) indicates that there is tissue compartmentalization of testosterone metabolism in the rat ventral prostate gland. That is, a portion of the blood-borne testosterone enters a compartment of the rat ventral prostate containing no 5α-reductase. Conversely, more than half of the circulating [^{3}H]testosterone enters a compartment containing 5α-reductase that instantaneously converts T into DHT. This tissue compartmentalization model is inferred from the essentially constant DHT/T ratio observed following a single aortic injection (Fig. 1).

The concept of compartmentalization of testosterone metabolism in prostate gland in vivo is supported by biochemical data [3] and provides a setting for regulation of the DHT/T ratio within the gland by blood-borne SHBG. The studies shown in Figures 2 and 3, in conjunction with previous observations [15], provide an explanation for the mechanism by which SHBG reduces the DHT/T ratio in prostate gland. Previous studies showed that the restricted availability of testosterone to 5α-reductase does not occur in the intracapillary compartment, since SHBG undergoes a

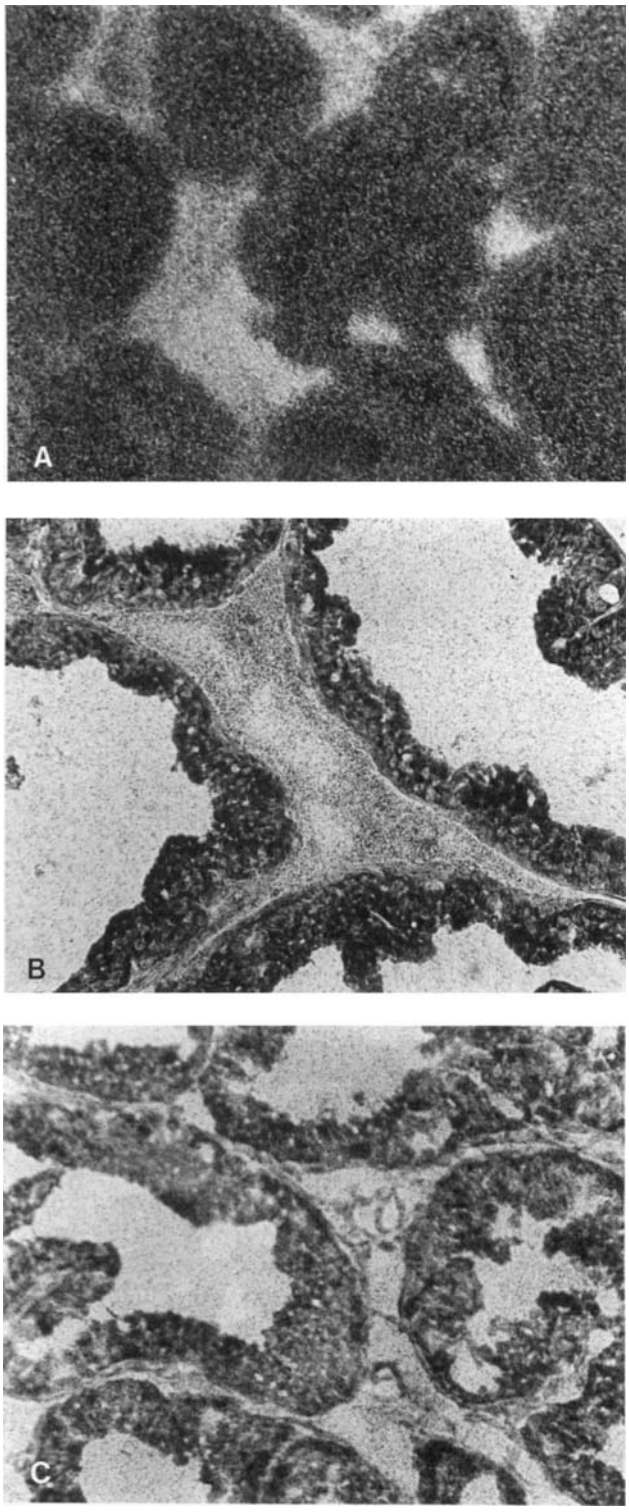

Fig. 5.

90% extraction across prostatic microvascular barriers on a single pass. That is, SHBG does not reduce the availability of circulating testosterone to the prostate gland as a whole. Therefore, the restricted access of circulating testosterone to 5α-reductase caused by SHBG must occur distal to the microvascular barrier [15]. The autoradiography studies in Figure 5 showing sequestration of testosterone in the stromal compartment following aortic infusion of radiolabeled testosterone mixed in HPS indicate that the plasma protein restricts access of testosterone to 5α-reductase by sequestering the steroid in the stromal compartment. Since 5α-reductase is found in the intracellular pools of stromal cells, and, in particular, the nuclear fraction [28], it is likely that the SHBG sequesters blood-borne testosterone in the extracellular compartment of the stromal space. Under these circumstances, the availability of blood-borne testosterone to stromal 5α-reductase would be greatly restricted and may be limited to the uptake of a testosterone-SHBG complex by the stromal cells, perhaps via the SHBG receptor recently identified in human prostate gland [29]. A receptor-mediated uptake mechanism may be necessary since the dissociation constant (K_D) of SHBG binding of testosterone, approximately 2 nM [16], is much lower than the Km of 5α-reductase for testosterone, which ranges from 11 to 78 nM [4,7]. Moreover, the uptake of stromal-derived DHT by the glandular epithelium, which has the highest concentration of androgen receptor [30], may also be limited by the concentration of SHBG in the stromal extracellular space. However, the K_D values of the androgen receptor and SHBG are comparable [16].

The conclusions regarding the role of SHBG in limiting androgen availability in the prostate gland and the interpretation of the experiments in Figures 2, 3, and 5 rest on the assumption that the major testosterone-binding protein in HPS is, in fact, SHBG. There is evidence in favor of this in previous studies showing that 95% of testosterone is bound to SHBG in HPS [19]. In addition, the studies shown in Figure 3, wherein there is no significant difference between heat-treated HPS and 5 g/dl HSA, are also evidence that the primary factor in HPS causing the inhibition of 5α-reductase is SHBG. The heat treatment employed in these experiments (60°C, 30 min) is known to denature SHBG selectively and not other androgen-binding proteins such as albumin [24]. The experiments performed with heat-inactivated HPS (Fig. 3) also indicate that the inhibition of testosterone conversion to dihydrotestosterone by the serum is not due to the presence of inhibitors, e.g., progesterone, of 5α-reductase in the infused serum. The mild heat treatment (60°C, 30 min) would not alter the inhibitory effect of HPS if this were caused by a diffusible, low-molecular-weight substance such as progesterone.

Finally, the present studies also show that rat or human albumin partially restrict the availability of circulating testosterone to 5α-reductase in rat ventral prostate gland in vivo (Fig. 3). However, for albumin to sequester circulating testosterone within stromal extracellular space, this plasma protein must have ready access to the pros-

Fig. 5. Dark-field (**A**) and bright-field (**B**) photomicrographs of rat ventral prostate obtained 60 sec after a descending aortic infusion of [^{3}H]testosterone and human pregnancy serum. These studies show sequestration of the radioactive steroid hormone and metabolites within the stromal compartment of the prostate gland, whereas the ^{3}H radioactivity is uniformly distributed among the stromal and epithelial compartments following aortic infusion of [^{3}H]testosterone dissolved in Krebs-Henselite buffer (**C**). × 100.

tatic gland extravascular compartment. Previous studies have shown that albumin is extracted 40% on a single pass by rat prostatic gland capillaries in vivo [13]. This is a relatively high extraction fraction for a plasma protein such as albumin (68,000 daltons) and indicates that prostatic gland capillaries are highly permeable to circulating plasma proteins. Since the extraction of albumin was comparable to that of transferrin or inulin [13], it is possible that circulating plasma proteins such as albumin are transported across prostatic microvascular barriers via rapid fluid-phase transcytosis pathways. The egress of SHBG from the prostatic vascular compartment is much faster than for albumin, inulin, or transferrin and may be receptor-mediated. Therefore, SHBG receptors in prostate gland may exist both at the microvascular barrier and at the epithelial or stromal plasma membranes in prostatic tissue in vivo.

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