Subcellular Distribution of Androgen Receptors in Human Normal, Benign Hyperplastic, and Malignant Prostatic Tissues: Characterization of Nuclear Salt-resistant Receptors

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ABSTRACT

Two populations of nuclear androgen receptors have been characterized in human prostatic tissue, and the levels and proportions of each were found to differ in normal prostates, benign hyperplastic prostates (BPH), and malignant prostates. A significant percentage (35 to 50%) of total nuclear androgen receptors was associated with the salt-resistant nuclear matrix fraction. The remainder were easily extracted from nuclei by 0.6 M KCl. Optimal conditions for measuring receptors in both compartments involved the use of an inhibitor of proteolysis (phenylmethylsulfonyl fluoride) and the omission of dithiothreitol from buffers. In the presence of dithiothreitol, most of the nuclear salt-resistant receptors were rendered salt extractable. Cytosol androgen receptor levels were not significantly different in normal, BPH, or malignant prostatic tissues. In contrast, the levels and distribution of nuclear salt-extractable and salt-resistant androgen receptors exhibited characteristic patterns. Compared to normal prostatic tissue, nuclear salt-extractable receptors were significantly elevated in both BPH and cancer, whereas nuclear salt-resistant receptors were elevated in BPH but not in cancer. The ratio of salt-extractable to salt-resistant receptors was approximately 1:1 in both normal and BPH tissues and 2:1 in cancer. In addition, a microassay has been developed for the measurement of androgen receptors in the three subcellular compartments of needle biopsy specimens of prostatic cancer. Studies are in progress to determine whether the measurement of both nuclear salt-extractable and salt-resistant receptors may improve the usefulness of receptor levels to predict the hormonal responsiveness of prostatic cancer.

INTRODUCTION

Assays have been developed for the quantitation of androgen receptors in the cytosol and soluble KCl extracts of nuclei prepared from human prostatic tissue (2, 22, 25, 27, 30, 38, 40, 45, 59, 61, 68, 78). Although information on both populations of receptors may be necessary to increase our understanding of the role of androgen receptors in benign and malignant diseases of the prostate, it is recognized that the interaction of steroid hormones and their specific receptor proteins with the nucleus of target tissues is a prerequisite for the modulation of hormonal responses in target tissues [see reviews (28, 33, 39, 64)]. Thus, a great deal of effort has been directed at correlating hormonal responses with the levels of steroid-binding activity in nuclei (19, 73). In the dog, BPH is characterized by increased levels of nuclear androgen receptor (67). In addition, levels of nuclear androgen receptor in human foreskin increase during the time of growth (24). With regard to studies of the human prostate, a major fraction of the cellular androgen receptor is localized to the nuclear compartment (30, 45, 66). Moreover, recent studies suggest that androgen receptor levels in the human prostatic nucleus, but not in the cytosol, correlate with the duration of response and survival following treatment with hormonal therapy in men with advanced prostatic cancer (69). It is apparent, therefore, that further studies of nuclear androgen receptors in the human prostate may be of significant value in identifying those patients who might benefit from hormone-ablative therapy.

Until now, however, nuclear androgen receptors in human prostatic tissue have been characterized only following their solubilization from nuclei by 0.3 to 0.6 M KCl or NaCl (13, 27, 30, 38, 40, 45, 58, 59, 61, 66, 68, 69). This procedure is based on the early observation that nuclear receptors can be dissociated from nuclei by the use of high-ionic-strength conditions (14, 34, 52). However, in many different target tissue systems, a significant amount of steroid-binding activity in the nucleus remains resistant to solubilization by salt (reviewed in Ref. 4; see also Refs. 3, 5, 6, 9, 12, 18, 20, 21, 31, 35, 50, 54, 56, 57, 71, and 75). Moreover, there is evidence that these salt-resistant nuclear steroid receptors represent a physiologically meaningful compartment of nuclear receptors (4, 18, 31, 54, 55, 57, 63) and deserve more study. For example, Clark and Peck (18) have demonstrated that estrogen-induced long-term growth of the rat uterus, which involves both hypertrophy and hyperplasia, depends on the specific interaction of estradiol:receptor complexes with nuclear salt-resistant binding components. In addition, Ruh and Baudendistel (54, 55) observed that treatment of immature rats with estradiol results in the appearance of both salt-extractable and salt-resistant nuclear estradiol receptors, whereas treatment with antiestrogens, which stimulate only limited uterine growth, results in the appearance of only salt-extractable nuclear receptors. Ruh and Baudendistel (55) concluded that the nuclear salt-resistant

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estradiol receptors may be involved in the mechanism of replenishment and processing of receptors, an event required for continued growth response to estrogen stimulation. Nuclear salt-resistant steroid hormone receptors have also been implicated in the mechanism of induction by estrogens of Leydig cell tumors in mice, since in strains that are susceptible to tumor induction the Leydig cells contain salt-resistant nuclear receptors, whereas Leydig cells of resistant mice contain only salt-extractable nuclear estradiol receptors (57). The presence of nuclear salt-resistant receptors for glucocorticoids in dexamethasone-sensitive murine leukemic myeloblasts, but not in certain clones of dexamethasone-resistant cells (Ref. 31; compare with Refs. 10 and 63) further suggests that nuclear salt-resistant steroid hormone receptors may be involved in the responsiveness of normal and neoplastic cells to specific steroid hormones.

Additional evidence that nuclear salt-resistant hormone-binding sites may be involved in the mechanism of action of hormones is that high-affinity, tissue-specific nuclear acceptor sites for steroid:receptor complexes have been described in residual fractions of chromatin that resist dissociation by 0.5 to 2 M NaCl or 4 to 5 M guanidine HCl. These salt-resistant acceptor sites have been characterized for androgen receptors in the rat prostate (75), testis (36), and Sertoli cell (71) and for progesterone receptors in the chick ovotestis (64). The nuclear acceptor sites are believed to represent the specific target sites in the nucleus where nuclear events such as gene expression are modulated by steroid hormone:receptor complexes (28, 39, 64).

Finally, of interest in this regard is the demonstration that a considerable proportion (45 to 65%) of the nuclear steroid hormone receptors of both androgen- and estrogen-responsive tissues in the rat and chicken (rat prostate, rat uterus, hen liver) are associated with the nuclear matrix, which is a discrete chromatin- and lipid-depleted salt-insoluble skeletal framework of the nucleus (3–6). There is growing evidence that the nuclear matrix plays a fundamental role in many dynamic aspects of nuclear function, including DNA replication and RNA synthesis, processing, and transport (4, 7, 60). Since steroid hormones affect these processes in specific target tissues, the specific binding of steroid receptors to this insoluble nuclear matrix structure suggests that the nuclear matrix is intimately involved in hormone action and that receptors associated with this salt-resistant structure may reveal new insight into the relationship between receptors and biological response.

For these reasons, we felt that it was important to determine whether human prostatic tissue contained a nuclear salt-resistant population of androgen receptors. In the present study, we have characterized and quantitated specific, high-affinity androgen receptors in the salt-resistant nuclear matrix fraction of normal peripheral prostate, BPH, and prostatic cancer tissues and compared these receptors to salt-extractable and cytosol androgen receptors. In addition, we have developed and evaluated a method for quantitating androgen receptors in the 3 subcellular compartments using biopsy-sized specimens of tissue. Data are presented on levels of androgen receptors in prostatic cancer biopsies obtained from patients with metastatic disease prior to their treatment by hormonal manipulation. It is anticipated that more detailed information on androgen receptors in these subcellular and subnuclear compartments may provide additional insight into the potential value of measuring receptors to identify patients with metastatic prostate cancer who might benefit from hormone-ablative therapy.

**Materials and Methods**

**Tissues.** Normal peripheral prostatic tissue was dissected from prostate removed at cystectomy. BPH tissue was obtained at open simple prostatectomy. Prostatic cancer tissue was obtained during radical prostatectomy performed on patients with clinical Stage B1 prostatic cancer (74). Prostatic cancer biopsies were obtained by percutaneous perineal needle biopsy from patients with evidence of metastatic carcinoma of the prostate (Stage D) prior to treatment with hormonal therapy (orchietomy or estrogen therapy). Following their removal, all tissues were placed immediately in 0.9% NaCl solution in ice, examined by a pathologist, and transported to the laboratory where they were weighed and stored in liquid nitrogen. Only tissues in which the diagnoses were confirmed by histology were used in these studies. Informed consent was obtained from all patients.

**Subcellular Fractionation.** The following procedure is a modification of the method described by Barrack and Coffey (3) for the isolation of nuclear matrix. All procedures were carried out at 4°C, and all buffers contained 1 mM PMSF (Sigma Chemical Co., St. Louis, Mo.), which was added from a stock solution (0.1 M PMSF in anhydrous n-propyl alcohol) immediately before use (32). Tissues were powdered with a tissue pulverizer (Thermovac Industries Corp., Copiague, N. Y.) and homogenized in about 10 volumes of buffer in a Dounce glass:glass homogenizer ( Kontes Co., Vineland, N. J.) with a motor-driven pestle (1250 rpm) for three 10-sec periods, alternating with 60-sec cooling periods on ice. The homogenizing buffer was TEG plus 1 mM PMSF (TEG plus PMSF buffer). The homogenate was centrifuged at 750 x g for 20 min. The supernatant was adjusted to contain 25 mM sodium molybdate and 1 mM dithiothreitol and then centrifuged at 104,000 x g for 1 hr to yield the soluble cytosol fraction. The 750 x g crude nuclear pellet was suspended in TEG plus PMSF buffer and extracted with Triton X-100, at a final concentration of 1%, for 10 min. The nuclear fraction was pelleted and washed twice in TEG plus PMSF buffer (750 x g, 15 min). The washed nuclear pellet was resuspended in 10 mM Tris (pH 7.5), 5 mM MgCl2, and 1 mM PMSF and incubated with pancreatic DNase I (20 μg/ml final concentration; Worthington Corp., Freehold, N. J.) for 30 min on ice. The nuclei were pelleted and extracted with 10 mM Tris (pH 7.4), 1.5 mM EDTA, 0.6 M KCl, and 1 mM Na2EDTA for 30 min at 4°C. The soluble salt extractions were then adjusted with 25 mM sodium molybdate and 1 mM dithiothreitol and then centrifuged at 104,000 x g for 20 min; the salt-resistant pellet was washed in 10 mM Tris (pH 7.4), 1.5 mM EDTA, and 1 mM PMSF and resuspended in this buffer. Aliquots of homogenate and washed nuclei (prior to DNase digestion) were taken for DNA analysis. The recovery of nuclei from the homogenate, based on DNA analysis, averaged about 70%.

In some experiments, where noted, nuclei were not extracted with Triton X-100. In other experiments, sodium molybdate and dithiothreitol were omitted from the preparation of the cytosol fraction. To study the effects of dithiothreitol on the subcellular distribution of receptors, some experiments (as indicated in text and legends) were carried out in which buffers containing 1 mM dithiothreitol were used throughout.

**Measurement of Androgen Receptors.** Androgen receptors were quantified by an in vitro exchange assay, essentially as described earlier (30, 45), using tritiated R1881 (87 Ci/mmol; New England Nuclear, Boston, Mass.). Duplicate or triplicate aliquots (0.2 mL) of cytosol, nuclear salt extract, or nuclear salt-resistant fractions were incubated in vitro in a total volume of 0.25 mL containing 0.07 nM to 5 or 10 nM [3H]R1881 alone (to measure total binding) or together with an excess (1 μM) of unlabeled R1881 (to measure nonspecific binding). In order to inhibit the binding of [3H]R1881 to cytosol progesterone receptors, assays of both total and nonspecific R1881 binding to cytosol fractions also contained 10 μM unlabeled triamcinolone acetonide (2, 30, 68, 78). When only small aliquots of tissue (100 to 200...
mg) were available, the binding assays were carried out at a single concentration of \[^{3}H\]R1881 (5 or 10 nM) with and without 1 μM unlabeled R1881 (30). Specific binding was calculated by subtracting nonspecific from total binding. Binding assays were carried out at 4°C for 20 to 24 hr, conditions that allow maximal labeling of occupied and unoccupied androgen receptors (30, 45, 66). All assays were terminated by the addition of 0.5 ml of a hydroxylapatite slurry [50% v/v, in 10 mM Tris (pH 7.4); 1.5 mM EDTA; Bio-Gel HTP; Bio-Rad, Richmond, Calif.], followed by incubation for 15 min at 4°C on a rotary platform shaker. Samples were pelleted at 5000 × g, 5 min (4°C), washed 3 times with 1.5 ml Tris:EDTA buffer, and extracted with 1 ml absolute ethanol at 30°C for 30 min. The ethanol extract was collected by centrifugation and added to 10 ml of liquid scintillation fluid (ACS, Amersham, Arlington Heights, Ill.). All measurements of androgen binding have been expressed in terms of fmol/mg DNA equivalent. Because of the wide range of nuclear recoveries in different experiments, it was important to calculate cytosol binding data using homogenate-derived DNA assays and to calculate nuclear binding data using nuclear DNA determinations. Statistical analysis was performed using the unpaired Student t test, designed to accept groups of unequal size.

Chemical Determinations. DNA was extracted and assayed by the diphenylamine method (15), using calf thymus DNA as a standard. Insoluble precipitates were solubilized in 0.1 N NaOH at 70°C for 15 min and assayed for protein by the method of Lowry et al. (41), using bovine serum albumin as a standard. The protein concentration of soluble extracts was assayed either by the Bio-Rad method (11), using bovine γ-globulin as a standard, or by the method of Lowry et al. (41).

RESULTS

Characterization and Quantitation of High-Affinity Binding in Salt-resistant and Salt-extractable Nuclear Fractions of Human BPH. Following a modification of the method used to characterize nuclear matrix-associated androgen receptors in the rat ventral prostate (3), we have demonstrated the presence of specific, high-affinity androgen receptors in the salt-resistant compartment of human prostate nuclei. Nuclei from human BPH tissue were digested briefly with DNase I and extracted with 0.6 μM KCl; specific androgen binding was then quantitated in both the salt-extractable and salt-resistant compartments by conventional in vitro exchange assay methodology, using \[^{3}H\]R1881 as the labeled probe ("Materials and Methods"). In addition to the classical salt-extractable receptor component [277 ± 23 (S.E.) fmol/mg DNA equivalent; n = 5], a similar number of androgen receptors was found associated with the salt-resistant nuclear fraction of human BPH tissue (250 ± 32 fmol/mg DNA equivalent; n = 5). Specific binding of \[^{3}H\]R1881 to the salt-resistant nuclear fraction was saturable and of high affinity, with an apparent equilibrium dissociation constant, \(K_d\), of 1.9 ± 0.1 nM. A representative experiment is shown in Chart 1. PMSF, an inhibitor of proteolysis (32), was added to all buffers used in the subcellular fractionation since preliminary experiments revealed lower levels of binding activity when PMSF was omitted. Similar observations have been reported for the measurement of androgen receptors in rat prostate nuclear matrix (3, 4) and human prostate cytosol (68).

The pretreatment of nuclei with DNase I allows the subsequent solubilization of greater than 95% of the total nuclear DNA with KCl and prevents the formation of a gelatinous pellet which otherwise is obtained when prostatic nuclei are suspended in concentrated salt solutions. Therefore, it is unlikely that the association of specific androgen receptors with the salt-insoluble fraction of prostate nuclei merely represents nuclear salt-extractable receptors that have been trapped in the residual pellet.

Nuclear androgen receptors were resistant to Triton X-100. When nuclei were washed with the neutral detergent Triton X-100 (1%) to extract phospholipids, the numbers of specific, high-affinity androgen-binding sites in the nuclear salt-extractable or salt-resistant fractions were not significantly different (p > 0.2) from those of nuclei that had not been treated with the detergent (Table 1). Because Triton treatment has been suggested to be helpful in reducing nonspecific binding, nuclei were washed in 1% Triton X-100 in all subsequent experiments.

The high-affinity receptors for \[^{3}H\]R1881 in the nuclear salt-
Table 1

Specific binding of $[^3H]$R1881

<table>
<thead>
<tr>
<th>Treatment of nuclei</th>
<th>No. of experiments</th>
<th>fmol/mg DNA equivalent</th>
<th>$K_a$ (nM)</th>
<th>fmol/mg DNA equivalent</th>
<th>$K_a$ (nM)</th>
<th>Total nuclear (fmol/mg DNA equivalent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Triton</td>
<td>5</td>
<td>277 ± 23*</td>
<td>2.0 ± 0.1</td>
<td>250 ± 32</td>
<td>1.9 ± 0.1</td>
<td>528 ± 50</td>
</tr>
<tr>
<td>+ Triton</td>
<td>9</td>
<td>242 ± 10</td>
<td>2.5 ± 0.1</td>
<td>232 ± 13</td>
<td>2.2 ± 0.2</td>
<td>475 ± 18</td>
</tr>
</tbody>
</table>

*Mean ± S.E.

Table 2

Steroid-specific inhibition of $[^3H]$R1881 binding to nuclear androgen receptors in BPH

<table>
<thead>
<tr>
<th>Competing steroid</th>
<th>Salt-extractable</th>
<th>Salt-resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1881</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>125</td>
<td>80</td>
</tr>
<tr>
<td>Testosterone</td>
<td>24</td>
<td>38</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;1</td>
<td>0.8</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5α-Androstan-3α,17β-diol</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Relative binding affinity is the ratio of the concentration of unlabeled R1881 required to inhibit specific binding of $[^3H]$R1881 by 50% to the concentration of another unlabeled competitor required to produce the same inhibition, multiplied by 100%.

When 1 mM dithiothreitol was added to all buffers used in the subcellular fractionation procedure, the subnuclear distribution of androgen receptors was altered dramatically. Table 3 compares the effect of the absence or presence of dithiothreitol on the total number and subcellular distribution of androgen receptors in cytosol, nuclear salt-extractable, and nuclear salt-resistant compartments. The numbers of cytosol, total nuclear, or total cellular specific androgen receptors were not significantly different in the absence or presence of dithiothreitol. However, when dithiothreitol was used, the number of salt-extractable androgen receptors was almost 2-fold greater (505 ± 65 fmol/mg DNA equivalent) than in control experiments (no dithiothreitol: 277 ± 23 fmol/mg DNA equivalent). Concomitantly, the number of salt-resistant nuclear androgen receptors decreased from 250 ± 32 fmol/mg DNA equivalent (control, no dithiothreitol) to 135 ± 33 fmol/mg DNA equivalent. In the absence of dithiothreitol, salt-extractable and salt-resistant nuclear androgen receptors were distributed approximately equally (53 ± 2% and 47 ± 2% of total nuclear receptors, respectively). In contrast, the addition of dithiothreitol to all buffers resulted in the subsequent solubilization by KCl of 80% of the total number of nuclear androgen receptors. The ratio of specific androgen receptors in the nuclear salt extract to that in the salt-resistant nuclear fraction was 1.2 ± 0.1 in the absence and 4.0 ± 0.6 in the presence of dithiothreitol (Table 3). Dithiothreitol also had the unexpected effect of decreasing the apparent affinity of all 3 subcellular binding components for R1881 ($K_a$, nM, in the absence or presence, respectively, of dithiothreitol were: cytosol, 2.7 ± 0.3 and 8.0 ± 1.5, p < 0.05; salt-extractable, 2.0 ± 0.1 and 4.9 ± 0.7, p < 0.01; salt-resistant, 1.9 ± 0.1 and 3.5 ± 0.4, p < 0.01).

Based on studies of androgen receptors in rat ventral prostate nuclei, it appears that the effect of dithiothreitol is to render components of the intranuclear ribonucleoprotein network of the nuclear matrix, as well as its associated salt-resistant receptors, susceptible to disruption and solubilization by high-ionic-strength solutions (3, 4). In all subsequent experiments, therefore, reducing agents were routinely omitted.

Influence of Sodium Molybdate on Cytosol Androgen Receptor Levels. Optimal conditions for quantitating cytosol androgen receptors have been reexamined. Based on reports that cytosol glucocorticoid, estrogen, and progesterone receptors can be protected by sodium molybdate from inactivation (1, 8, 17, 37, 43, 46, 65), several investigators have extended the use of sodium molybdate to studies of the androgen receptor in prostate tissue (26, 29, 49, 62, 68, 76). However, experiments carried out in this laboratory have demonstrated
that, when 25 mM sodium molybdate is added to the homogenization buffer, nuclear receptor levels are dramatically reduced while cytosol receptor levels increase, suggesting that there is an apparent redistribution of androgen receptors from the nuclear fraction into the cytosol (68). In agreement with these findings, Murakami and Moudgil (48) have shown that sodium molybdate can extract glucocorticoid receptors from isolated liver nuclei. Consequently, because sodium molybdate reduces nuclear androgen receptor content and because nuclear androgen receptor levels appear to correlate with the hormonal regulation of prostatic growth (3, 4, 39, 67, 69, 73), we did not think that sodium molybdate should be added to the homogenization buffer.

On the other hand, however, experiments carried out on rat ventral prostate androgen receptors indicated that, when sodium molybdate was added to the pre-cytosol supernatant (obtained by centrifuging the tissue homogenate at 750 × g), prior to the isolation of the 100,000 × g cytosol supernatant, then the number of specific, high-affinity androgen receptors in the cytosol was significantly greater than if molybdate had been omitted. The number of cytosol receptors was not increased when sodium molybdate was added only to the final cytosol preparation.

Similar experiments were carried out using human BPH tissue (Table 4). With delayed addition of 25 mM sodium molybdate, with or without 1 mM dithiothreitol, to the pre-cytosol 750 × g supernatant fraction, the final cytosol preparation contained 372 ± 17 fmol/mg DNA equivalent. These receptor levels represented a 60% increase over the number of cytosol androgen receptors measured under control conditions (231 ± 17 fmol/mg DNA equivalent; see Table 4). The addition of molybdate in this way does not interfere with the subnuclear distribution of receptors. The number of total cellular androgen receptors was 19% higher when molybdate was added to measure cytosol receptors (823 ± 37 fmol/mg DNA equivalent versus 694 ± 39 fmol/mg DNA equivalent (Table 4)). Although dithiothreitol appeared to afford no additional protection, both 25 mM sodium molybdate and 1 mM dithiothreitol were added routinely, in delayed fashion, to the pre-cytosol 750 × g supernatant, in all subsequent experiments. Whether the increased level of cytosol androgen receptors that is associated with the use of sodium molybdate under these conditions is due to protection of the androgen receptor from inactivation by a labilizing activity (or activities) present in the 750 × g supernatant has not been elucidated. The molybdate-induced increase in the level of cytosol androgen receptors, however, does not represent nuclear receptors, since the initial 750 × g centrifugation step precipitates >95% of the nuclei, based on recovery of DNA.

Comparison of Subcellular Levels of Androgen Receptors in Normal, Benign Hyperplastic, and Malignant Prostatic Tissues. Having demonstrated conditions for quantitating androgen receptors in human BPH tissue, similar experiments were undertaken to investigate the subcellular distribution of androgen receptors in normal peripheral prostatic tissue and in prostatic adenocarcinoma. All samples were processed as described above for BPH tissue. PMSF, at a final concentration of 1 mM, was added to all buffers, and sodium molybdate and dithiothreitol were added, in delayed fashion, only to the pre-cytosol supernatant. Dithiothreitol was not used at any other step in the procedure. Androgen receptors in the cytosol, nuclear salt-extractable, and nuclear salt-resistant fractions were quantitated by saturation and Scatchard analysis and were expressed as fmol/starting mg DNA equivalent. The cumulative data from 7 normal and 11 cancer specimens are shown in Table 5, as well as data obtained with BPH tissue. Also shown are the levels of signifi-

Table 3
Dithiothreitol affects the subnuclear distribution of androgen receptors in human BPH tissue

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Salt-extractable (SE)</th>
<th>Salt-resistant (SR)</th>
<th>SE:SR</th>
<th>Total nuclear</th>
<th>Cytosol</th>
<th>Total cellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>277 ± 23 ( ^b )</td>
<td>250 ± 32</td>
<td>1.2 ± 0.1</td>
<td>528 ± 50</td>
<td>232 ± 31</td>
<td>697 ± 87</td>
</tr>
<tr>
<td>+ 1 mM dithiothreitol ( ^c )</td>
<td>505 ± 65</td>
<td>135 ± 33</td>
<td>4.0 ± 0.6</td>
<td>640 ± 92</td>
<td>280 ± 98</td>
<td>840 ± 44</td>
</tr>
</tbody>
</table>

\( ^a \) Control buffer consisted of TEG plus 1 mM PMSF.
\( ^b \) Mean ± S.E. of 4 or 5 individual experiments.
\( ^c \) Indicates that all buffers contained 1 mM dithiothreitol, in addition to TEG plus PMSF.

Table 4
Effect of sodium molybdate on cytosol androgen receptors in human BPH tissue

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. of experiments</th>
<th>fmol/mg DNA equivalent</th>
<th>( K_d ) (nM)</th>
<th>% of total cellular</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Control (no molybdate)</td>
<td>7</td>
<td>231 ± 17 ( ^a )</td>
<td>2.4 ± 0.3</td>
<td>33 ± 2</td>
<td>694 ± 39</td>
</tr>
<tr>
<td>B. Delayed addition of molybdate + dithiothreitol</td>
<td>5</td>
<td>372 ± 25</td>
<td>1.7 ± 0.2</td>
<td>45 ± 2</td>
<td>823 ± 37</td>
</tr>
</tbody>
</table>

\( ^a \) Mean ± S.E.
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Androgen receptors in normal, benign hyperplastic, and malignant prostatic tissues

Normal peripheral prostatic tissue was dissected from prostates removed at cystectomy. BPH and prostatic cancer tissues were obtained at open prostaticctomy. Prostatic cancer specimens were obtained from patients with clinical Stage B1 prostatic cancer. All tissues were homogenized in TEG plus PMSF buffer. Molybdate (25 mm) and dithiothreitol (1 mm) were added only to the 750 x g preeytosol supernatant, prior to the isolation of the final cytosol fraction. Specific binding in all compartments was quantitated by saturation and Scatchard analysis.

Androgen receptors (fmol/mg DNA equivalent)

<table>
<thead>
<tr>
<th></th>
<th>Cytosol</th>
<th>Salt-extractable</th>
<th>Salt-resistant</th>
<th>Total nuclear&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Total cellular&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 7)</td>
<td>387 ± 26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>157 ± 17</td>
<td>170 ± 28</td>
<td>327 ± 27</td>
<td>713 ± 38</td>
</tr>
<tr>
<td>BPH (n = 18)</td>
<td>426 ± 35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>241 ± 12</td>
<td>240 ± 11</td>
<td>481 ± 19</td>
<td>872 ± 32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cancer (n = 11)</td>
<td>364 ± 26</td>
<td>227 ± 20</td>
<td>128 ± 13</td>
<td>356 ± 23</td>
<td>719 ± 34</td>
</tr>
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</table>

Level of significance (p)<sup>d</sup>

<table>
<thead>
<tr>
<th></th>
<th>Normal vs. BPH</th>
<th>Normal vs. cancer</th>
<th>BPH vs. cancer</th>
</tr>
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<tbody>
<tr>
<td>Normal vs. BPH</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>Normal vs. cancer</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BPH vs. cancer</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cumulative data on nuclear receptors in BPH tissue are presented from experiments in which nuclei either had or had not been extracted with 1% Triton X-100, since Triton had no significant effect on levels of nuclear binding (see Table 1). All normal and prostatic cancer nuclear preparations were washed with 1% Triton X-100.

<sup>b</sup> Total nuclear receptor levels represent the sum of receptors in the nuclear salt-extractable and salt-resistant subfractions for each individual experiment.

<sup>c</sup> Total cellular receptor levels represent the sum of receptors in the cytosol, nuclear salt extract, and nuclear salt-resistant compartments for each individual experiment.

<sup>d</sup> Mean ± S.E. of the number of specimens indicated in parentheses in Column 1.

<sup>e</sup> NS, not significantly different.

Results indicated that when only one concentration of ligand was used, 5 nM provided a more reliable, although underestimated, measurement of androgen receptor content.

The number of specific binding sites in cytosol, nuclear salt-extractable, and nuclear salt-resistant fractions estimated at 10 nM [3H]R1881 (with and without 1 μM unlabeled R1881), relative to the B<sub>max</sub> value estimated from Scatchard plots, averaged 95 ± 4% (n = 58; coefficient of variation, 28%). In comparison, the number of binding sites estimated at 5 nM [3H]R1881 averaged 73 ± 1% (n = 58; coefficient of variation, 13%) of the Scatchard-derived B<sub>max</sub> value. Thus, measurements at 10 nM R1881 were more variable than those at 5 nM (coefficients of variation, 28 and 13%, respectively). The more consistent relationship between 5 nM and B<sub>max</sub> estimates of binding than that between 10 nM and B<sub>max</sub> estimates is represented graphically in Chart 2. There was a better linear relationship between 5 nM and B<sub>max</sub> estimates (r = 0.93) than between 10 nM and B<sub>max</sub> estimates (r = 0.80). In summary, therefore, we concluded that, when only a single concentration of ligand is used for androgen receptor determinations, 5 nM provides a more reliable, although underestimated, measurement of receptor content.

In order to approximate the lower limit of sensitivity of microassay androgen receptor measurements, samples of each of the 3 subcellular compartments, obtained from a BPH specimen, were heated at 45° for 1 hr prior to the exchange assay. Apparent specific binding, measured at 5 nM [3H]R1881, was calculated to be 15 fmol/μg DNA equivalent for each receptor compartment (data not shown). Thus, measurements of androgen receptor at or below this level should be considered receptor negative.

We have applied this microassay method to the analysis of androgen receptors in cytosol, nuclear salt-extractable, and nuclear salt-resistant fractions in 26 biopsy specimens obtained from patients with Clinical Stage D prostatic carcinoma (Chart 3). Because measurements were carried out at 5 nM R1881, we assume that the receptor levels presented in Chart...
Chart 2. Relationship between androgen receptor measurements at single steroid concentration and by saturation analysis. Cytosol, nuclear salt-extractable, and nuclear salt-resistant fractions from BPH and prostatic cancer (Stage B1) tissues were assayed for specific R1881 binding by saturation analysis over a range of ligand concentrations. The total number of specific androgen receptors at saturation ($B_{max}$) in each subcellular compartment, determined by extrapolating the Scatchard plot to the x-intercept, was plotted against the amount of specific binding measured at either 5 nM $[^3H]R1881$ (A) or 10 nM $[^3H]R1881$ (B). All binding data are expressed as fmol/mg starting DNA equivalent. The best straight line fit to the data was derived by the method of least squares. A: correlation coefficient, 0.93; B: correlation coefficient, 0.80.

3 represent approximately 75% of the true receptor content. Indeed, the average receptor levels in these Stage D cancer biopsy specimens (228 ± 42, 181 ± 87, and 95 ± 60 fmol/mg DNA equivalent in cytosol, nuclear salt-extractable, and salt-resistant fractions, respectively) are approximately 72% (range, 63 to 80%) of the respective $B_{max}$ levels of receptors in Stage B1 prostatic cancer shown in Table 5; this close correspondence adds further to the validity of the 5 nM single-point assay.

The greater range of receptor levels observed in Stage D prostatic cancers may not be surprising in view of the heterogeneity of prostatic cancer, with respect to both the range of histological states of differentiation and the clinical course of these patients (duration of response and survival following hormonal therapy). However, since the object of studying biopsy material is to define some parameter(s) that will be related to the varied and unpredictable duration of response to hormone-ablative therapy, it is apparent that invariant parameters would be expected to yield little discriminating information. Clinical follow-up of the patients described in Chart 3 will be needed to elucidate the potential significance of these data. These studies are currently in progress.

**DISCUSSION**

We have identified a salt-resistant subpopulation of nuclear androgen receptors in the human prostate that account for approximately 50% of total nuclear androgen receptor content in normal and BPH tissues and about 35% of total nuclear receptor content in prostatic cancer. In previous studies of human prostatic androgen receptors, the majority of these salt-resistant nuclear androgen receptors may have been classified as part of the nuclear pool of salt-extractable androgen receptors, since in these prior investigations a reducing agent, dithiothreitol or -mercaptoethanol, was incorporated into the buffer solutions used for subcellular fractionation. We have shown in the present study (Table 3) that dithiothreitol allows the subsequent solubilization by 0.6 M KCl of 80% of the nuclear androgen receptors.

With regard to the observed effect of dithiothreitol on the redistribution of nuclear receptors to the salt-extractable fraction, it is important to note that dithiothreitol treatment alone does not extract steroid-binding sites; it only renders them capable of being solubilized by subsequent or simultaneous treatment of nuclei with high concentrations of salt (3, 4). Indeed, this effect of dithiothreitol may explain why some investigators have not found significant amounts of salt-resistant nuclear receptors (14, 16, 70).

Until more information is obtained on the relationship between androgen receptors and the hormonal responsiveness of human prostatic tissues in different benign and malignant disease states, it may prove useful to study the 3 subpopulations of cellular and nuclear androgen receptors as individual compartments. Indeed, as shown in Table 5, receptor levels in any one compartment were not sufficient to distinguish the 3 types of tissues from each other. For example, on the basis of only the nuclear salt-extractable receptors, it would appear that nuclear receptor levels were the same in BPH and cancer. However, when the nuclear salt-resistant receptors are also taken into account, differences between all 3 tissues become apparent (i.e., normal versus BPH, normal versus cancer, and BPH versus cancer).
Although all data are presented in this study in terms of fmol/mg DNA equivalent, we have also calculated our binding data in terms of fmol/g tissue and fmol/mg protein. However, there are large variations in the recovery of nuclei (42 to 93%), and large differences among specimens in the amount of DNA per g of tissue (1.61 to 5.89 mg homogenate DNA per g tissue), which may be due to differences in ploidy, cell size, cell number, or the loss of water content during storage of tissues in liquid nitrogen. As a result, receptor levels expressed as fmol/g tissue are much more variable than when expressed as fmol/mg DNA. Thus, 2 specimens may yield similar values of receptor content per g of tissue but different levels per mg DNA, and vice versa. Therefore, we feel that expressions of receptor content per g of tissue are less revealing than receptor content per mg DNA. Nevertheless, conclusions based on data expressed per g tissue were generally similar to those based on data expressed per mg DNA. Binding data expressed as fmol/mg protein are inferior in conveying information, since one cannot then calculate relative subcellular and subnuclear distribution of receptors.

With regard to subcellular levels of androgen receptors in normal tissue, BPH, and cancer (Table 5), it is of interest to consider various other ways of expressing the data, such as the number of receptors per nucleus. Since the amount of DNA per nucleus is reported to be the same in normal and BPH tissues (53, 79), the magnitude of the increases in nuclear salt-extractable and salt-resistant androgen receptor levels in BPH relative to normal prostatic tissue (Table 5) would be the same whether the data were expressed as fmol per mg DNA or number of receptor molecules per nucleus. In contrast, however, several studies have shown that the degree of ploidy (i.e., amount of DNA per nucleus) is generally higher in cancer than in normal prostatic tissue (13, 23, 53, 79). If this were the case in the present study, then the increase in the number of nuclear salt-extractable androgen receptors in prostatic cancer tissue relative to normal peripheral prostatic tissue based on fmol per mg DNA equivalent (Table 5) would be even more pronounced if the data were expressed as molecules of receptor per nucleus. In addition, cytosol, nuclear salt-resistant, total nuclear, and total cellular receptor levels appear to be similar in cancer and normal tissues when expressed as fmol per mg DNA equivalent (Table 5); however, if there was more DNA per cell in cancer than in normal, then the receptor levels in each of these compartments, expressed as number of receptor molecules per nucleus or cell, would actually be higher in cancer than in normal tissue.

In summary, then, based on the assumption that cancer cells, but not BPH cells, have more DNA than do normal cells, a comparison of receptor levels in BPH or cancer, relative to normal tissue, would indicate that cytosol androgen receptors were elevated in cancer but not in BPH; nuclear salt-extractable receptors would be elevated in both BPH and cancer, but more markedly in cancer; and nuclear salt-resistant receptors would be elevated in both BPH and cancer.

Of course, all comparisons are based on the assumption that all nuclei in a given specimen have the same binding capacity. We cannot distinguish between the possibilities that receptor levels per nucleus (or per cell) are different versus whether the percentage of cells containing nuclear (or cytosol) receptors is different. These considerations nevertheless point out that the various methods of expressing receptor levels may give important insight into the relationship between receptor levels and biological response.

In an earlier report from this laboratory (66), no significant differences in nuclear salt-extractable androgen receptor levels were found in normal peripheral versus hyperplastic prostatic tissues. These previous observations are clearly different from the data obtained in the present study. It is important to note that there have been several changes and improvements in our methodology, as reported herein, thereby making direct comparisons of data obtained by different methods very difficult. In the present study, perhaps as a result of some of these changes, interassay variation has been reduced markedly. Moreover, the subfractionation of nuclear receptors into discrete salt-extractable and salt-resistant fractions, as described in the present investigation, may have allowed the resolution of differences that otherwise might have been masked.

The finding of elevated nuclear androgen receptor levels in human BPH is of considerable interest. In both naturally occurring and experimentally induced BPH in the dog, increased cellular levels of dihydrotestosterone (47) and increased nuclear levels of androgen receptor (67) appear to be important and related events in the etiology of BPH. Wilson (77) has suggested that the pathogenesis of human BPH might be mediated by similar mechanisms. Indeed, the appearance of elevated nuclear levels of dihydrotestosterone in human BPH (13, 44) together with the data obtained in the present study of increased nuclear androgen receptor levels in human BPH support this hypothesis.

It should be noted that salt-extractable and salt-resistant nuclear receptors are operationally defined, and it has not yet been demonstrated whether these receptors have different biological functions. Nevertheless, the evidence available thus far in a number of different systems (4, 10, 18, 31, 54, 55, 57, 63) suggests that important distinctions between hormone responsiveness and resistance may be elucidated from analysis of both soluble and insoluble nuclear steroid hormone receptors. The differences reported in this study between normal, benign hyperplastic, and malignant prostatic tissues may further our understanding of the role of androgen receptors in the human prostate.

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Subcellular Distribution of Androgen Receptors in Human Normal, Benign Hyperplastic, and Malignant Prostatic Tissues: Characterization of Nuclear Salt-resistant Receptors

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