Human Prostate Androgen Receptor Quantitation: Effects of Temperature on Assay Parameters

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ABSTRACT

When cytoplasmic extracts of human prostatic tissues were split to permit quantitation of total androgen receptor (RCT) content by saturation analysis at 15° and 2°, we observed that 30% (10 of 32) of the specimens yielded statistically increased values for RCT following incubation at 15° as compared to 2°. Considering only those specimens (13 of 32) showing statistically differentiated RCT yield, 77% (10 of 13) yielded greater RCT content following incubation at 15°. The families of association constants (Kₐ) obtained for RCT determinations at 2° and 15° were not statistically differentiated. The increased yield of RCT content determined at 15° was 95% (mean) and 20 to 350% (range). Nuclear androgen receptor content determined at 15° was greater for 25% (2 of 8) of the patient specimens when compared to split determinations performed at 2°. Incubation of nuclear extracts at 15° resulted in a significant 3-fold reduction in receptor Kₐ for methyltrienolone (R1881). This did not appear to affect assay precision. These studies showed that incubation at 15° is preferable to incubation at 2° for quantitation of RCT and nuclear androgen receptor content by saturation analysis.

Single saturating dose determinations of RCT consistently yielded underestimates. The extent of underestimate was variable from specimen to specimen and was both ligand concentration and assay temperature dependent. Our data suggest that results of single saturating dose determinations of RCT require cautious interpretation.

INTRODUCTION

During the past 9 years, numerous investigators studying prostate physiology have determined prostatic androgen receptor content as one characteristic of physiological properties. Methods used for separation of receptor-bound ligand from non-receptor-bound and solution-free ligand principally have included sucrose density gradient centrifugation (15, 19), agar:gel electrophoresis (12, 30, 31), polyacrylamide gel isoelectric focusing (2), solid-phase anti-5α-dihydrotestosterone antibodies (11), and DCC adsorption (8, 10, 22, 23, 27).

Separation procedures that did not involve DCC were principally used to separate 5α-dihydrotestosterone binding to androgen receptor from binding to plasma sex hormone-binding globulin. Introduction of the synthetic androgen methyltrienolone (R1881) initially appeared to resolve difficulties resulting from 5α-dihydrotestosterone metabolism and binding to SHBG during quantitation of human prostate androgen receptors (4, 5). Subsequently, it was shown that R1881 bound to androgen and progesterone receptors with comparable affinity (1, 6) and also bound to a progestin-like binding component present in some human prostate specimens (6, 9, 22). We (21, 22) and others (8) established that R1881 binding to the progestin-like component was eliminated by incubation at 15°. Others subsequently showed that this could also be achieved by inclusion of excess TA in the incubation mixture during assay at 2° (10, 32).

Although assay protocols using DCC appear to be the current procedures of choice, selection of incubation temperature remains controversial. Incubation temperature selection has been dictated by individual laboratory findings, based on limited observations, of androgen receptor recovery and completeness of exchange of probe with endogenous steroid (7, 10, 21, 27). In this report, we describe the results of a systematic evaluation, using multiple human prostate specimens, of the effect of incubation temperature on androgen receptor quantitation.

MATERIALS AND METHODS

Chemicals. [17α-methyl-3H] R1881 (specific activity, 87 Ci/mmol) and radioinert R1881 were obtained from New England Nuclear (Boston, Mass.). Sodium molybdate, PMSF, and DTT were purchased from Sigma Chemical Co. (St. Louis, Mo.). Human γ-globulin (fraction II) was from Calbiochem-Behring Corp. (La Jolla, Calif.), Norit was from Fisher Scientific Co. (Fairportown, N. J.), and Dextran T70 was from Pharmacia Fine Chemicals, Inc. (Piscataway, N. J.). All other chemicals were reagent grade and were used as obtained from the manufacturer. Solutions were prepared from water that had been distilled, deionized, and redistilled from glass.

Patient Specimens. All tissues were obtained from consenting patients at the time of diagnostic or palliative surgery. Tissue was dissected by a pathologist immediately following excision and, when possible, specimens of grossly normal (N) and carcinomatous (C) prostate were selected. Specimens were placed into ice-cold Earles medium (Grand Island Biological Co., Grand Island, N. Y.) and transported to the research laboratory on ice. Tissues were blotted with filter paper, cut into pieces of approximately 125 cu mri, and packaged (0.5 to 1 g) in individual heat-sealed packets (Kapak Corp., Blooming- ton, Minn.), which were then frozen between blocks of dry ice and stored at −90°. Representative specimens of all tissues were proc-
Tissue Fractionation. All procedures were performed in a refrigerated room at 2°-4°C. Frozen tissue was finely minced with a scalpel and homogenized with a Dual glass-glass homogenizer in 5 volumes of TES buffer. After removal of an aliquot (100 µl) for DNA determination, the homogenate was centrifuged 5 min at 2° in 50,000 × g using a Sorvall SS-24 rotor. The resultant supernatant was brought to 20 mM sodium molybdate, 20 mM DTT, and 1 mM PMSF by addition of the appropriate volume of stock solutions of 1 mM Na₂MoO₄ and 1 mM DTT in TES buffer and 100 mM PMSF in isopropyl alcohol. Cytosol was prepared by centrifuging the resultant solution for 30 min at 2° at 200,000 × g using a Beckman SW 50.1 rotor. Cytosol was treated with a DGCC pellet to remove endogenous unbound androgen (22), the DGCC-treated cytosol was brought to 1 mM in TA, and TA containing cytosol was used for quantitation of androgen receptor content.

The crude nuclear pellet obtained by centrifugation at 50,000 × g was washed and then extracted 3 times with 10 mM Tris:1.5 mM EDTA:0.5 mM β-mercaptoethanol:600 mM KCl, pH 7.4, to provide a nuclear KCl extract as reported previously (22).

Androgen Receptor Quantitation. We quantitated RC and RCT content using our standard assay protocols (3, 22) with the following modifications. The assay mixture consisted of DGCC-treated cytosol (100 µl) containing TA (1 µM) and R1881 in a total volume of 220 µl TES buffer. For quantitation of RCT, the concentrations of ligand used were 2.5, 5.0, 10.0, 15.0, 20.0, 30.0, 40.0, 50.0, 80.0, and 100 nM R1881. Incubations for quantitation of RCT were 20 hr at 2° or 15°. RC was quantitated by incubation with R1881 for 2 hr at 2°. The ligand concentrations used were 0.5, 1.0, 2.0, 5.0, and 10.0 nM. Comparative determinations of RCT during incubation at either 2° or 15° or determinations of RC were performed on the same day by using different portions of the same cytosol preparation.

Nuclear androgen receptor content was quantitated as described previously by incubating nuclear KCl extracts (100 µl) with R1881 for 20 hr at either 2° or 15°. Assays were performed in a total volume of 220 µl Tris:1.5 mM EDTA:0.5 mM β-mercaptoethanol:600 mM KCl, pH 7.4, using the following ligand concentrations: 0.5, 1.0, 2.0, 3.0, 5.0, and 10.0 nM. Comparative incubations at 2° and 15° were performed on the same day by using different portions of the same nuclear KCl extract.

Nonspecific binding of radioligand was evaluated by parallel incubation in the presence of 1 µM radioinert R1881. All incubations were terminated by equilibration of assay tubes at 2° and separation of bound and “free” R1881 by treatment with DGCC:ethanol, cytoplasmic assays, or DGCC, nuclear assays, as described previously (3, 22). Aliquots, 200 µl of the resultant supernatants were generally counted to 2% precision, at the 95% confidence interval, in a Beckman 7500 scintillation spectrometer. After correction for nonspecific binding, data were displayed as Scatchard (20) and double reciprocal plots (22).

Other Methods. Protein was determined by the procedure of Lowry et al. (14) using bovine serum albumin as standard. Interference due to DTT in tissue extracts was eliminated with Iodogen (Pierce Chemical Co., Rockford, Ill.) by using a minor modification of the procedure reported by McClard (16). Aliquots of Iodogen dissolved in CHCl₃ (0.3 ml; 2 mM) were dried in disposable culture tubes (10 x 75 mm) after which cytosol (50 µl) was added and incubated 30 min at room temperature prior to removal of an aliquot (5 µl) for protein determination. This procedure eliminated interference by solutions containing up to 30 mM DTT. Quantitation of DNA was performed as described previously (22), and salmon testis DNA was used as standard.

Statistical Analysis of Saturation Data. We exclusively used the double reciprocal plot format, where the independent variable ([R1881]⁻¹ was the abscissa value and the dependent variable (fmol bound)⁻¹ was the ordinate value, for statistical analysis of saturation data. The double reciprocal plot is the preferred representation because neither the ordinate value, bound/free, nor the abscissa value, bound, of the Scatchard representation is an independent variable which is a requirement for simple linear regression (18). Because replicate saturation analyses indicated that there was no definable relationship between the independent variable ([R1881]⁻¹ and the variance of the dependent variable (fmol bound)⁻¹, we used unweighted linear regression (18) to determine receptor site concentration and R1881 binding affinity. We estimated variance for the slope and intercept (18) and used these estimates to calculate 95% confidence limits.

To determine whether a regression defined a line that passed through the origin, we estimated the variance of the regression and used the single-tailed t test of significance (26). To determine the significance of differences for ordinate intercepts (site content), we estimated the variance of the individual regressions and used the combined variances to perform a 2-tailed t test of significance (18). Differences among group means were evaluated by one-way analysis of variance, and a posteriori contrasts were determined by the Student-Newman-Keuls procedure (17).

RESULTS

Effect of Incubation Temperature on Quantitation of Prostatic RCT Content: Cytoplasmic androgen receptor content of 32 different patient specimens is reported in Charts 1 and 2. RCT content determined at 2° and 15° was significantly different (p < 0.05) for 13 of the 32 specimens (Chart 1). RCT content determined at 2° and 15° was statistically differentiated. Bars, 95% confidence limits (p < 0.05) for 13 of the 32 specimens (Chart 1). RCT content determined at 2° and 15° was significantly different (p < 0.05) for 13 of the 32 specimens (Chart 1).
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Chart 2. Androgen receptor content of prostate specimens for which RCT quantitated at 15° and 2° was not statistically differentiated. Definitions and procedures of analysis were as described in the legend to Chart 1.

Patient Number

content was greater at 15° for 10 (77%) of this group of 13 patient specimens. RCT content determined at 2° was greater (p < 0.05) than that determined at 15° for 3 (23%) of this group of 13 patient specimens. Notably, one of these latter patient specimens, Patient 30N, had essentially undetectable levels of cytoplasmic androgen receptors as determined during incubation for 2 hr at 2°, available sites, or during incubation for 20 hr at 15°, total sites (Chart 1). The mean percentage increase in RCT detected at 15° compared to 2° was 95%, and the range was 20 to 350% (Chart 1).

The data of Chart 2 show there was no statistically significant difference for RCT detected at 2° and 15° for 19 (59%) of the 32 patient specimens analyzed. Real differences, which were statistically rejected because of the quality of saturation data, may exist for 2 patient specimens, 92 and 71C. Results for Patient 48 (Chart 2) were similar to those for Patient 30N (Chart 1). There was a subgroup of patient specimens for which the upper limit of the 95% confidence interval could not be defined because the value of (fmol bound)^-1 was negative. This result was obtained for 6 of 33 and 2 of 33 patient specimens during incubation for 20 hr at 15° and 2°, respectively (Chart 2). Excluding the data for Patient 48, temperature-dependent increased RCT yields, which were not statistically differentiated, characterized the results for 9 patient specimens during incubation at 2°, whereas comparable results were obtained for 4 patient specimens during incubation at 15°.

The mean association constants for cytoplasmic receptor binding of R1881 during incubation for 2 hr at 2°, 20 hr at 2°, and 20 hr at 15°, respectively, were 11.0 x 10^8 (M)^-1, 5.74 x 10^8 (M)^-1, and 1.91 x 10^8 (M)^-1 (Chart 3). The data define families of association constants which are not statistically differentiated for incubations at either 2° or 15° for 20 hr. The family of association constants characteristic of human prostate cytosols incubated at 2° for 2 hr (quantitation of RC) was statistically different from that obtained during quantitation of RCT (Chart 3). The value of RCT obtained during incubation at 2° was poorly correlated with that obtained during incubation at 15° (Chart 4). Analysis of variance revealed that the origin was not included among the population of values defining the regression equation. The data (Chart 4) imply a tendency to underestimate RCT during incubation at 2°.
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RCT concentration determined at a SSD was highly correlated with RCT concentration determined by full saturation analysis (Chart 5). Analysis of variance revealed that the origin was not included among the population of values defining the regression equation for determinations of RCT performed at either 2° or 15°. This result implies that SSD analyses underestimate RCT. The mean underestimate of RCT by SSD at 10 nm R1881 was 30%, 2° incubation, and 45%, 15° incubation, whereas the mean underestimates at 20 nm R1881 were 15%, 2° incubation and 25%, 15° incubation.

Effect of Incubation Temperature on Quantitation of Prostatic Nuclear Androgen Receptor Content. Nuclear androgen receptor content was significantly greater for incubation at 15° for 2 of 8 patient specimens examined (Chart 6). The increased receptor yield for both specimens was 40%. The results for the other 6 patient specimens showed that receptor contents determined at 2° and 15° were not statistically differentiated. Increasing incubation temperature to 15° significantly decreased ($p < 0.05$) the association constant of R1881 for the nuclear receptor (Chart 7). The mean values for the 8 paired

**DISCUSSION**

In this study, we show that 30% (10 of 32) of human prostate specimens yield statistically greater RCT content for incubation...
at 15° compared to that for incubation at 2°. By contrast, only 10% (3 of 32) of the specimens yielded statistically greater RCT following incubation at 2°. Considering only those specimens which showed statistically differentiated RCT yield, 77% (10 of 13) yielded greater RCT content during incubation at 15°. The increased yield of RCT at 15° cannot be attributed to measurement of an R1881-binding component other than the prostatic androgen receptor because of the following considerations. Scatchard plots of the data obtained for paired analyses of RCT performed at 15° and 2° were linear and thus did not provide evidence for measurement of multiple classes of binding sites. Steroid specificity of R1881 binding to human prostate androgen receptors during incubation at 15° (20 hr) in the absence of TA (8, 21) is comparable to that during incubation at 2° (20 hr) in the presence of TA (10). The families of association constants (K_a) obtained for split cytoplasmic preparations incubated for 20 hr at 2° and 15° are not statistically differentiated (Chart 3). Finally, both receptor site content and the family of association constants for R1881 obtained in the current study are comparable to those reported previously by us (21, 23) and others (8, 28, 29). This suggests that the minor assay modifications introduced in the current studies have not significantly altered assay parameters.

An enhanced yield of nuclear androgen receptors during incubation at 15°, as compared to 2°, was characteristic of 25% (A2 and A5, 2 of 8) of patient specimens examined (Chart 6). Although incubation at 15° significantly reduced K_d of the nuclear receptor for R1881 (Chart 7), this change did not appear to significantly affect assay precision (Chart 6). Hicks and Walsh (10) have reported identical recovery of nuclear androgen receptor when human prostate KCI fractions are incubated at either 2° or 15° with 10 nM R1881. We have previously shown increased yield of nuclear androgen receptors during incubation at 15° (24). The reason nuclear androgen receptor yield at 15° is not consistently increased relative to yield at 2° (Chart 6) is not apparent.

The use of a SSD of ligand for analysis of RCT has been considered an attractive alternative when small samples must be analyzed (10) and is a common feature of some assay protocols (2, 11, 12, 30, 31). We now report that although RCT content determined by the SSD procedure is highly correlated with RCT content determined by full saturation analysis (Chart 5), the SSD procedure underestimates RCT content in most specimens. The extent of underestimate of RCT is ligand-concentration dependent and highly variable. In our experience, SSD underestimates of RCT at 2° were 30% (mean; range, 0 to 60%) for 10 nM R1881 and 15% (mean; range, 0 to 55%) for 20 nM R1881, whereas underestimates of RCT at 15° were 45% (mean; range, 0 to 98%) for 10 nM R1881 and 25% (mean; range, 0 to 80%) for 20 nM R1881. This variability is not unexpected when the families of R1881 association constants that characterized these cytoplasmic preparations (Chart 3) are considered. If single concentration determinations of cytoplasmic receptor content are necessitated by limited availability of tissue, our data (see above and Chart 5) suggest that underestimates would be minimized by performing the exchange incubation at 2°. Our data indicate that SSD determinations of human prostate RCT content require cautious interpretation. It is perhaps notable that each complete saturation analysis for RCT described in this report required the equivalent of 200 mg of tissue.

Our current studies (Chart 8) confirm a previous report (23) that the majority of prostatic androgen receptors are not located in the nuclear compartment. Our studies do not confirm the frequent assumption that most prostatic androgen receptors are localized in the nucleus due to occupancy by endogenous androgen (13, 17). In fact, the current studies fail to establish a relationship between cytoplasmic receptor content and/or occupancy and nuclear receptor content (Charts 1 and 2). Furthermore, we have shown that androgen-stimulated accumulation of rat prostate nuclear androgen receptor is not associated with depletion of cytoplasmic receptor (3). These considerations suggest that prostatic cytoplasmic and nuclear androgen receptors may have independent functions and that both should be quantitated when examining the relationship between prostate physiology and androgen receptor content.

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