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Effects of Antiandrogens (Cyproterone Acetate and Flutamide) on the Activity of Nuclear Protein Phosphokinases and Phosphatases of Rat Ventral Prostate

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SUMMARY

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The effect of *in vivo* administration of the antiandrogens flutamide and cyproterone acetate on the activities of prostatic chromatin-associated protein phosphokinases (toward endogenous and exogenous substrates) and nuclear phosphatase activities was studied. Intact male rats (315-335 g) were treated daily for up to 48 hr with either flutamide or cyproterone acetate producing both time- and dose-dependent declines in acidic-phosphoprotein kinase activity (dephosphophosvitin as protein substrate). At a daily dose of 10 mg of antiandrogen for 48 hr, the decline in activity was 33% for cyproterone acetate and 36% for flutamide. The ability of the antiandrogens to compete with testosterone in relation to effects on prostatic chromatin-associated acidic-phosphoprotein kinase activity and phosphorylation of endogenous chromosomal proteins was examined by simultaneous treatment of castrated rats with antiandrogen and testosterone *in vivo*. A dose of 0.01 mg testosterone propionate/100 g body wt/day was found sufficient to maintain the chromatin-associated acidic-phosphoprotein kinase activity at the level of the intact animal. Both antiandrogens were effective in competing with the androgen. At a dose 100-fold greater than testosterone propionate (on a molar basis), flutamide gave 55% and cyproterone acetate 27% reduction in acidic-phosphoprotein kinase activity compared with the control treated with testosterone and oil vehicle. Under the same treatment regimen, phosphorylation of endogenous proteins in chromatin was decreased 69% with flutamide and 65% with cyproterone acetate. Histone kinase activity of prostatic chromatin, compared with kinase activity toward dephosphophosvitin, was affected less by orchiectomy or by antiandrogen treatment of intact animals. Protein phosphatase activities, measured with ^{32}P-labeled phosvitin or lysine-rich histone as substrates, demonstrated a small decline in activity following treatment with antiandrogens (10-mg dose) for 48 hr, if these activities were expressed per unit of nuclear protein. A decline of about 50% in activity was evident when specific activity was expressed per unit nuclear DNA. This effect on nuclear protein phosphatase specific activities partially reflects the decline in the protein:DNA ratio observed following antiandrogen treatment. The alkaline phosphatase activity of the prostatic nucleus increased over twofold following 48 hr antiandrogen treatment (10-mg dose), as was also found 48 hr after orchiectomy. The use of antiandrogens produces an effect comparable to orchiectomy on the protein phosphokinase reactions of prostatic chromatin, especially those involving acidic protein or endogenous protein substrates, which suggests that these enzymes are under the control of events mediated via the 5α-dihydrotestosterone–receptor complex system.

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INTRODUCTION

The androgen-dependent growth of male accessory sexual organs such as the prostate and seminal vesicle

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can be inhibited by a number of antagonistic agents with antiandrogenic activity (1–3). Two potent antiandrogens which have now received considerable attention are cyproterone acetate (1,2α-methylene-6-chloro-17α-acetoxypregna-4,6-diene-3,20-dione) and flutamide (α,α,α-trifluoro-2-methyl-4-nitro-*m*-propionotoluidine). The former is a steroidal compound, a 17α-hydroxyprogesterone derivative, whereas the latter is a nonsteroidal antiandrogen. It appears that flutamide, in contrast to cyproterone acetate, must be metabolized to a second form, probably to hydroxylated flutamide (α,α,α-trifluoro-2-methyl-4′-nitro-*m*-lactotoluidine), in order to be an effective antagonist (4–6). Cyproterone acetate and flutamide do not appear to interfere with 5α-dihydrotestosterone formation but exert their effects by competition for dihydrotestosterone binding to the receptor and in particular inhibiting the nuclear binding of the androgen–receptor complex (7–12).

In recent years, there has been accumulating evidence that implicates the phosphorylation of chromosomal nonhistone proteins in a regulatory role in the control of gene activity (13–15). We have previously shown that in the prostate, phosphorylation of nuclear nonhistone proteins and activities of protein phosphokinases in chromatin and nucleolus are greatly influenced by altered androgenic status of the animal (16–18). Since phosphorylation of prostatic chromosomal proteins may have an important role in mediation of androgen effects in the chromatin, a study of the effects of antiandrogens which prevent occupancy of nuclear acceptor sites by 5α-dihydrotestosterone–receptor complex was undertaken on phosphorylation of endogenous chromatin proteins and various protein phosphokinase reactions using exogenous protein substrates.

MATERIALS AND METHODS

Chemicals. Lysine-rich histones were purchased from Worthington Biochemical Corporation (Freehold, N. J.) and $^{32}P_i$ from International Chemical and Nuclear (Irvine, Calif.). Testosterone propionate was obtained from Sigma Chemical Company (St. Louis, Mo.). The antiandrogens were generously supplied to us as gifts; flutamide by Dr. Rudolph Neri, Schering Corporation (Bloomfield, N. J.), and cyproterone acetate from Schering AG (Berlin). Omnifluor scintillant was obtained from New England Nuclear Corporation (Boston, Mass.). All other reagents were of the highest purity available.

Animals. Adult male Sprague–Dawley rats (ARS Sprague–Dawley, Madison, Wisc.), 315–335 g, were maintained on a standard laboratory diet and water *ad libitum*, and were kept on a 12.5-hr-light and 11.5-hr-dark cycle. Orchiectomy was performed under light ether anesthesia via the scrotal route. Testosterone propionate, cyproterone acetate and flutamide were suspended in sesame oil and appropriate doses were given subcutaneously in 0.20 ml of the oil.

Preparation of nuclei and chromatin. Nuclei (from pooled ventral prostates of 12–20 rats) were isolated according to the procedure detailed previously (19) and were used for the preparation of Triton X-100-washed nuclear sonicates (20) or chromatin (17).

Protein phosphokinase assays. Protein phosphokinase assays were performed by the following procedures established previously utilizing dephosphophosvitin (17) and lysine-rich histone (21) as protein substrates. Chromatin (5 to 20 μg of DNA) was incubated for 30 min at 37° in a reaction medium containing 5 mM $MgCl_2$, 200 mM NaCl, 1 mM dithiothreitol, 1 mg dephosphophosvitin, 3 mM γ-^{32}P-ATP and 30 mM Tris–HCl (pH 7.4 at 37°) in a final volume of 0.5 ml. Specific radioactivity of the ATP in the reaction was 2600–3000 dpm/nmol ATP. Histone kinase activity was assayed by incubating chromatin for 30 min at 37° in a 0.5-ml reaction volume containing 8 mM $MgCl_2$, 120 mM NaCl, 1 mM dithiothreitol, 2.0 mg lysine-rich histone, 0.1 mM γ-^{32}P-ATP (specific radioactivity 8000–11,000 dpm/nmol ATP), and 30 mM Tris–HCl, final pH 8.1 at 37°. The rate of phosphorylation of endogenous chromatin proteins was measured as previously described (22). The reaction mixture contained 30 mM Tris–HCl, pH 7.6 at 37°, 5 mM $MgCl_2$, 1 mM dithiothreitol, 24 mM NaCl, 0.1 mM γ-^{32}P-ATP (30,000 dpm/nmol) and approximately 50 μg of chromatin protein in a final volume of 0.50 ml. The reactions were started by the addition of chromatin and terminated with 0.50 ml of 30% (w/v) trichloroacetic acid containing 2 mM P_i and 3% $Na_4P_2O_7$. The precipitated protein was washed and prepared for the measurement of radioactivity as described earlier (17, 21). Suitable controls to take into account the incorporation of radioactivity into the chromatin, as well as controls for the nonspecific binding of radioactivity to substrates, were included in the experiments. These values were subtracted from the total to calculate the radioactivity incorporated into the experimental substrates. All protein kinase assays were done in duplicate or triplicate and variability within an assay was less than 5%.

Phosphatase assays. The alkaline phosphatase and protein phosphatase activities of sonicates of Triton X-100-washed prostatic nuclei were measured as described previously (20, 23, 24). The alkaline phosphatase assay was performed by the incubation of nuclear sonicates (30–55 mg protein) for 40 min at 37° in a reaction volume of 0.50 ml containing 1 mM $MgCl_2$, 50 mM sodium carbonate/bicarbonate buffer and 3 mM *p*-nitrophenyl phosphate (Tris salt), final pH 9.68 at 37° (20). The histone phosphatase assay was performed in a reaction volume of 0.25 ml containing 3 mM 2-mercaptoethanol, 40 mM Tris–HCl (final reaction mixture pH 7.1 at 37°) and 0.5–0.8 mg/ml ^{32}P-labeled lysine-rich histone (23). The activity of the nuclear acidic-phosphoprotein phosphatase activity was measured in a reaction volume of 0.25 ml containing 1 mM $MgCl_2$, 32 mM imidazole–HCl (final reaction pH 6.7 at 37°) and 0.8 mg ^{32}P-labeled phosvitin (24). The histone phosphatase and acidic-phosphoprotein phosphatase assays were incubated for 30 min at 37° following which the amount of $^{32}P_i$ released from the phosphorylated protein substrate was measured. All phosphatase assays were done in triplicate and variability within an assay was less than 5%.

Other methods. Phosvitin from chicken egg yolk was prepared and partially dephosphorylated as previously described (25). The protein content of chromatin was

estimated by the method of Lowry *et al.* (26) using bovine serum albumin as standard. DNA was assayed by the method of Burton (27) with calf thymus DNA as standard. The procedures for the preparation and purification of γ-^{32}P-ATP have been detailed earlier (28).

RESULTS

Comparison of the effects of antiandrogens and orchiectomy on chromatin-associated protein phosphokinase reactions. The effects of *in vivo* treatment of intact animals with cyproterone acetate or flutamide to compete with endogenous androgen were compared with those of androgen deprivation via orchiectomy with respect to chromatin-associated protein phosphokinase activities using dephosphophosvitin or lysine-rich histone as protein substrates. At 48 hr postorchiectomy, there was a marked reduction (67%) in protein kinase activity (Table 1) toward dephosphophosvitin as substrate (acidic-phosphoprotein kinase) whereas histone kinase was less affected (17% decrease). The acidic-phosphoprotein kinase activity demonstrated a dose–response relationship to either cyproterone acetate or flutamide treatment. At a daily 10-mg dose (48 hr), a decline in activity of 33% was found for cyproterone acetate and 36% for flutamide. A dose–response relationship was not clearly established for the histone kinase activity, but inhibition of activity was observed at the higher concentration of antiandrogen and the effect appeared greater following flutamide treatment. With either antiandrogen (10-mg dose) as well as with castration, there was a decline in the chromatin protein to DNA ratio.

TABLE 1

The effect of antiandrogens administered to intact rats on the activities of protein phosphokinases of ventral prostate chromatin

Rats (12 per treatment) were treated subcutaneously with the given dose of antiandrogen in sesame oil or of sesame oil alone at the beginning of the experiment and 24 hr later. The animals were sacrificed at 48 hr and nuclei and chromatin were prepared. The data of the antiandrogen studies are presented as individual experiments in which a group of animals was divided into the subgroups for the respective treatment. Mean value (± SD) of protein phosphokinase activities for intact and 48-hr castrated rats are given for comparison with the antiandrogen-treated animals and to present the relative variation among individual chromatin preparations. The kinase values of the intact and castrated rats are means of individual chromatin preparations with 12–20 rats per preparation. The protein kinase activities determined with dephosphophosvitin and lysine-rich histones as substrates are expressed as nmol ^{32}P/mg DNA/hr. The numbers in parentheses are the percentage decline in activity compared with the oil-treated control.

Treatment	*N*	Acidic-phosphoprotein kinase	Histone kinase	Protein/DNA
Intact	14	196.0 ± 68.0	7.8 ± 2.0	1.92 ± 0.20
Castration 48 hr	6	97.8 ± 29.2	6.4 ± 1.6	1.75 ± 0.34
Cyproterone acetate				
Experiment 1				
Oil		314.2	8.0	2.02
5 mg cyproterone acetate		263.3 (−26)	7.2 (−10)	1.99
10 mg cyproterone acetate		199.4 (−36)	7.6 (−5)	1.89
Experiment 2				
Oil		278.7	7.5	1.96
5 mg cyproterone acetate		242.5 (−13)	—	1.87
10 mg cyproterone acetate		196.2 (−30)	7.4 (−1)	1.83
Flutamide				
Experiment 1				
Oil		281.6	7.6	1.98
5 mg flutamide		216.1 (−23)	6.1 (−20)	1.93
10 mg flutamide		181.3 (−35)	5.8 (−28)	1.77
Experiment 2				
Oil		323.1	7.9	1.92
5 mg flutamide		237.7 (−26)	6.0 (−24)	1.86
10 mg flutamide		201.4 (−38)	5.8 (−27)	1.81

We previously demonstrated that as early as 9 and 18 hr postorchiectomy, there was a decline of 29 and 67%, respectively, in prostatic chromatin-associated acidic-phosphoprotein kinase activity (18). At 18 hr of antiandrogen treatment (10-mg dose) a decline in activity toward dephosphophosvitin (data not shown) was found for either cyproterone acetate (16%) or flutamide (17%). This effect was accentuated with 48 hr (daily) of treatment (Table 1). In addition, the decline in activity at 18 hr of treatment preceded any decline in the protein:DNA ratio (data not shown), a response that was evident at 48 hr postorchiectomy or antiandrogen therapy (Table 1). An effect on the histone kinase activity at early times of antiandrogen treatment was not as distinctive as that of the protein kinase active toward the acidic-phosphoprotein substrate; the histone kinase activity, however, was also not as sensitive to androgen deprivation (17% decline at 48 hr orchiectomy). Thus, the effects of antiandrogen treatment on prostatic chromatin-associated acidic-protein kinase are commensurate with those observed in response to androgen deprivation (18).

Competition studies between antiandrogens and testosterone in castrated animals: effects on phosphorylation of endogenous chromatin proteins and chromatin-associated acidic-phosphoprotein kinase activity. The ability of flutamide or cyproterone acetate to compete with exogenously administered testosterone was investigated with respect to maintenance of the androgen-dependent acidic-phosphoprotein kinase activity in castrated animals. A dose of 0.01 mg testosterone propionate/100 g body wt was fully effective in maintaining the chromatin-associated acidic-phosphoprotein kinase activity at the level of the intact animal. As described in Table 2, castrated rats were maintained for 48 hr with 0.01 mg testosterone propionate/100 g body wt while simultaneously being treated with antiandrogens. A decrease of 64% in kinase activity was observed in castrated animals treated with oil only when compared with the testosterone-treated controls. Castrated, testosterone-maintained animals treated with either cyproterone acetate or flutamide at a 10- or 100-fold higher dose (on a molar basis) demonstrated an effective competition of the antiandrogen with androgen with respect to chromatin-associated protein phosphokinase activity. The effect on acidic-phosphoprotein kinase activity was greater at the higher dose of antiandrogen where flutamide gave a 55% reduction in activity and cyproterone acetate 27%, when compared with the testosterone control. The phosphorylation of endogenous chromatin proteins by associated protein kinases in these chromatins prepared for the testosterone–antiandrogen competition studies was

TABLE 2

The effect of antiandrogen competition with testosterone propionate in the castrated rat on chromatin-associated acidic-phosphoprotein kinase and histone kinase activities and phosphorylation of endogenous chromatin proteins

In preliminary experiments, orchiectomized rats were maintained with testosterone propionate by subcutaneous treatments immediately upon castration and at 24 hr following castration. They were sacrificed at 48 hr postorchiectomy. A comparison of doses of 0.01 to 1.0 mg testosterone propionate/100 g body wt was made. The dose of 0.01 mg/100 g body wt was as effective as the others in maintaining the chromatin-associated acidic-phosphoprotein kinase activity at the level of the intact animal. For the purposes of the androgen–antiandrogen competition studies, the 0.01 mg/100 g body wt dose was thus chosen and the experiments were carried out as follows. Animals were orchiectomized and injected at two independent subcutaneous sites with oil alone; oil + 0.01 mg testosterone propionate (TP)/100 g body wt; 0.01 mg TP/100 g + flutamide; or 0.01 mg TP/100 g + cyproterone acetate. Two doses of flutamide or cyproterone acetate were given, one at 10-fold and the other at 100-fold higher concentration than the testosterone propionate dose on a molar basis. Injections of oil, hormone, or antiandrogens were given immediately upon castration, at 24 hr, and at 48 hr just prior to sacrifice. Nuclei and chromatin (12 rats per preparation) were prepared as described under Materials and Methods. Each experiment was done in parallel with the subgroups of testosterone- and antiandrogen-treated castrated animals derived from the same pool of rats. The data are presented as these individual experiments. The numbers in parentheses are the percentage decrease in activities compared with the testosterone propionate + oil-treated controls.

Treatment	Dephosphophosvitin	Lysine-rich histones	Endogenous phosphorylation
Cyproterone acetate			
Experiment 1			
Testosterone + oil	308.4	5.9	9.6
Oil + oil	114.1 (−63)		3.5 (−63)
Testosterone + 10× cyproterone acetate	231.0 (−25)	6.4 (+8)	7.0 (−27)
Testosterone + 100× cyproterone acetate	213.1 (−31)	5.4 (−10)	4.4 (−54)
Experiment 2			
Testosterone + oil	351.1	7.7	11.6
Testosterone + 10× cyproterone acetate	290.5 (−17)	6.8 (−12)	7.8 (−33)
Testosterone + 100× cyproterone acetate	269.3 (−23)	6.3 (−18)	5.0 (−57)
Flutamide			
Experiment 1			
Testosterone + oil	343.9	6.6	10.6
Oil + oil	123.2 (−64)		3.9 (−63)
Testosterone + 10× flutamide	294.6 (−19)	6.8 (+3)	6.9 (−35)
Testosterone + 100× flutamide	188.8 (−51)	4.6 (−30)	4.2 (−60)
Experiment 2			
Testosterone + oil	315.0	6.2	10.2
Testosterone + 10× flutamide	270.5 (−9)	6.1 (−8)	6.6 (−35)
Testosterone + 100× flutamide	191.7 (−39)	5.1 (−18)	4.3 (−58)

also measured (Table 2). Oil-treated, castrate controls showed a 63% decline in the rate of phosphorylation of endogenous chromatin proteins as compared with testosterone-treated, castrate controls. Antiandrogen treatment of testosterone-maintained castrated animals also resulted in a decreased rate of phosphorylation of chromatin proteins, an effect that was dose dependent. Both antiandrogens, at each dose used, produced a greater decline in endogenous phosphorylation than in kinase activity toward dephosphophosvitin. This implies that castration or antiandrogen treatment may result in a loss of both kinase activity and the endogenous proteins of chromatin that serve as substrate proteins for phosphorylation reactions.

Comparison of the effects of antiandrogens and orchiectomy on nuclear phosphoprotein phosphatase and alkaline phosphatase activities. Protein phosphatases that dephosphorylate ^{32}P-labeled phosvitin or lysine-rich histone were altered little following orchiectomy when their activities were expressed per unit of nuclear protein (Table 3). However, a small decline (about 30%) was found if these activities were expressed per unit of DNA. Treatment of intact animals with cyproterone acetate or flutamide produced little change in protein phosphatase activities at the 5-mg dose, but resulted in nearly a 30% decline at 10 mg when the data were given per unit of nuclear protein. This decline was about 50% based per unit of nuclear DNA. These data suggest that the protein phosphatase activity per nucleus declines with orchiectomy or antiandrogen treatment, and this reduction in activity is partly related to the loss of total protein from the nucleus (as judged by a decline in protein/DNA ratio). The prostatic nuclear alkaline phosphatase activity was elevated following antiandrogen treatment but did not follow a dose–response pattern in the dose range utilized. This rise in activity was found whether activity was expressed per unit of nuclear protein or DNA and is in concordance with the increased activity observed following orchiectomy (20).

DISCUSSION

The cellular regression and cytoplasmic changes in the prostate induced by cyproterone acetate (29) or flutamide (30) are markedly similar to those that occur postorchiectomy. These antiandrogens have also been shown to inhibit growth-related and androgen-dependent prostatic nuclear biosynthetic events such as *de novo* DNA (30, 31) and RNA formation (9, 10, 32, 33). We have now demonstrated that the androgen-sensitive chromatin-associated acidic-phosphoprotein kinase (17) and histone kinase (21) activities, as well as phosphorylation of endogenous proteins of prostatic chromatin, are decreased upon *in vivo* treatment with cyproterone acetate or flutamide. This effect on the nuclear protein kinase system

TABLE 3

The effects of antiandrogen treatment of intact rats on the activities of nuclear phosphatases of the ventral prostate

The given dose of antiandrogen was administered subcutaneously in sesame oil to intact rats at the beginning of the experiment and at 24 hr. The animals were sacrificed at 48 hr and nuclei were prepared (see Materials and Methods). The data of these studies are presented as individual experiments in which a group of animals was divided into subgroups for the respective treatment. Mean values (± SD) of the protein phosphatases and the alkaline phosphatase activities for intact and 48-hr castrated rats are given for comparison with the antiandrogen-treated animals and to present the relative variation among individual nuclear preparations.

Treatment	N	Acidic phosphoprotein phosphatase (nmol P_i/hr)		Histone phosphatase (nmol P_i/hr)		Alkaline phosphatase (nmol *p*-nitrophenol/hr)		Protein/DNA
		per mg protein	per mg DNA	per mg protein	per mg DNA	per mg protein	per mg DNA	
Intact	10	393 ± 69	1027 ± 224	12.7 ± 0.9	35.6 ± 2.8	168 ± 42	448 ± 120	2.55 ± 0.32
Castration								
48 hr	6	342 ± 52	723 ± 173	12.9 ± 1.1	28.9 ± 3.7	416 ± 89	914 ± 187	2.12 ± 0.27
Cyproterone acetate								
Experiment 1								
Oil		387	971	13.4	33.6	187	469	2.38
5 mg		409	892	15.7	34.2	357	778	2.08
10 mg		197	380	10.7	20.6	428	826	2.01
Experiment 2								
Oil		391	930	12.8	30.5	161	383	2.38
5 mg		373	776	13.5	28.1	303	630	2.08
10 mg		211	424	9.1	18.3	458	920	2.01
Flutamide								
Experiment 1								
Oil		411	1076	12.5	32.8	158	414	2.62
5 mg		357	853	13.0	31.1	574	1371	2.39
10 mg		237	488	11.9	24.5	484	997	2.06
Experiment 2								
Oil		379	974	14.1	36.2	193	496	2.57
5 mg		393	884	15.4	34.6	502	1129	2.25
10 mg		293	580	10.7	21.2	460	912	1.98

by antiandrogens is similar to that also observed for nuclear RNA polymerase activities (10). The acidic-phosphoprotein kinase activity which decreases more rapidly than that of the histone kinase upon castration responded in a similar manner to these antiandrogens. Likewise, some decrease in activities of the acidic-phosphoprotein phosphatase and histone phosphatase of prostatic nuclei was observed following antiandrogen treatment, an effect also noted following orchiectomy (23, 24). The finding that antiandrogens decrease nuclear protein kinase activity without a concomitant increase in protein phosphatase activity further supports the view that the phosphorylation step in the control of androgen-dependent prostatic nuclear phosphoprotein metabolism may be more open to regulation than the phosphatase-mediated dephosphorylation steps (24, 25). An alkaline phosphatase of the prostatic nucleus which is substantially elevated in activity upon orchiectomy (20) was also stimulated by cyproterone acetate and flutamide treatment. The role of this phosphatase in the prostate nucleus is not understood; its activity may be related to an actomyosin-type ATPase of the nucleus.

The mode of action by which the antiandrogens produce diminished nuclear protein phosphokinase activities would appear to be mediated through the 5α-dihydrotestosterone–receptor system. In both types of experiments utilized in the present study, antiandrogen administered to intact rats or given to castrated rats simultaneously with testosterone, the antiandrogen can be viewed as competing for binding sites on the receptor molecule and preventing its sequestering in the nucleus (4–6). Thus, as acceptor sites become available following release of hormone–receptor complex, they would not be reoccupied. In view of current hypotheses that binding of the 5α-dihydrotestosterone–receptor to chromatin acceptor sites results in gene activation (13, 34), this would imply that androgen-stimulated nuclear protein kinase activity involves the elaboration of a new gene product (this may be the enzyme itself or some other regulatory molecule). The involvement of 5α-dihydrotestosterone in the maintenance of the nuclear protein kinase system is important with respect to present concepts of the control of prostatic metabolism since different cellular regulatory functions have been proposed for different metabolites of testosterone in target tissues; e.g., stimulation of cell division by 5α-dihydrotestosterone and cellular secretion by 5α-androstan-3β,17β-diol (35). The sensitivity of nuclear protein kinase activities to altered androgenic status suggests that nuclear protein phosphorylation may be an important early molecular event in androgen-mediated cell growth and proliferation.

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